Abstract

Carrozzino, Jennifer Marie. Drug Partitioning and Solvation Environments in Lipid Bilayers. (Under the direction of Morteza G. Khaledi.)

The main goal of this research project was to investigate various factors influencing solute partitioning and solvation in lipid bilayer membranes using liposomes as biomembrane models and applying a combination of spectroscopic and capillary electrophoresis techniques.

The first area of investigation involved using a series of polarity sensitive solvatochromic indicators to probe the dipolarity of various phospholipid and synthetic surfactant vesicles. A homologous series of probes of varying hydrophobicity allowed a systematic probing of the dipolarity in the interfacial region of the vesicles and resulted in very specific polarity information for various regions or microenvironments depending on the probes positions. This dipolarity was examined in terms of vesicle size and composition, in addition to indicator partitioning behavior.

To develop Liposome Electrokinetic Chromatography (LEKC) as a method for rapidly determining liposome - water distribution coefficients, the effects of various parameters on the retention of basic drugs in liposomes were examined. This included characterizing the electrostatics of interactions between charged drugs and charged lipid membranes by examining the effect of membrane and buffer compositions. Additionally, LEKC was used to determine the effect of pH on the partitioning of basic drugs into liposomes composed of
lipids which mimic the composition of natural cell membranes. Drug partitioning as a function of pH is examined in detail in terms of the fractions of charged and neutral drug forms in the aqueous and lipid phases. An increase in pH results in a smaller degree of ionization of the basic drugs and consequently leads to a lower degree of interaction with the negatively charged membranes.

Finally, LEKC retention was used in QSAR studies for the evaluation of membrane permeability and intestinal absorption. LEKC retention factor data was correlated with human oral absorption in comparison with other methods such as octanol-water partitioning, total number of hydrogen bonding groups, and polar surface area. LEKC retention data was also related to Caco-2, MDCK, and human jejunal permeability in comparison with the standard model, octanol-water partitioning.
Drug Partitioning and Solvation Environments in Lipid Bilayers

by

Jennifer Marie Carrozzino

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirement for the Degree of Doctor of Philosophy

Chemistry

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Approved By:

__________________________  __________________________
Edmond F. Bowden             Charles B. Boss

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Daniel L. Feldheim           Morteza G. Khaledi
                                      Chair of Advisory Committee
This work is dedicated with much love to my parents.

You raise me up to more than I can be.
Biography

Jennifer Carrozzino was born on April 4, 1977 to Donald and Eileen Carrozzino and was raised in West Chester, Pennsylvania. Jennifer received dual Bachelor of Arts degrees in Chemistry and French from Bucknell University in Lewisburg, Pennsylvania, in the spring of 1999. Following this undergraduate education, Jennifer pursued a Ph.D. in Analytical Chemistry at North Carolina State University under the guidance of Dr. Morteza Khaledi.
Acknowledgements

There are many people I would like to thank who have helped me in different ways throughout the past few years. Most importantly I would like to thank my parents. They taught me that girls can be anything, encouraged me to reach for my goals, and always believed in Dr. DeeBee. Without their encouragement, motivation, and most importantly love and support, this would not be possible. Thank you for always being there for me. Additionally, there is also a special place in my heart for all of my family and friends and I would like to express my gratitude and love for each and every one of them.

Many thanks to Mr. Kamin's for sparking my interest in chemistry. If I had not been in his chemistry class my senior year of high school, I probably would not have studied chemistry. Also, thank you to Timothy Strein for being a mentor and encouraging me to pursue my PhD.

A special thank you to Morteza Khaledi for his support and encouragement. I would also like to acknowledge and thank all current and former group members who have helped me in many different ways, from fixing broken instruments to "tasty treat Friday" and interesting conversations, and especially to Suzie for her friendship and laughter.

Last, but certainly not least, I would like to thank Dave. You have been my constant source of support and strength throughout this process; my shoulder to lean on, always making me smile, providing laughter, support, encouragement, and love. I am so lucky to have met you and I look forward to our future together.
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LIST OF SYMBOLS, ABBREVIATIONS, AND TERMS

ADME………………………………………………Absorption, Distribution, Metabolism, Excretion
CE……………………………………………………………………Capillary Electrophoresis
Chol…………………………………………………………………………………Cholesterol
CZE………………………………………………………………………………….Capillary Zone Electrophoresis
DHP……………………………………………………………………Dihexadecyl Phosphate
DLS…………………………………………………………….Dynamic Light Scattering
$D_{LW}$………………………………………Liposome - water distribution coefficient
DNAP………………………………………………………………………………di-n-alkyl-p-nitroaniline
HTS…………………………………………………………High - Throughput Screening
IAM……………………………………………………..….Immobilized Artificial Membrane
ILC……………………………………………………Immobilized Liposome Chromatography
$k$………………………………………………………………………………..Retention factor
$K_{LW}$…………………………………………………Liposome-water partition coefficient
LEKC…………………………………………….Liposome Electrokinetic Chromatography
LSER………………………………………………….Linear Solvation Energy Relationship
MDCK…………………………………………………Madin Derby Canine Kidney
MEKC…………………………………………….Micellar Electrokinetic Chromatography
MLV…………………………………………………Multilamellar Vesicle
PC…………………………………………………………………………Phosphatidylcholine
PCS…………………………………………………………...Photon Correlation Spectroscopy
PE…………………………………………………………...Phosphatidylethanolamine
PG…………………………………………………………….Phosphatidylglycerol
PI……………………………………………………………… Phosphatidylinositol
$P_{ow}$……………………………………………………….Octanol-water partition coefficient
PS…………………………………………………………….Phosphatidylserine
QSAR……………………………………………Quantitative Structure Activity Relationship
SEC……………………………………………………….Size Exclusion Chromatography
SPH……………………………………………………….Sphingomyelin
SUV…………………………………………………… Small Unilamellar Vesicle
VEKC……………………………………………Vesicular Electrokinetic Chromatography
$\Phi$…………………………………………………………….Phase ratio
Chapter 1

Introduction
Liposomes

Liposomes are composed of phospholipids and cholesterol. When dispersed in an aqueous solution, the phospholipids form a lipid bilayer where the polar headgroups align on the exterior and the hydrocarbon chain tails of the lipids align on the interior of the bilayer due to the hydrophobic effect. Figure 1-1 shows a portion of a lipid bilayer containing cholesterol and two types of phospholipids. The lipid bilayer has two regions including the polar headgroups, which are in contact with the aqueous solution and the non-polar bilayer interior. The lipid bilayer found in a liposome is much like the lipid bilayer found in biological cell membranes.

Phospholipids are the major lipid component of most cell membranes; however the exact composition varies from one cell type to another. Likewise, liposomes can be prepared using various types of phospholipids and different additives including cholesterol or even proteins. The bilayer portion shown in Figure 1-1 consists of two double chain phospholipids including phosphatidylcholine (PC) and phosphatidylglycerol (PG). The two carbon chains on the phospholipids used in this work consist of 16 carbons (C\textsubscript{16}) each.

There are many different types (classes) of lipids varying in structure including zwitterionic, anionic, or even cationic headgroups. Additionally, each phospholipid class can have considerable variation in acyl chain composition, including chain length and degree of saturation. All of these lipid properties will influence aggregation properties in liposomes. The C\textsubscript{16}, C\textsubscript{18}, and C\textsubscript{20} fatty acids account for more than 80 % of the acyl chains in most biological membranes (1).
The phospholipids used in liposome preparations are biologically relevant phospholipids, meaning they are actual cell membrane components. PC and phosphatidylethanolamine (PE) are the major phospholipids of mammalian membranes, with PC being the predominant lipid. For example, the percentage of PC in human liver and lung tissues is 45.2 % and 41.7 %, respectively (1). Human liver tissue also has a significant amount of PE (28.4 %) (1). PC is one of the most commonly used phospholipids in liposome preparations due to its large presence in biological membranes, and because it is a well-established and highly reproducible system.

Phosphatidylserine (PS) and phosphatidylinositol (PI) are common lipids with a net negative charge at physiological pH. Many membranes have a significant component of anionic lipid. Human lung tissue, for example, has 3.8 % PI and 7.4 % PS (1). PG, a synthetic lipid, is the most commonly used phospholipid in this work. PG is preferred in this work over PS or PI due to the lower cost of the synthetic lipid, while retaining similar liposome properties.

Through the careful selection of lipids and membrane additives, such as cholesterol or membrane proteins, the simulation of various cell environments is accomplished. Generally cholesterol is incorporated in the liposomes because it is an important component of biological membranes. Cholesterol is localized on the interior of the lipid bilayer incorporated between the phospholipid chains (2). Additionally, cholesterol provides stability to the liposomes (3). A specific cell membrane can be mimicked by the selection of lipids present in the membrane and a very simple liposome is formed to model the significantly more complicated cell membrane environment (4). For example, in Chapter 5 for studies on
drug transport, liposomes are prepared to simulate the lipid bilayer environment in Caco-2 cells by selecting the lipids present in the Caco-2 cells.

The lipid bilayer formed by the phospholipids creates a spherical vesicle that encapsulates an aqueous interior while also having an aqueous environment surrounding the vesicle. Liposome preparation is accomplished by first mixing the appropriate phospholipids and additives in an organic solvent. Once the solvent is removed and the remaining lipid film is dried, it is then hydrated with an aqueous buffer. Upon hydration, multilamellar vesicles (MLVs) are formed. MLVs have layer upon layer of lipid bilayer and are described as "onion-like". These MLVs are processed to large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs), both of which contain only a single lipid bilayer encapsulating an aqueous interior and only differ based on vesicle diameter (5).

The processing of MLVs to SUVs was initially accomplished in this lab by sonication methods, which results in very small liposomes (~ 30 nm) with high polydispersity. This high polydispersity caused reproducibility problems and band broadening in capillary electrophoresis (CE) experiments. A high polydispersity of liposome solutions has been shown to cause a wide peak width in CE due to a differential migration of liposomes of different sizes (6).

Extrusion is the preferred method of SUV preparation used in this work. Extrusion involves passing MLVs through membranes containing progressively smaller pore sizes. This extrusion results in a decrease in the size of the vesicles according to the size of the pores and SUVs are formed (7). When the phospholipids are subjected to sonication or other preparation methods such as extrusion, the initially formed multilamellar vesicles (MLVs)
are disrupted by physical forces and the resulting phospholipid fragments find an intermediate configuration as SUVs. SUVs are more stable (lower energy) than the fragments, though less stable over the long term than MLVs, which are the lowest energy arrangement for the aqueous lipid dispersion (8). Extrusion results in very reproducible vesicles, with a narrow size range and low polydispersity. The size range of the vesicles is controlled by the size of the pores in the membrane used for extrusion. Typical SUVs are prepared in the 50 or 100 nm size ranges.

Octanol - Water versus Liposome - Water Partitioning

Meyer and Overton initially suggested the use of olive oil and water as a simple chemical model for the measure of solute - membrane interactions (9-12). Ever since, much work has been done to identify a more accurate in- vitro chemical model for cell membranes and to quantify solute affinity for lipid environments (termed lipophilicity). As a result, the octanol - water partition coefficient has become the most widely used scale for lipophilicity and a model for drug interactions with cell membranes (13-18).

The octanol - water partition coefficient is a measure of the equilibrium concentration of a compound between octanol and water. The partition coefficient, \( P_{ow} \), is defined as \( \frac{C_o}{C_w} \), such that \( C_o \) and \( C_w \) are the concentrations of the species in the octanol and aqueous phases, respectively. Similarly, for a charged solute, this is termed distribution coefficient \( (D_{ow}) \). A distribution coefficient must be measured as a function of pH for the interaction of a charged solute between the two phases.
Octanol-water partition coefficients are typically determined using the shake-flask method. Determining octanol-water partition coefficients is quite time-consuming, cumbersome, and not applicable for high-throughput applications. However, methods exist for predicting partition coefficients into octanol (ClogP<sub>ow</sub>, calculated octanol-water partition coefficient) based on solute structure, which is a significant advantage (16).

Much as a partition or distribution coefficient is determined for the octanol-water system, the partitioning between a liposome and water phase may be determined as the liposome-water partition coefficient (K<sub>LW</sub>). In this case, K<sub>LW</sub> will be the ratio of the concentration of the solute in the lipid (C<sub>L</sub>) versus aqueous (C<sub>W</sub>) phase given as C<sub>L</sub> / C<sub>W</sub>.

There are obvious differences between partitioning into octanol and partitioning into a lipid bilayer environment. Octanol-water is a simple biphasic system where solutes merely transfer between the two bulk phases. The structure of octanol saturated with water is thought to be a cluster of water molecules surrounded by 16 octanol molecules, with the polar groups pointing towards the water clusters, all connected by a network of hydrogen-bonds (19). While the octanol-water system involves a bulk phase partitioning, the partitioning into liposomes involves interaction with a complex interfacial lipid bilayer region. The properties of interfacial region will of course be influenced by the selection of lipids. Additionally, liposomes possess the fluid lipid bilayer form found in cell membranes, contrary to the environment in octanol.

The octanol-water model especially falls short when it comes to the interaction of charged solutes. Octanol can only interact with solutes by hydrophobic and hydrogen-bond interactions. Thus it fails to mimic the interfacial character of lipid bilayers and the ionic
interactions between membrane phospholipids and charged drugs. Liposomes are quite different, because in addition to hydrophobic and hydrogen-bonding interactions, they can also have electrostatic interactions. This results in important differences in the interactions of the ionized forms of drugs with phospholipids compared with octanol (20,21).

Despite these significant differences, octanol-water partitioning has remained an important tool in studies of drug interactions. One of the reasons the octanol system remains attractive is methods exist to predict partition coefficients. However, recent reports from this laboratory have demonstrated the possibility of calculating liposome-water partition coefficients from solute structure (22). Predicting the partitioning or lipophilicity behavior into lipid bilayer environments will be a huge advantage in drug development.

Factors Influencing Partitioning

There are numerous factors that influence partitioning of a drug into a liposome. This includes both properties of the drug and properties of the model lipid bilayer membrane. These properties include hydrogen bonding, dipolarity, ionization state, molecular size and shape, pH, temperature, membrane fluidity, etc. Linear solvation energy relationship (LSER) models have been used to unravel the contributions of various types of interactions on the partitioning of solutes into liposomes revealing that size and hydrogen bonding are very important (23).

One of the important characteristics of liposomes and lipid bilayers is the interfacial region. In bulk phases, such as octanol, physical properties are uniform throughout.
Partitioning of solutes into bilayers, which are interfacial phases, is of a fundamentally different nature than partitioning into bulk phases such as octanol (24). The interfacial region in a lipid bilayer is the region spanning the bulk aqueous to the lipid bilayer headgroup area. This interfacial region is the first area a drug will experience upon partitioning into the lipid bilayer.

In interfacial phases, physical properties vary with distance from the interface. For example, in the lipid bilayer interfacial region there is a significant change in dielectric constant from the very high value of 78 in the bulk aqueous to a very low value of 2 in the lipid bilayer core, with a gradient of dielectric constant spanning these two regions (19). Similarly, there is a gradient of dipolarity in the headgroup region of the lipid bilayer. This complex environment is probed in terms of the dipolarity in Chapter 2 by solvatochromic studies. The same sort of gradient in dipolarity is seen with probes that reside in different depths of the headgroup region.

The lipid bilayer interfacial region can be manipulated due to the variation of surface charge density. This can be adjusted, for example, through the selection of phospholipid type (charge state and molar ratio), temperature, and cholesterol incorporation. Characterizing the interfacial region is essential for drug partitioning studies. Drugs will interact primarily with the headgroup region of the lipid bilayer, or penetrate only partially into the lipid bilayer.
**Liposome Electrokinetic Chromatography**

Liposome Electrokinetic Chromatography (LEK C) is a capillary electrophoresis (CE) technique that uses phospholipid vesicles as a pseudostationary phase to provide sites of interaction for solutes of varying properties. Determining distribution coefficients for the binding of charged solutes to liposomes can be accomplished via LEKC much in the same manner as it is done for micelles via Micellar Electrokinetic Chromatography (MEKC) (25,26). The exception is that liposomes constitute the pseudostationary phase instead of micelles. In EKC, solutes interact with certain sites on the pseudostationary phase and are separated based on their differential partitioning into the phase. LEKC has previously been shown as a simple approach to determining liposome - water partition coefficients for a series of organic solutes and drugs (23,27). LEKC is the method of choice in this work for probing drug – liposome interactions.

The retention factor, k, for neutral solutes is calculated from the LEKC data using the retention times, including the retention time of the solute, the retention time of the electroosmotic flow marker, and the retention time of the liposomes. The migration of ionizable solutes in LEKC is much more complicated than those of neutral compounds. The retention behavior of charged drugs must be determined from a combination of LEKC and capillary zone electrophoresis (CZE) techniques. In addition to partitioning into the liposomes, these compounds will possess their own electrophoretic mobility due to their charge.
The retention of a cationic solute is shown schematically in Figure 1-2, where $\mu_{EOF}$ marks the electroosmotic flow, $\mu_{BH^+}$ is the mobility of the charged form of the solute, and $\mu_{lip}$ is the mobility of the liposomes as marked by the mobility of decanophenone. The arrows indicate the direction of mobility. The liposomes are shown with a net negative charge, and therefore will have a negative mobility with the voltage applied as shown in Figure 1-2. $K_{lip,B}$ and $K_{lip,BH^+}$ are the partition and distribution coefficients of the neutral and charged drug forms, B and BH$^+$, respectively. Both of these forms will interact with the liposomes to a different extent.

In LEKC, the observed mobility of a cationic solute will be the weighted average of the mobility of the solute in the liposome phase and in the aqueous phase. The uncharged form, B, has zero mobility in the aqueous phase, and only has mobility when associated with the vesicle (when it will then travel at the liposome mobility). The charged form, BH$^+$ has its own positive electrophoretic mobility in the aqueous phase and has the mobility of the liposome when associated with the lipid bilayer phase. The mobility and association with the liposomes as a function of drug ionization are discussed in Chapter 5.

Advantages and Limitations of LEKC

Drug - membrane interactions are a crucial step to evaluate during the development of new pharmaceutically active compounds. As a result, numerous methods have been developed for studying drug - membrane interactions using liposomes or immobilized lipid bilayers (28), a few of which are discussed in the following paragraphs.
Equilibrium dialysis is a traditional method used in the determination of liposome-water distribution coefficients (29,30). In equilibrium dialysis experiments, a liposome solution is separated from a pure aqueous phase by a semi-permeable membrane that allows the passage of the drug but retains the liposome. The distribution coefficient is then calculated from the drug concentration in both compartments after equilibrium is reached (31).

A pH-metric technique based on a two-phase potentiometric titration is also frequently used in the determination of distribution coefficients (32,33), specifically of charged solutes. As a result of the potentiometric titration, the apparent ionization constant of a solute in the presence or absence of liposomes is used to calculate the distribution coefficient between the liposome and water phases. However, these techniques (equilibrium dialysis and pH-metric titration) are time-consuming and not useful for high throughput applications. In addition they require large solute concentrations and a high level of purity.

There are many advantages to developing chromatographic methods for modeling drug partitioning. Chromatographic methods for measuring or predicting liposome-water partition coefficients include Immobilized Artificial Membranes (IAMs), Immobilized Liposome Chromatography (ILC), both HPLC techniques, and of course LEKC, a CE method.

IAMs are monolayers of phospholipid analogs covalently bonded to the surface of silica particles. IAM has been used in the study of drug-membrane interactions and has been used to predict liposome-water partition coefficients (34). IAM has successfully been used in
studies for the evaluation of blood brain barrier (BBB) permeability (35,36), Caco-2 permeability (37), and intestinal absorption (34).

Using chromatographic methods to study the interaction of charged solutes or drugs with liposomes or immobilized lipids has many significant advantages. Compared to the titration and dialysis methods discussed above, chromatographic methods only require a very small sample size, at a very low concentration. This is an advantage in drug discovery when only small amounts of drug may be synthesized in early stages of drug development. Additionally, no measurement of solute concentration is required, because the only measurement needed is migration times. Solute impurities can potentially be separated from the solute of interest, therefore not having the rigid purity requirements of the non-chromatographic methods. Finally, these technique allows the simultaneous measurement of multiple solutes (i.e. injecting mixtures of solutes in CE or HPLC. This rapid analysis is also aided in LEKC by the very short run time. The main feature in LEKC for high-throughput analysis is the possibility of running parallel analyses using multiple capillaries, such as with a commercially available 96-capillary CE system. None of the other techniques has this capability.

IAM has many of the same advantages as LEKC; however, there are a few significant differences between the two systems. IAM uses covalently bonded monolayers of lipids as opposed to the fluid lipid bilayers that are found in cell membranes (and liposomes). In addition, changing liposome compositions is easier and less costly in LEKC. IAM requires a separate column for each composition investigated, while LEKC simply involves rinsing the capillary with the new bilayer solution.
An important advantage in using LEKC (compared to IAM) for determining partition coefficients concerns the phase ratio ($\Phi$). The retention factor in LEKC is directly proportional to the partition/distribution coefficient ($K_{lw}$) as $k = K_{lw} \Phi$. Similarly, the retention factor in IAM is directly proportional to the partition/distribution coefficient. The phase ratio in IAM is difficult to determine, varies from column to column, and for a given column, will change over time. On the other hand, the phase ratio in LEKC is constant for a given composition of liposomes and buffer. Phase ratio in LEKC does not vary between capillaries or from lab to lab, therefore allowing the determination of a consistent and reproducible scale of partition coefficients which can be transferred or compared between labs. This is not possible with IAM due to the difficulties inherent in the phase ratio.

Some of the problems that arise in using LEKC for the determination of partition/distribution coefficients include difficulty with very hydrophobic drugs. In LEKC, there is a defined retention window identified by the $t_{co}$ and $t_{lip}$ markers. Some of the more hydrophobic drugs which have a large interaction with the liposomes will elute very close to the $t_{lip}$ marker resulting in a large amount of error or even coelute with the $t_{lip}$ peak.

**Liposomes in QSAR Studies**

Liposomes are an excellent tool in Quantitative Structure Activity Relationship (QSAR) studies due to their fluid lipid bilayer phase, which mimics a biological cell membrane. Liposomes have successfully been used in various QSAR studies. Rogers et al. determined QSARs using liposome partition coefficients of $\beta$-adrenoceptor blocking agents and their
pharmacokinetic properties and corneal penetrations (38). The authors have also used the apparent partition coefficients of a series of imidazoline $\alpha$-adrenoceptor agonists in liposome-buffer systems in QSAR studies looking at biological activities (hypertensive and hypotensive activities) and $\alpha$-adrenoceptor receptor binding affinities (39). Additionally, Rogers et al. used the partitioning into liposomes of a series of nitroimidazoles in QSAR studies with pharmacokinetic parameters (40). Liposomes either immobilized or in solution, have been used in studies correlating liposome partitioning with human passive intestinal absorption (41-43) and Caco-2 permeability (44).

In this work, liposomes are used for partitioning studies to gain insight into the process of drug interactions with model cell membranes. An ongoing area of work in this laboratory has been to identify more biologically relevant models to study this partitioning. This model is found in liposomes. Liposomes have shown better correlations with percent oral absorption and permeability in QSAR studies compared with micelle and microemulsion systems (45).

**Intestinal Absorption and Membrane Permeability**

Partitioning into lipid bilayers is generally considered the first step in membrane permeability, specifically the passive transcellular transport through membranes. This partitioning (K) and permeability (P) is shown schematically in Figure 1-3. In order for the drug to pass through the membrane, the first step is an initial partitioning into the membrane, or interaction with the membrane. This initial interaction is followed by a passive diffusion through the lipid bilayer, and finally a partitioning out of the membrane on the other side of
Drug transport across membranes is given by the membrane permeability coefficient, $P_m$. $P_m$ is linearly related to the membrane partition coefficient, $K_m$ according to Equation 1-1, where $D_m$ is the diffusion coefficient and $L$ is the thickness of the lipid bilayer membrane.

$$P_m = \frac{D_m K_m}{L}$$

Due to the ease of administration and patient compliance, the oral route is the preferred method of drug administration. Once a drug is administered orally, it must travel through the digestive tract and possess adequate solubility properties. Approximately 90% of all absorption in the gastrointestinal tract occurs in the small intestine region (19). A layer of epithelial cells separate the lumen of the small intestine from the circulatory system. Once a drug is solubilized in the stomach contents, it must cross the intestinal epithelium (i.e. uptake of the drug through the cell membrane). Drugs are absorbed through the epithelial cell layers by diffusion across the lipid bilayer of the cell membrane (transcellular diffusion). Additionally drugs can be transported by other mechanisms including paracellular or active transport by membrane proteins.

Cell lines such as Caco-2, a human colon adenocarcinoma, and Madin-Derby canine kidney (MDCK), a canine epithelial cell line, are used as models for studying intestinal epithelial transport (46-48). Typically, a monolayer of cells is grown on a membrane filter, which is mounted in diffusion chambers. The permeability of a drug through the membrane is determined after introducing the drug on one side of the filter and monitoring the rate of appearance of the drug in the receiver compartment. These cells are generally accepted as a
primary absorption - screening tool for pharmaceutical companies, a time consuming process that produces widely varying results.

Favorable permeability and absorption characteristics are essential to the success and efficacy of a drug. Therefore these properties must be carefully screened during all stages of drug discovery. Due to the disadvantages mentioned above, and the significant inter- and intra- laboratory variability of some of the methods, simple in- vivo models are desired to aid in the process. Developing simple biomembrane models composed of phospholipid vesicles which model the Caco-2 or MDCK cell lines are therefore quite advantageous when investigating drug partitioning in relation to drug transport. Liposome Electrokinetic Chromatography (LEKC), as explored in this work, can be developed as a screening method for liposome - water partition coefficients which then can be applied in QSAR studies.

One of the major trouble areas in drug discovery is the profiling in the early discovery stages of a drug candidate's physicochemical and physiological properties including lipophilicity, absorption, permeability, solubility, etc. (49). Large numbers of drug candidates are produced in combinatorial chemistry libraries. The problem arises when it comes to screening the important properties of these potential drug candidates. As a result, it is widely recognized that there is a need for better, faster, and easier methods for an early screening of these properties to enhance drug success rates.

LEKC has the potential to be a great tool for QSAR applications if applied early in drug discovery in combinatorial chemistry laboratories for the screening of the lipophilicity of large numbers of potential drug compounds. The liposome - water partition coefficient can also be used in QSAR studies with human oral intestinal absorption and cell membrane
permeability. This approach can be used to detect drugs that are likely to pose problems in these areas in later stages of drug development. The potential application of LEKC in these areas is discussed in Chapter 5. In addition to the many advantages of LEKC methods discussed above, the use of a commercially available 96 capillary CE system would provide a significant increase in the high-throughput capabilities of the method.
Figure 1-1. Fraction of a lipid bilayer containing PG, PC, and Cholesterol.
**Figure 1-2.** Diagram of a basic solute interacting with a liposome using LEKC. $\mu_{EOF}$ marks the electroosmotic flow marker, $\mu_{BH^+}$ is the mobility of the cationic solute, and $\mu_{lip}$ is the mobility of the liposomes as marked by the mobility of decanophenone. The mobility arrows mark the direction of the mobility. $K_{lip,B}$ and $K_{lip,BH^+}$ are the partition and distribution coefficients of the neutral and charged drug forms, $B$ and $BH^+$, respectively.
**Figure 1-3.** Diagram of partitioning (K) versus permeability (P).
References


Chapter 2

Characterization of Small Unilamellar Vesicles Using
Solvatochromic π* Indicators and Particle Sizing

Abstract

A suite of small unilamellar vesicles (SUVs) composed of mixtures of phospholipids and cholesterol or the synthetic surfactant dihexadecyl phosphate (DHP) and cholesterol was investigated using a homologous series of solvatochromic $\pi^*$ indicators coupled with size exclusion methods and photon correlation spectroscopy (PCS). The solvatochromic method, which is based on the measurement of solvent-dependent shifts in $\lambda_{\text{max}}$ from UV-Vis spectra of solubilized indicators, was used to quantify the dipolarity and polarizability ($\pi^*$) of probe solvation environments. The partitioning of the series of individual di-n-alkyl-$p$-nitroaniline $\pi^*$ indicators in PG$_{24}$PC$_{46}$Chol$_{30}$ and DHP$_{70}$Chol$_{30}$ SUVs was examined as a function of the head group structure as well as the method of dye-vesicle preparation. Solubilization of the larger more hydrophobic probes in the bilayer portion of PG$_{24}$PC$_{46}$Chol$_{30}$ SUVs was aided through physical entrapment. Such physical methods were not needed for the smaller indicators or for the range of indicators in the DHP$_{70}$Chol$_{30}$ dispersions. Extrusion and size-exclusion chromatographic methodologies for the preparation of physically entrapped dopants in SUVs of fixed size range demonstrated that the larger (longer alkyl chain) dopants in the series resided in PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes with a wider range of sizes, while the smaller more polar solutes tended to be entrapped in smaller vesicles with a narrower size range.
**Introduction**

Small unilamellar vesicles (SUVs) are comprised of phospholipids and cholesterol, which assemble in aqueous solution due to hydrophobic interactions among the individual lipid and cholesterol molecules. The double chain hydrophobic tails of the phospholipids line up on the inside of the bilayer leaving the polar head groups along the outside. The resulting bilayer with an encapsulated inner aqueous cavity is called a liposome. Other SUVs may be comprised of synthetic double chain surfactants, which also form vesicles (1).

Liposomes are often used as models of biological membranes (2,3). In pharmaceutical applications, they can be made to entrap drugs having a wide range of lipophilicities, thus creating powerful tools for drug delivery. In particular, drugs can be incorporated into the liposomes either in the phospholipid bilayer, entrapped within the liposome in the aqueous core, or they may reside at the outer bilayer - solution interface. The location and solvation environment of drugs in these systems is made more complex by the fact that vesicle dispersions consist of a distribution of different sized aggregates, which may have a differing affinity for organic solutes. Optimizing the use of liposomes as encapsulation systems or model membranes necessitates not only a detailed characterization of liposome and bilayer structure and permeability, but also an understanding of the affinity of drugs of different size and polarity for different sized vesicles.
Probing Microenvironments in Micelle and Bilayer Systems

There are several methods that can be used to examine the polarity of bilayers and micelles. Electron Spin Resonance (ESR) has been applied to the study of hydration in lipid bilayers (4), and to the location of polarity-indicating probes within a bilayer. Steady-state and time-resolved fluorescence experiments have been used to investigate the polarity of bilayers of egg phosphatidylcholine (5), and to study the interfacial polarity of other phospholipid vesicles based on Stokes shifts (6). Different types of polarity-sensitive molecular probes have also been applied. For example, dimerizing cationic dyes have been used to study phospholipid membranes and surfactant micelles by means of their absorption spectra (7). In particular, UV-Vis spectral features resulting from inter- and intramolecular charge transfer interactions with solubilized reagents were used as polarity-indicating parameters to investigate the polarity and microenvironment of vesicles (8). Thermally induced variations in the polarity of phospholipids and surfactant vesicles also have been monitored using a probe that forms an intermolecular charge-transfer complex (9).

Application of Solvatochromic Dyes

Probe molecules whose UV-Vis absorption bands exhibit shifts in response to changes in solvent polarity are termed solvatochromic. Several solvent polarity scales have been developed on the basis of this phenomenon, allowing various polarity-related parameters to be estimated for a variety of systems including those containing micelles and bilayers.
The solvatochromic comparison method, developed by Kamlet, Abboud, and Taft (10-12) allows the determination of specific solvent polarity parameters corresponding to dipolarity - polarizability ($\pi^*$) (10,13), hydrogen - bond donor (HBD) acidity ($\alpha$) (11) and hydrogen - bond acceptor (HBA) basicity ($\beta$) (12). The $\pi^*$ parameter, which is the subject of this work, describes the non-hydrogen bonding contribution to solvent polarity. In particular, $\pi^*$ can be used to characterize the non-specific portion of van der Waals interactions for a solvation medium (10,13).

Values of $\pi^*$ are calculated from the relative positions of UV-Vis absorption bands of selected solvatochromic indicators, and are related to the energy of transition (or frequency of maximum absorption) through the following linear solvation energy relationship (LSER).

\begin{equation}
\nu_{\text{max}} = \nu_0 + s\pi^*
\end{equation}

In Equation 2-1, $\nu_{\text{max}}$ is the frequency of maximum absorption ($10^3 \text{ cm}^{-1}$) for the indicator in the solution of interest and $\nu_0$ is the frequency of maximum absorption of the indicator in cyclohexane. The $s$ term reflects the magnitude of the spectral shift of the indicator between two reference solvents, cyclohexane and DMSO. These solvents have $\pi^*$ values of 0.00 and 1.00, respectively (10). Individual $\pi^*$ indicators may be ranked according to their -s values (10) to give an idea of the relative spectral shifting capability of the probe molecules. Indicators with larger values of -s (e.g. in the range of 2.8 - 3.5) are better able to resolve small differences in polarity that may be associated with heterogeneous bilayer containing systems.
The attractiveness of the solvatochromic comparison method lies in its ability to resolve the different contributions of $\alpha$, $\beta$ and $\pi^*$ to the total polarity of a system. To that end, the LSER in Equation 2-1 could be expanded to include the additional terms $\alpha$ and $\beta$ which could be obtained from indicators that respond to both hydrogen bonding and solvent dipolar effects. The determination of these latter parameters has been previously described (10-12). The significance of using the $\pi^*$ scale alone, as in this work, is that a single indicator may be used to obtain specific polarity related information about a system. For the case of a complex solution (e.g. containing vesicles or liposomes) having multiple solubilization sites, a suite of indicators with similar solvatochromic properties (i.e. -s) but variable sorption capability is desirable. In this work, we will examine the hypothesis that a homologous series of seven N,N-dialkyl-\(p\)-nitroaniline $\pi^*$ indicators (of varying size and hydrophobicity) (Table 2-1) (10,14-16) may be used in a comparative manner to better probe solvation sites in solutions of vesicles and liposomes.

The Polydispersity of Vesicle Containing Systems

The complexity of vesicle dispersions suggests that the use of molecular probes alone may not be sufficient for characterizing the solvating properties of those systems, and that information from these probes (e.g. $\pi^*$ values) may be more fully interpreted when complementary techniques are applied. Previous applications of the di-n-alkyl-\(p\)-nitroaniline (DNAP) indicators to micellar systems have suggested that the size of the indicator relative to the size and structure of the micelle is important for optimizing indicator probing
capability (15). Where liposome containing systems are known to be polydisperse (17), methods that can fractionate the particles with respect to aggregate size may reveal how the different indicators (Table 2-1) are dispersed within the system.

In this work, size exclusion column chromatography (SEC) and photon correlation spectroscopy (PCS) were applied to the characterization of the vesicle dispersions used in the solvatochromic studies. By examining average particle size as a function of aggregate structure and probe solvation environment ($\pi^*$), we can learn about the solvation characteristics of SUVs in a distribution of aggregates.

**Materials and Methods**

**Reagents**

N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] and N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], sodium salt (HEPES buffer) were purchased from Sigma. Tris[hydroxymethyl]aminomethane (TRIS buffer) was purchased from Acros Organics. Cholesterol (Chol), L-α-phosphatidylcholine dipalmitoyl (C16:0), (PC); L-α-Phosphatidic Acid, dipalmitoyl (C16:0), (PA) and L-α-Phosphatidyl Serine, dipalmitoyl (C16:0), (PS) were obtained from Sigma. 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (PG) was from Avanti Polar Lipids, Inc. Dihexadecyl phosphate (DHP) was purchased from Aldrich Chem. Co. Sepharose CL-4B was purchased from Fluka. The indicators N,N-dimethyl-p-nitroaniline, N,N-diethyl-p-
nitroaniline were obtained from Pfaltz & Bauer and Frinton Laboratories, respectively. Synthesis of the indicators N,N-dipropyl-p-nitroaniline, N,N-dibutyl-p-nitroaniline, N,N-dipentyl-p-nitroaniline, N,N-dioctyl-p-nitroaniline, and N,N-didecyl-p-nitroaniline has been previously described (14-16). The structures and parameters \( (v_0 \text{ and } -s) \) for the indicators are given in Table 2-1 (10,14,18).

**Vesicle Preparation**

Headgroup structures for the surfactants and phospholipids are given in Table 2-2. The phospholipid molar ratios that were examined are PC\(_{70}\)Chol\(_{30}\), PG\(_{10}\)PC\(_{60}\)Chol\(_{30}\), PG\(_{24}\)PC\(_{46}\)Chol\(_{30}\), PG\(_{35}\)PC\(_{35}\)Chol\(_{30}\), PG\(_{70}\)Chol\(_{30}\), PS\(_{24}\)PC\(_{46}\)Chol\(_{30}\), PA\(_{24}\)PC\(_{46}\)Chol\(_{30}\), where the subscripts represent the molar percentage of the individual lipid and cholesterol components. The procedure for preparation of the liposomes is illustrated by the preparation of 6 mM PC\(_{70}\)Chol\(_{30}\) as follows: a 7:3 molar ratio mixture of L-\(\alpha\)-phosphatidylcholine dipalmitoyl and cholesterol was dissolved in a 9:1 volume mixture of chloroform and methanol (respectively). The organic solvent was removed under reduced pressure using a rotary evaporator in a water bath maintained at 70°C. The thin lipid film was hydrated with 8 mL of 25 mM HEPES buffer, pH 7.5. For liposomes prepared according to the sonication method, the hydrated vesicles were sonicated for 30 minutes with a titanium - tipped probe sonicator in a temperature - controlled water bath maintained at 70°C. The total sonication time was 30 minutes, with 3 seconds of sonication followed by 2 seconds of pause. For vesicles prepared with DHP/Chol, 8 mL of a 50 mM Tris buffer, pH 9.0 was added to the appropriate amount.
of DHP and cholesterol and the SUVs were formed according to the same sonication procedure. Sonicated vesicle preparations were equilibrated to ambient temperature and passed through a 0.45 µm filter prior to use.

For liposomes prepared according to the extrusion method, the hydrated vesicles were subjected to a freeze - thaw procedure five times. This procedure involved freezing the vesicles by immersion in liquid nitrogen, followed by thawing the solution in 70°C water. The vesicles were then processed to unilamellar vesicles by extrusion through double - stacked 200, 100, and 50 nm polycarbonate membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada) maintained at a temperature of 60°C by a circulating water bath. The vesicles were extruded through the 200 and 100 nm filters a total of five times and ten times through the 50 nm filters.

**Preparation of Probe Containing Vesicles and Liposomes**

Liposome solutions containing an indicator were prepared by adding a small amount of the probe dissolved in the chloroform and methanol mixture to the phospholipids and cholesterol and then further diluted with the chloroform and methanol mixture. The rest of the procedure for liposome preparation was the same. In all cases, the indicator was added to give a concentration such that the measured absorbance was about 0.5 (in the range of 10\(^{-5}\) - 10\(^{-4}\) M, approximately), a concentration which, for practical purposes, represents infinite dilution of the dopant.
In some cases, liposomes were completely prepared according to the previous method, without adding any of the indicators dissolved in chloroform and methanol. Instead, a small amount of the probe dispersed in the aqueous buffer was added to the preformed liposome suspension following sonication and filtering. This method was employed for experiments in which the effect of the method of preparation was examined. In this latter approach, the probe was allowed to equilibrate with the liposome from the adjacent solution. In all manners of dye-liposome preparation, UV-Vis wavelength of maximum absorption ($\nu_{\text{max}}$) for individual probe dyes did not change from 1 hour to 1 day following the addition of the probe.

*Spectroscopic Methods*

UV-Vis spectra of the liposome solutions were measured both before and after fractionation using the Sepharose CL-4B column. All spectra were collected using a Hewlett Packard 8453 spectrophotometer equipped with a thermostated cell holder. The temperature was maintained at 25.0°C, using a Fisher Scientific Isotemp Refrigerated Circulator (Model 90), which is below the phase transition temperature for the vesicle preparations. The gel-to-liquid crystalline phase transition temperature of the DHP is around 45°C (19), and approximately 41°C for the liposomes (20). Separate spectra were taken of the background solvent and of the dye-solvent system. For the liposomes, the background solution was the liposome solution without any added indicator. The spectral data were digitized and imported into an Excel spreadsheet. Background spectra were directly subtracted from that of the dye.
containing solutions. \( \lambda_{\text{max}} \) values were taken as the midpoint between the two positions where the absorbance is 90% of the maximum absorbance, according to the method of Kamlet, Abboud and Taft (10). This was done to reduce the effects of spectral anomalies in the determination of \( \lambda_{\text{max}} \).

For an indicator to be usable (i.e. for calculation of \( \pi^* \)) a value of \( -s \) had to be estimated for the indicator. The value was estimated using a seven solvent LSER (Equation 2-1) in accordance with the methods of Kamlet et. al. (10). The solvents used for determination of \( v_0 \) and \( -s \) were cyclohexane, DMSO, n-heptane, 1-methyl-2-pyrrolidinone, trichloroethylene, carbon tetrachloride, and pyridine. Other previously determined \( -s \) values were repeated so as to obtain a set of values for the indicators that would be internally consistent. Table 2-1 lists available \( -s \) and \( v_0 \) values for 1a-g plus those that were repeated and/or estimated here. All values of \( \pi^* \) were calculated from Equation 2-1 using the pre-determined values of \( -s \) for the indicator in question.

\[ \text{Size Exclusion Methods} \]

The liposomes were prepared according to the previously mentioned sonication method where the indicator is dispersed with the phospholipids and cholesterol prior to sonication. For experiments involving fractionation of an SUV dispersion, a small amount of the liposome preparation was added to a Sepharose CL-4B column (2.5 x 30 cm) at room temperature and eluted using 25 mM HEPES buffer, pH 7.5. Fractions that were
approximately 6 mL (in volume) were collected to obtain a narrow size distribution of vesicles. An absorbance spectrum was measured for each fraction. Liposome size distributions were determined (via PCS) for each fraction. This process was performed a total of three times for each indicator reported to obtain an average size and π* value for each individual fraction.

**Determination of Liposome Size**

Average sizes of the liposome dispersions were determined using Photon Correlation Spectroscopy (PCS) using a Zetasizer 1000HS (Malvern Instruments Ltd, Malvern UK) with a 5 mW Helium Neon laser at 633 nm. The scattered light was collected at an angle of 90°. All measurements were made at 25°C. The Malvern PCS software algorithm chosen to analyze the data was Contin and the size distribution profiles were analyzed using the method of volume. Average vesicle sizes were obtained from at least three repeat measurements of the mean diameter of the vesicle - probe system.

**Results and Discussion**

The results of this work build on previous studies of micelles involving DNAP π* indicators (15) and on recent LSER studies of micelles and vesicles (21-24), where the latter provides a framework for understanding the solute - solvent interactions that govern probe solvation and partitioning in phospholipid bilayers. In this section, we report on the behavior
of 1a-g in vesicles of different size, composition, and head group structure. Some key findings are the importance of the size of the probe relative to the size and composition of the vesicles being probed, and the notion that the manner in which vesicles are prepared affects the solvation microenvironment of the captured molecule.

Partitioning of DNAP Indicators into PG$_{24}$PC$_{46}$Chol$_{30}$ Liposomes

Figure 2-1 illustrates $\pi^*$ values calculated from UV-Vis spectra of 1a-g solubilized in dispersions of PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes, where the data are shown as a function of lipid concentration. These probes were incorporated by sonication of the probe with the lipids during the formation of the liposomes.

The most noticeable feature of the plot is that as the probes become more hydrophobic, moving from 1a through 1g, the measured $\pi^*$ values decrease. This is true at all concentrations, however most noticeable at 6 mM, where 1a has the highest value of 1.16 ($\pm$ 0.02) compared to the lowest value of 0.50 ($\pm$ 0.03) for 1g. The decrease in $\pi^*$ values indicates the probes are residing in a less polar environment in the interfacial region of the liposomes. The increase in partitioning with increasing hydrophobic character of the dye is supported by partition coefficients that were measured for dyes 1a-d in the PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes; Log $K_{LW} = 2.14 - 3.12$ for 1a-d, respectively. These Log $K_{LW}$ values were determined using Liposome Electrokinetic Chromatography (LEKC) according to the procedure outlined in reference 24.
The $\pi^*$ values for $1f$ and $1g$ are on the order of the average measured $\pi^*$ value determined using all eight indicators for octanol or octanol saturated with water, with values of 0.55 ($\pm$ 0.02) and 0.58 ($\pm$ 0.02), respectively. $1g$ resides in a less polar region of the liposomes, in an environment of similar dipolarity as octanol. This is significant since octanol - water partitioning is the traditional system used for studying drug partitioning.

An important feature of Figure 2-1 is that for the two shorter alkyl chain dyes ($1a$-$b$), $\pi^*$ decreases markedly with increasing lipid concentration, suggesting that not all of the probe was equilibrated with the bilayer at the lower lipid concentrations (6 and 15 mM). The measured $\pi^*$ is a composite value for dye molecules that are in equilibrium between the bilayer and the adjacent solution. With increasing liposome concentration, the equilibrium shifts such that a larger fraction of the probe is partitioned into the vesicle resulting in a lower apparent $\pi^*$. For example, the smallest indicator ($1a$) exhibited a $\pi^*$ of 1.16 ($\pm$ 0.02) for the 6 mM PG$_{24}$PC$_{46}$Chol$_{30}$ dispersion, implying that the probe resided largely in the adjacent aqueous phase ($\pi^* = 1.09$ for water; Kamlet et al. (10)). Small moderately polar molecules (or short chain indicators) have previously been found to associate with or bind to bilayers predominately at the water - amphiphile interface (25). The $\pi^*$ (for $1a$) at 30 mM PG$_{24}$PC$_{46}$Chol$_{30}$ was 0.90 ($\pm$ 0.01). A shift in $\pi^*$ with increasing vesicle concentration also was seen for $1b$ ($\Delta\pi^* = 0.13$). For the largest (most hydrophobic) indicators $1d$-$g$, values of $\pi^*$ were lower ($e.g.$ 0.69 $\pm$ 0.03 and 0.50 $\pm$ 0.03, respectively) and essentially constant, across the range of lipid concentrations, implying a relatively high affinity between these probes and the PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes, and an equilibrium that was well shifted towards the bound
form. The slight increases in $\pi^*$ from 15 to 30 mM in Figure 2-1 is still within the error of the measurements.

There are two methods of preparing the dye - liposome suspensions, (1) by adding the individual dyes with the phospholipids during SUV preparation process, a physical mode of encapsulation, and (2) by adding the individual indicator to the preformed vesicle dispersion. The trends in behavior begin to deviate where both methods of dye - liposome preparation are considered.

Figure 2-2 shows the difference in $\pi^*$ values between the two preparation methods for 1a-g at a given lipid concentration of 15 mM. 1a-e show no difference between the two methods since the probe resides primarily in the aqueous phase or in the outer headgroup region of the liposomes. For the smaller dye molecules (1a and 1b) there were no differences between the two preparation methods for 6 and 30 mM liposome concentrations as well. The larger probes did not exhibit such similarity in behavior. 1d and 1e show lower $\pi^*$ values when the probe is added to the preformed dispersions while 1f and 1g show a different trend of having much higher $\pi^*$ values when added to the preformed liposome dispersions, according to Figure 2-2. These trends are seen also with 6 and 30 mM liposome concentrations.

The $\pi^*$ values for 1g under the conditions of requiring the probe to partition without sonication imply a relatively polar solvation environment, ($\pi^* = 0.89 \pm 0.01$) and that the indicator is solvated largely in the outer polar portions of the liposomes. Yet where 1g is incorporated during the SUV formation process (i.e. via sonication), the probe equilibrates to
a much more nonpolar solvation environment. π* values for 1g across the range of lipid concentrations (6 - 30 mM) are constant for both preparation methods and suggest that when the dye is added to the preformed vesicle dispersion, the probe’s bulky character and long alkyl chains prevent it from partitioning very deeply into the bilayer (i.e. from the adjacent solution).

These results may be explained in terms of known mechanisms of vesicle formation (26). During sonication (or other physical encapsulation methods such as extrusion), the initially formed multilamellar vesicles (MLVs) are disrupted by physical forces. The resulting phospholipid fragments find an intermediate configuration as SUVs, which are more stable (lower energy) than the fragments, though less stable over the long term than MLVs, which are the lowest energy arrangement for the aqueous lipid dispersion (26). The reorganization following sonication enables the long alkyl chain indicators (1f-g) to more easily align with the hydrocarbon component of the PC and PG phospholipid chains, a process that is enhanced with heating of the dispersion during sonication. The probes are most likely arranged such that the aromatic headgroup is aligned with the phospholipid headgroup, and the probe chains are oriented inward positioned between the phospholipid chains. A longer chain probe would simply pull the molecule further into the bilayer region. This is accomplished by the energy added during the sonication and formation of the liposomes. As a result, favorable sorption sites that are inaccessible (to the larger solute) once the SUV is formed become accessible when the dye is added during the SUV formation process.
Solute partitioning into interfacial regions such as lipid bilayers requires a rearrangement of the alkyl chains for the creation of a cavity within the bilayer that is proportional to the solute size. This process is energetically unfavorable because it would require entropic energy. Thus, Dill discussed the partitioning of solutes into lipid bilayers and the expulsion of the solute due to entropic effects based on the ordering among the phospholipid chains (27-29). This “entropic expulsion” is more pronounced as the solute size increases, or as the ordering of the bilayer increases. For the partitioning of a very long chain probe (1f-g) that would typically reside in a more ordered region of the bilayer, this “entropic expulsion” could cause the probe to sit flat or absorb at the interface region. A probe sitting at the surface of the liposome would result in a much higher $\pi^*$ value than one which is embedded in the lipid bilayer.

**Effect of Headgroup Structure**

The role of phospholipid headgroup structure is also important for understanding solute partitioning and the behavior of solvatochromic DNAP indicators in SUVs. As DNAP indicators are polar molecules, the more polar outer portions of the bilayer can be expected to be the primary sorption site for these dopants (i.e. as opposed to the hydrocarbon region). Previous studies of nitroaniline solvatochromic dyes in micellar dispersions suggest that the nitro group is oriented towards the outer polar portion of a micelle (15, 30).

The significance of headgroup structure was explored in experiments involving liposomes prepared from varying ratios of different phosphatidyl phospholipids (Table 2-2).
In particular, we identified the solvatochromic dye(s) (within the DNAP series) whose level of hydrophobicity (e.g. alkyl chain length) allowed for maximum probing of headgroup structural differences in dispersions of fixed liposome concentration, i.e. the indicator environment will mostly reflect the polar portion of the liposome environment as opposed to the adjacent bulk solution.

Table 2-3 gives values of $\pi^*$ for 1a, 1c, 1e, and 1g over a range of SUV headgroup structures, where the liposomal dispersions were prepared by sonication of the probe with the liposomes and lipid concentrations were held constant at 6 mM. The headgroup region was varied by using different ratios of the phosphatidyl phospholipids in Table 2-2. For indicator 1a, $\pi^*$ appears relatively constant over the range of vesicle types indicating this dye cannot effectively probe the head group differences (for the experimental conditions as used). 1c and 1g show some variation with headgroup but not as much as 1e, the dipentyl-\textit{p}-nitroaniline. 1e is solvated in such a way as to be most sensitive to the structural variation in the outer polar component of the bilayer. Values of $\pi^*$ obtained from spectra of 1e, across the range of vesicle types, are sensitive to the ratio of PG to PC, where $\pi^*$ increases with increasing $[\text{PG}]/[\text{PC}]$.

For equal molar proportions of PS, PA, and PG in the vesicle composition, the inclusion of PS results in greater dipolarity of the headgroup solvation environment as probed by 1e and the inclusion of PA results in the slightly lower dipolarity of the headgroup region. Since PS, PG, and PA all have the same net charge, this difference in $\pi^*$ could be due to the differences in headgroup size of the lipids. PA has the smallest headgroup, which could allow
the probe molecules to sit deeper in the headgroup region compared with the bulkier PS and PG headgroups. LSER models showed that changing the liposome composition in the same manner showed little variation in the system coefficient representing the dipolarity of the liposomes (31). The advantage to using this homologous series of solvatochromic dipolarity indicators is the ability to probe various depths of the headgroup region to better detect differences in liposome dipolarity as seen by probe 1e.

The partitioning of solutes into the outer polar portion of the bilayer may also be affected by the magnitude of charge on the outer functional groups and extent of headgroup repulsion (32). In this work we prepared 15 mM vesicle dispersions of dihexadecylphosphate (DHP) and cholesterol (CH) (e.g. DHP\textsubscript{70}Chol\textsubscript{30}) at pH 9.0 in addition to the PG\textsubscript{24}PC\textsubscript{46}Chol\textsubscript{30} phospholipid liposomes (also 15 mM) buffered at pH 7.5. Note that both types of SUVs contained the same amount of cholesterol, a component known to affect bilayer rigidity and permeability (33).

As with the PG\textsubscript{24}PC\textsubscript{46}Chol\textsubscript{30} liposomes, dispersions of DHP\textsubscript{70}Chol\textsubscript{30} were prepared with indicators 1a-g using the two previously described methods, i.e. (1) physical encapsulation via sonication, and (2) addition of individual dyes to preformed SUVs. Figure 2-3 shows $\pi^*$ values as a function of the preparation method for indicators in the DHP\textsubscript{70}Chol\textsubscript{30} dispersions.

The DHP\textsubscript{70}Chol\textsubscript{30} vesicles have a significantly higher $\pi^*$ than the liposomes as probed by 1a-c, reaching a highest $\pi^*$ value of 1.25 for probe 1a. For probes 1d and 1e, the probes are solubilized in similar environments in the PG\textsubscript{24}PC\textsubscript{46}Chol\textsubscript{30} liposomes and the
DHP$_{70}$Chol$_{30}$ vesicles, indicated by similar $\pi^*$ values. For probes 1f-g, the DHP containing vesicles have higher $\pi^*$ values than the liposomes.

A key aspect of Figure 2-3 is that the $\pi^*$ values (black lines for the physically encapsulated indicators; gray lines for dyes added to the preformed dispersion) are very similar across the range of indicators regardless of the method of dye incorporation. This includes the larger probes, 1f and 1g. This is different than the results in the liposome dispersion (Figure 2-2), where $\pi^*$ differs significantly between the two incorporation methods. We suggest that for the DHP$_{70}$Chol$_{30}$ SUVs, the more open configuration of the headgroup (due to repulsion of negatively charged headgroups) relative to that of the PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes enables more effective solute partitioning regardless of the size and hydrophobicity of the dye and manner of dye incorporation. SUVs containing interacting charged moieties but with no net charge, such as the zwitterionic phosphatidylcholines (PC), have a tight packing of lipid molecules in the headgroup region and the vesicle as a whole is smaller and tighter (32).

The DHP$_{70}$Chol$_{30}$ vesicles would have a net negative charge on the outer phosphate groups resulting in repulsion of the headgroups on the bilayer and a more open, less tight structure (in the headgroup region). Such structure - dependent packing within the headgroup can affect the permeability to organic solutes in the adjacent outer solution. For the “tighter” structures where the probe is less able to pass through the headgroup on its own, inclusion of the solute during SUV preparation (i.e. via sonication) should enhance the ability of the dye to find more nonpolar sorption sites deeper in the vesicle. For the less tight structure, such
differences in preparation method would not affect the final solvation site of the probe due to
the greater ease with which the probe re-equilibrates within the bilayer. The lack of variation
in $\pi^*$ for DNAP indicators (among the two preparation methods) in the DHP$_{70}$Cho$_{30}$ vesicles
may be due to this latter phenomenon. This is also supported by the data comparing the $\pi^*$
values with various ratios of PG to PC, where the more tightly packed, net neutral liposomes
(32), PC$_{70}$Cho$_{30}$ have a much lower $\pi^*$ than the net negatively charged PG$_{70}$Cho$_{30}$
liposomes.

Solvatochromic Dyes and Particle Sizing

It has been shown thus far that probe partitioning studies in which different preparation
methods are considered (e.g. physical encapsulation vs. solubilization) can shed light on
factors affecting the indicator’s equilibrium solvation environment. For the PG$_{24}$PC$_{46}$Cho$_{30}$
liposomes, the use of physical encapsulation appears especially useful in solubilizing the
larger more hydrophobic DNAP indicators (1f-g). In this section, we examine the role of
vesicle size in that physical entrapment and sorption process.

The importance of solute size vs. size of the partition medium (e.g. the lipid aggregate)
has been emphasized in previous studies. For example, earlier investigations of 1a-e and the
di-n-hexyl-$p$-nitroaniline showed that the longer chain di-n-butyl and di-n-hexyl probes yield
lower $\pi^*$ values and deeper partitioning for larger micelles of the nonionic surfactant Triton
X-114 as compared to the smaller more tightly packed micelles of the anionic sodium
dodecylsulfate (SDS) (15). The importance of solute size also has been emphasized in LSER
studies of micelles and vesicles where it was found that the solvent cavity - formation term is an important contributor to the magnitude of measured retention factors \((k)\) in micellar, vesicular, and liposome electrokinetic chromatography (MEKC, VEKC, LEKC) (21-23); \(k\) is directly related to the liposome - water partition coefficient \((K_{LW})\). In this work we examine measured diameters (in nm) of PG24PC46Chol30 liposomes in relation to the size of the captured probe, where an important difference relative to the earlier surfactant studies is that the probes (in this study) were physically encapsulated into the vesicles.

Dye incorporated liposomes were prepared in three size ranges using two physical encapsulation methods (Table 2-4). Extrusion was used to prepare liposomes in the 50 and 100 nm diameter ranges. This method, which produces populations of liposomes with smaller polydispersity than sonication, permits selection of a liposome size range by the pore size of the extrusion filter (34). The membranes had pore diameters of 50 and 100 nm respectively. The liposome diameters (from both preparation methods) were further characterized by PCS (Table 2-4). Sonication followed by size exclusion chromatography was used to create fractions of dye - encapsulated PG24PC46Chol30 liposomes with average diameters ranging from 24.4 \((\pm 0.5)\) to 38.6 \((\pm 4.7)\) nm. We note that in all manners of preparation, there was no evidence of precipitation of the indicators, and the vesicle size (as determined by PCS) remained unchanged before and after the addition of the indicators.

Table 2-5 lists values of \(\pi^*\) for 1a-b, d, g incorporated in liposomes prepared via extrusion, where the average measured liposome diameters were 62.6 and 100.1 nm, respectively for vesicles prepared using the 50 and 100 nm pore size membranes. The
observation that liposomes extruded through the smaller pore sizes (50 nm pore size membranes) have a higher size than the membrane pore size has been explained by the method of vesicle formation by extrusion involving lipid aggregate fragmentation followed by reassembly into vesicles (35). 10 mM was the highest concentration of liposomes that were used with extruded liposomes. At higher liposome concentrations the baseline absorbance of the liposomes is too high resulting in distorted peaks and an inaccurate $\pi^*$ determination. If we compare $\pi^*$ values for the smaller probes 1a-b (as encapsulated in the extruded liposomes at 10 mM PG$_{24}$PC$_{46}$Chol$_{30}$, Table 2-5), we find that lower $\pi^*$ values are associated with the smaller of the two liposome sizes ($\pi^* = 0.95$ and 0.85 for 1a and 1b respectively in dispersions created with the 50 nm pore size membrane; $\pi^* = 1.09$ and 0.93 for 1a-b in liposomes prepared with the 100 nm extrusion filter). These results suggest that the smaller less hydrophobic indicators are in a slightly more polar microenvironment likely due to differences in vesicle curvature and water penetration in the smaller sized vesicles.

Conversely, the larger indicators 1d and 1g exhibited $\pi^*$ values that were more uniform across the two sizes of 10 mM liposomes ($\pi^* = 0.45$ and 0.44 for 1d in 50 and 100 nm liposomes respectively; $\pi^* = 0.51$ and 0.52 for 1g in 50 and 100 nm liposomes). The longer chain probes experienced a similar microenvironment across the two sizes of liposomes. These differences in the behavior of the short chain and long chain probes is in agreement with Hof et al.’s findings that the differences in lipid packing between large and small vesicles did not affect the fluorescent quenching deep inside the bilayer, but the differences in curvature plays a role in the interface region of the bilayer (36).
Such trends in dye - liposome association also were seen for liposomes in the 25 to 45 nm size range that were prepared via sonication. In this case, the resulting dye - vesicle dispersions were individually fractionated on a size exclusion column so as to obtain the indicator that was physically isolated by vesicles in a particular size range (as characterized by PCS). Values of $\pi^*$ were obtained spectroscopically (for the dye in each individual fraction) and plotted against the average size of the vesicles in which they were located (Figure 2-4). The range of liposome sizes for each encapsulated probe ($1c$-$e$, $1g$) is given in Table 2-6 as well as in Figure 2-4. The two shortest chain probes are not included in this data. Upon size exclusion, these probes were found to elute primarily with the aqueous phase as opposed to the liposome phases. This is reasoned by their main association with the outer headgroup region of the liposomes, and therefore results in their preference for the aqueous phase upon separation.

As previously discussed, the more hydrophobic probes reside in less dipolar microenvironments of liposomes of the same size compared to the less hydrophobic probes. Comparing fraction numbers 11 and 12 between the different probes shows this. These fractions represent a very narrow range of sizes (approximately 25 nm), as opposed to the overall more polydisperse liposome solution. $1c$ results in the largest $\pi^*$ followed by $1d$, $1e$, and $1g$ in decreasing $\pi^*$ order.

Figure 2-4 shows that in addition to the decreasing values of $\pi^*$ exhibited by the larger more hydrophobic indicators (due to their stronger affinity for the lipid portions of the vesicles), the larger indicators were trapped and sorbed by liposomes with a wider range of
sizes. For example, \textbf{1d} was found in vesicles with sizes ranging from 24.4 (± 0.5) to 30.3 (± 0.6) nm while \textbf{1g} was found in liposomes with sizes ranging from 27.1 (± 2.4) to 38.6 (± 4.7) nm. Additionally, the larger dyes will partition into the larger sized fractions of liposomes. This is not seen with the shorter chain probes. For example, 25.8 (± 1.4) nm is the largest size fraction that probe \textbf{1c} was measured in, while \textbf{1g} was found in sizes up to 38.6 (± 4.7) nm.

The data in Figure 2-4 are significant because they portray solvatochromic parameters for individual probes as they are physically isolated by the different sized vesicles in liposome dispersions. For the PG$_{24}$PC$_{46}$Chol$_{30}$ vesicle systems, the data suggests that for solutes that are relatively small and polar, having less hydrophobic interaction with the bilayer, the size of the capturing medium (relative to the size of the solute to be captured) is more critical. Larger more hydrophobic solutes, with greater affinity for the lipid bilayer, can be captured to produce a wider range of sizes of dye - entrapped liposomes.

**Conclusions**

Solvatochromic probes can make a valuable contribution to the characterization of vesicles, especially when coupled with other spectroscopic techniques. $\pi^*$ values for dispersions of small unilamellar vesicles of varying composition were determined using a homologous series of N,N-di-n-alkyl-$p$-nitroanilines (\textbf{1a-g}). The partitioning of \textbf{1a-g} into PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes increased with both increasing vesicle concentration and increasing length of the alkyl chains on the probes. Photon correlation spectroscopy has
shown, for the PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes, that larger indicators reside predominantly in larger vesicles and smaller indicators are predominantly located in smaller sized vesicles. For the PG$_{24}$PC$_{46}$Chol$_{30}$ liposomes, addition of the indicators during vesicle preparation (via sonication) results in probe solvation sites that are not accessible where probes are merely added to the already formed vesicles. The advantage to using this homologous series of solvatochromic dipolarity indicators is the ability to probe various depths of the headgroup region to better detect differences in liposome dipolarity that might not be detectable by a single probe.

**Acknowledgements**

A research grant from the U.S. National Institutes of Health (GM 38738) is gratefully acknowledged.
Table 2-1. Chemical structures of the DNAP indicators and corresponding values of $\nu_0$ and $-s$ from a seven-point estimation and previous literature.

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**Table 2-2.** Structures for headgroups of key phospholipids; all phospholipids have two C<sub>16</sub> chains (not shown).

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-α-phosphatidylcholine Dipalmitoyl (PC)</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (PG)</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>L-α-Phosphatidyl Serine Dipalmitoyl (PS)</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>L-α-Phosphatidic Acid Dipalmitolyl (PA)</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>Dihexadecyl Phosphate (DHP)</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
</tbody>
</table>
Table 2-3. Values of $\pi^*$ for 1a, 1c, 1e, and 1g in 6 mM solutions of various SUV types.

<table>
<thead>
<tr>
<th>Vesicle Composition</th>
<th>1a</th>
<th>1c</th>
<th>1e</th>
<th>1g</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC$<em>{70}$Chol$</em>{30}$</td>
<td>1.01</td>
<td>0.72</td>
<td>0.62</td>
<td>0.41</td>
</tr>
<tr>
<td>PG$<em>{10}$PC$</em>{60}$Chol$_{30}$</td>
<td>1.08</td>
<td>0.78</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td>PG$<em>{24}$PC$</em>{46}$Chol$_{30}$</td>
<td>1.04</td>
<td>0.80</td>
<td>0.65</td>
<td>0.55</td>
</tr>
<tr>
<td>PG$<em>{35}$PC$</em>{35}$Chol$_{30}$</td>
<td>1.05</td>
<td>0.80</td>
<td>0.79</td>
<td>0.55</td>
</tr>
<tr>
<td>PG$<em>{70}$Chol$</em>{30}$</td>
<td>1.06</td>
<td>0.86</td>
<td>0.88</td>
<td>0.67</td>
</tr>
<tr>
<td>PS$<em>{24}$PC$</em>{46}$Chol$_{30}$</td>
<td>1.05</td>
<td>0.83</td>
<td>0.79</td>
<td>0.56</td>
</tr>
<tr>
<td>PA$<em>{24}$PC$</em>{46}$Chol$_{30}$</td>
<td>1.03</td>
<td>0.78</td>
<td>0.60</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Table 2-4. Values of average diameter (nm) (from three PCS measurements) for dye-encapsulated PG\textsubscript{24}PC\textsubscript{46}Chol\textsubscript{30} liposomes prepared via extrusion and sonication (before size exclusion).

<table>
<thead>
<tr>
<th>Method of SUV Preparation</th>
<th>Average Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrusion with 50 nm filter</td>
<td>62.6 (± 1.8)</td>
</tr>
<tr>
<td>Extrusion with 100 nm filter</td>
<td>100.1 (± 4.5)</td>
</tr>
<tr>
<td>Sonication</td>
<td>31.7 (± 0.9)</td>
</tr>
</tbody>
</table>
Table 2-5. Average π* and standard deviation for 1a-b, 1d, and 1g in PG₃₄PC₆₆Chol₃₀ liposomes (prepared via extrusion) at different PG₃₄PC₆₆Chol₃₀ concentrations; comparison of π* for dye-encapsulated liposome dispersions in two SUV size ranges.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>6 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nm filter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indicator</td>
<td>6 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>1a</td>
<td>1.04 ± 0.04</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>1b</td>
<td>0.88 ± 0.03</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>1d</td>
<td>0.48 ± 0.02</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>1g</td>
<td>0.51 ± 0.00</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>100 nm filter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indicator</td>
<td>6 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>1a</td>
<td>1.14 ± 0.01</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>1b</td>
<td>1.01 ± 0.04</td>
<td>0.93 ± 0.01</td>
</tr>
<tr>
<td>1d</td>
<td>0.55 ± 0.05</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td>1g</td>
<td>0.53 ± 0.02</td>
<td>0.52 ± 0.03</td>
</tr>
</tbody>
</table>
Table 2-6. Average $\pi^*$ and standard deviation for SEC fractions of dye - encapsulated PG$_{24}$PC$_{46}$Cho$_{30}$ liposomes; liposome - dye dispersions were prepared via sonication and fractionated on an SEC column; corresponding values of average SUV diameter as measured by PCS (for SUVs in each fraction) are given; data are plotted in Figure 2-4.

<table>
<thead>
<tr>
<th>Dye/SEC Fraction #</th>
<th>Average Size (nm)</th>
<th>Average $\pi^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c/ 11</td>
<td>25.8 ± 1.4</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>1c/ 12</td>
<td>24.6 ± 1.6</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>1d/ 9</td>
<td>30.3 ± 0.6</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>1d/ 10</td>
<td>27.3 ± 0.6</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>1d/ 11</td>
<td>26.4 ± 0.3</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>1d/ 12</td>
<td>24.4 ± 0.5</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>1e/ 9</td>
<td>32.7 ± 1.4</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>1e/ 10</td>
<td>29.5 ± 2.2</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>1e/ 11</td>
<td>26.7 ± 0.6</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>1e/ 12</td>
<td>24.3 ± 1.1</td>
<td>0.64 ± 0.02</td>
</tr>
<tr>
<td>1g/ 8</td>
<td>38.6 ± 4.7</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>1g/ 9</td>
<td>34.2 ± 2.7</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>1g/ 10</td>
<td>31.3 ± 1.8</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>1g/ 11</td>
<td>29.1 ± 1.3</td>
<td>0.53 ± 0.00</td>
</tr>
<tr>
<td>1g/ 12</td>
<td>27.1 ± 2.4</td>
<td>0.54 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 2-1. $\pi^*$ vs. PG$_{24}$PC$_{46}$Chol$_{30}$ concentration for seven indicators (1a-g) incorporated in liposome dispersions by physical encapsulation via sonication.
**Figure 2-2.** \( \pi^* \) for 1a-g incorporated in 15 mM PG\textsubscript{24}PC\textsubscript{46}Chol\textsubscript{30} liposomes with different preparation methods; gray line for dye - liposome dispersions prepared by adding the probe to the preformed SUV dispersion; black line for dye - liposome dispersions prepared by physical encapsulation via sonication.
Figure 2-3. \( \pi^* \) for 1a-g incorporated in 15 mM DHP\(_{70}\)Chol\(_{30}\) vesicles with different preparation methods; gray line for dye - liposome dispersions prepared by adding the probe to the preformed SUV dispersion; black line for dye - liposome dispersions prepared by physical encapsulation via sonication.
Figure 2-4. $\pi^*$ vs. vesicle size for DNAP indicators: 1c (◆), 1d (▲), 1e (●), and 1g (■) associated with 15 mM PG$_{24}$PC$_{46}$Chol$_{30}$ liposome dispersions that were fractionated by SEC; liposomes in the individual dye containing fractions were sized via PCS.
References


34. Northern Lipids Inc. 3650 Westbrook Mall, Vancouver BC Canada V6S2L2.


Chapter 3

Interaction of Basic Drugs With Lipid Bilayers Using Liposome Electrokinetic Chromatography (LEKC) 

Abstract

This study explores factors influencing the interactions of positively charged drugs with liposomes using Liposome Electrokinetic Chromatography (LEKC) for the development of LEKC as a rapid screening method for drug - membrane interactions. Liposomes were prepared and the retention factors were measured for a series of basic drugs under a variety of buffer conditions, including various buffer types, concentrations, and ionic strengths as well as using different phospholipids and liposome compositions. LEKC retention is compared with octanol - water partitioning. The interaction of ionizable solutes with liposomes decreased with increasing ionic strength of the aqueous buffer. The type of buffer also influences positively charged drug partitioning into liposomes. Varying the surface charge on the liposomes by the selection of phospholipids influences the electrostatic interactions between the positively charged drugs and the liposomes, causing an increase in retention with increasing percentages of anionic lipids in the membrane. Poor correlations are observed between LEKC retention and octanol - water partitioning. These studies demonstrate the overall buffer ionic strength at a given pH is more important than buffer type and concentration. The interaction of positively charged drugs with charged lipid bilayer membranes is selectively influenced by the pKₐ of the drug. Liposomes are more biologically relevant in- vitro models for cell membranes than octanol, and LEKC provides a unique combination of advantages for rapid screening of drug – membrane interactions.
Introduction

Partitioning of solutes (drugs, metabolites, toxins, etc.) into lipid bilayers of biological membranes plays a significant role in their uptake, transport, bioavailability, and distribution (1-5). As most drugs are administered orally, their ability to transport across the intestinal epithelium, a mono-layer of cells that line the interior of the intestine, is an important issue. The primary mechanism of gastrointestinal absorption of the vast majority of drugs is believed to involve initial partitioning into cell membranes followed by passive transmembrane diffusion (6-8). Other factors such as dissolution of drugs in the gastrointestinal (GI) fluid, metabolism, and active transporters (such as P-glycoprotein) could also influence the intestinal absorption of certain drugs.

Assessment of absorption and distribution of numerous drug candidates in combinatorial libraries is a crucial step in the early stages of drug discovery (9). Recent advances of combinatorial chemistry in drug discovery have provided the opportunity of rapidly synthesizing staggering numbers of drug candidates. This, however, has created enormous challenges and the need for development of High – Throughput Screening (HTS) of physico-chemical properties for characterization of pharmacokinetic properties that involve Absorption – Distribution – Metabolism – Excretion (ADME) of potential drug candidates in compound libraries (10-12). The most important solute property in such studies is lipophilicity, which is measured by a partition coefficient between an aqueous and organic phase. There exists a great deal of interest from both a scientific and practical standpoint in pharmaceutical research for a better understanding and quantification of solute partitioning between water and lipid bilayers of cell membranes.
Over a century ago, Meyer and Overton suggested the use of solute distribution between water and olive oil as a simple chemical model and a measure of solute – membrane interactions. Ever since, much work has been done to identify an accurate in-vitro chemical model for cell membranes and to quantify solute affinity for the lipid environments or lipophilicity.

Octanol – water partition coefficient (log $P_{ow}$) has become the most widely used scale for solute lipophilicity and a model for drug interactions with cell membranes (13-18). However, there exists a plethora of evidence that solute distribution between isotropic bulk solvents is quite inadequate in modeling solute partitioning into anisotropic, heterogeneous, and organized environments of lipid bilayers in cell membranes (19-22). A single scale such as $P_{ow}$ cannot possibly represent drug interactions with various membranes with wide range of compositions in absorption and distribution studies. The lack of electrostatic interactions in octanol – water partitioning is a serious misrepresentation of the actual drug affinity for membranes. A great majority of drug molecules have ionizable functional group(s); many of which are partially or fully charged under biological conditions. Finally, direct measurement of octanol – water partition coefficients of numerous new compounds in combinatorial libraries is a major obstacle. Nevertheless, in the absence of a more chemically relevant model, octanol – water partition coefficient (log $P_{ow}$) is presently the most widely used scale for estimating solute – membrane interactions.

Liposome Electrokinetic Chromatography (LEKC) provides distinct advantages for determination of lipophilicity in pharmacokinetic and Quantitative Structure Activity (QSAR) studies. LEKC is a Capillary Electrophoresis (CE) method where liposomes are
incorporated in the buffer solutions and serve as pseudo-stationary phases for separations of uncharged and charged molecules. Liposomes are spherical lipid bilayer microstructures that are made of phospholipids and closely resemble biological cell membranes (23). There are several significant advantages in using LEKC for assessment of drug–membrane interactions over the existing models such as octanol–water partitioning, retention in HPLC systems such as Immobilized Artificial Membrane (IAM-HPLC) (24-31), or Immobilized Liposome Chromatography (ILC) (32-36) as summarized below:

(a). Liposomes are more suitable models for the lipid bilayer environment of cell membranes than octanol or HPLC bonded stationary phases. Successful applications of liposome–water partition coefficients in QSAR ranging from correlations with intestinal absorption to pharmacokinetic parameters like binding to plasma proteins to have been demonstrated (22,37-40). However, partition coefficient data are scarcely available for lipid bilayers. LEKC provides an opportunity for rapid and high-throughput measurement of partition coefficients into liposomes for large compound libraries.

(b). LEKC is a powerful method for physico–chemical measurements and offers advantages such as speed, convenience, small sample size, and lack of sample purity requirement as compared to the existing techniques such as potentiometry, solid–phase extraction, dialysis, spectrophotometry, etc., where such measurements are simply too cumbersome and/or prohibitively time consuming for widespread use in drug discovery applications (41-51). In fact, LEKC is the only technique for measurement of partition coefficients with the capability of high-throughput screening of large compound libraries.
Multiplexed 96 – capillary CE systems with both absorbance and fluorescence detection are now commercially available.

(c). Using LEKC, it is possible to establish universal and consistent partition coefficient scales for drug – membrane interaction studies. A major shortcoming of HPLC methods (IAM or RPLC) is the lack of availability of a universal and consistent partition coefficient scale (for inter - laboratory use) for quantifying drug – membrane interaction. Retention factor in HPLC (k') depends on partition coefficient into the stationary phase (K) and phase ratio (Φ) as k' = K Φ. However, the uncertainties in measuring the phase ratio, and more importantly, the variability of phase ratio among different HPLC columns have hindered the development of a coherent, consistent, and reproducible partition coefficient scale for inter - laboratory use in drug – membrane interaction studies.

On the contrary, phase ratio in LEKC can be determined for a given pseudo - phase, and more importantly, phase ratio does not vary between instruments, capillaries, or with use (which is the case for HPLC). The LEKC phase ratio, Φ_{LEKC}, is defined as the ratio of the volume of the liposome phase (V_{lip}) over the aqueous phase (V_{aq}) and is related to the intrinsic properties of the phospholipids such as molar volume, v, and critical aggregation concentration (CAC) as well as concentration (C_{PL}, the concentration of phospholipids), and can be reproducibly determined with relatively high accuracy according to Equation 3-1.

Equation 3-1

Φ_{LEKC} = \frac{V_{lip}}{V_{aq}} = \frac{v(C_{PL} - CAC)}{1 - v(C_{PL} - CAC)}

The intrinsic characteristics of the liposomal pseudo - stationary phase remain constant for a given temperature and ionic strength, and do not depend on the CE system or the
capillary. This would make it possible to accurately determine $K_{lw}$ (the liposome-water partition coefficient) from retention factor in LEKC (using Equation 3-1 and 3-2) for a variety of liposome systems that could be readily utilized in different laboratories.

\[ K_{lw} = \frac{k}{\phi_{LEKC}} \]  
\[ \text{Equation 3-2} \]

The retention factor, $k$, for neutral solutes is calculated from the LEKC data using the retention times and Equation 3-3, where $t_R$ is the retention time of the solute, $t_{eo}$ is the retention time of the electroosmotic flow marker, methanol, and $t_{lip}$ is the retention time of decanophenone, the marker of the liposomes.

\[ k = \frac{(t_R - t_{eo})}{t_{eo} \left(1 - \frac{t_R}{t_{lip}}\right)} \]  
\[ \text{Equation 3-3} \]

Charged solutes will possess their own electrophoretic mobility in the aqueous phase in addition to partitioning into the liposomes and migrating at the liposome mobility. As a result, the migration of the charged solute in the bulk aqueous ($t_0$) needs to be included in the calculation of retention factor. Equation 3-4 is used to calculate the retention factors of charged solutes.

\[ k = \frac{(t_R - t_o)}{t_o \left(1 - \frac{t_R}{t_{lip}}\right)} \]  
\[ \text{Equation 3-4} \]

The liposome-water distribution coefficient ($D_{lw}$) of a charged solute can be substituted for the partition coefficient and calculated according to Equation 3-2.
(d). In LEKC, the pseudo - stationary phase is a part of the buffer solution, which renders the flexibility of controlling the composition of the lipid bilayer pseudo - phase. The composition of these “artificial membranes” can be carefully controlled to nearly mimic the properties of the natural membranes that are important in the trans - cellular (passive) diffusion. This is accomplished through adjustment of the type and mole fractions of phospholipids as well as incorporating “additives” such as cholesterol and even proteins. For example, in an attempt to emulate the in vivo situation for studying partitioning, Kramer et al. used liposomes composed of a complex lipid mixture extracted from MDCK cells (52,53). They studied the partitioning of propranolol into these unilamellar liposomes termed “MDCKsomes” using equilibrium dialysis. In this work, liposomes were prepared that mimic the composition of the intestinal epithelium cells.

Obviously, systems such as the “MDCKsomes” are much closer to the natural membrane than octanol or Immobilized Artificial Membrane stationary phases in HPLC (IAM columns) where a single phospholipid is chemically attached to silica gel. The IAM columns have recently become popular as an alternative to the octanol – water system; however, a phospholipid - bonded phase does not truly represent the dynamic and fluid environment of lipid bilayers in vesicular assemblies.

A large number of pharmaceutical compounds are basic drugs and consequently possess a positive charge at physiological pH. Therefore examining several factors influencing the retention of basic drugs in LEKC is important for the development of LEKC as a method for screening of drug - liposome interactions. Liposomes were prepared and the retention factors were measured for a series of basic drugs under a variety of buffer conditions, including
various buffer types, concentrations, and ionic strengths as well as using different phospholipids and liposome compositions.

**Materials and Methods**

**Reagents**

N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], sodium salt (HEPES buffer), 2-[N-Morpholino]ethanesulfonic acid (MES buffer), sodium phosphate monobasic (phosphate buffer), 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS buffer), Tris[hydroxymethyl]-aminopropane (TRIS buffer), and 2-(Cyclohexylamino)ethanesulfonic acid (CHES buffer) were purchased from Sigma. Cholesterol (Chol), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (PC), 1,2-Dipalmitoyl-sn-Glycero-3-(Phospho-L-Serine) (Sodium Salt) (PS), 1,2-Dipalmitoyl-sn-Glycero-3-(Phospho-rac-(1-glycerol)) (Sodium Salt), (PG), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine (PE), L-α-Phosphatidylinositol (Soy, Sodium Salt) (PI), and Sphingomyelin, (Egg, Chicken) (SPH) were obtained from Avanti Polar Lipids, Inc. All samples used in the LEKC studies were purchased from Aldrich.

**Vesicle Preparation**

Buffers containing multiple components and constant ionic strengths were prepared according to the software program developed by Okamoto which is used to determine the quantity of various buffer components required to achieve a certain pH at given ionic
strength and temperature conditions (54). A description and application of the buffer program is found in reference 54 by Okamoto. Dr Okamoto has graciously donated a copy of the software.

The appropriate amounts of phospholipids and cholesterol were dissolved in a 9:1 volume mixture of chloroform and methanol (respectively). The organic solvent was removed under reduced pressure using a rotary evaporator in a water bath maintained at 70°C. The thin lipid film was hydrated with the appropriate buffer. Liposomes were prepared according to the extrusion method where the multilamellar vesicles (MLVs) were processed to small unilamellar vesicles (SUVs) to achieve a uniform size distribution and smaller size liposomes. Extrusion was performed through polycarbonate membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada) maintained at a temperature of 60°C by a circulating water bath. Extrusion was performed a total of five times through 200 nm pores size filters, five times through filters with a pore size of 100 nm, and finally ten times through the smallest pore size filter, 50 nm.

*CE System*

The LEKC experiments were carried out on a laboratory built CE instrument. A Spellman SL30 high voltage power supply was used to apply a positive voltage over the length of the fused silica capillary (Polymicro Technologies, Phoenix, AZ), with an inner diameter of 50 µm and an outer diameter of 375 µm. The temperature of the system was maintained at 36°C using a circulating oil bath. The absorbance was measured at 214 nm.
using a SSI 500 variable-wavelength UV detector. Methanol was used as the electroosmotic flow marker, t<sub>eo</sub>, and decanophenone were used as the liposome marker, t<sub>lip</sub>.

**LEKC Methods**

At the beginning of each day, the capillary was rinsed in the following manner: 20 minutes with Milli-Q water; 20 minutes with 1.0 M NaOH; 10 minutes with Milli-Q water; 10 minutes with methanol; 10 minutes with Milli-Q water. For LEKC experiments, the capillary was then rinsed for 30 minutes with the liposome solution. Following this rinse procedure, a voltage was applied for approximately 30 minutes to further equilibrate the column with the liposomes before sample injections were performed. Typically liposome solutions were prepared simultaneously while the initial capillary rinse procedure was carried out. At the end of the day, the capillary was rinsed for 10 minutes with Milli-Q water.

As mentioned above and described by Equation 3-4, in order to determine retention factors, k, for charged solutes in LEKC, the migration times, t<sub>R</sub>, in the presence of liposome (LEKC condition) and in the absence of liposome, t<sub>0</sub>, (CZE condition) have to be determined. All CZE and LEKC solute mobilities are the average of 4 repeated measurements. The CZE data for each sample was collected immediately following the LEKC data by rinsing the capillary for 2 minutes with the buffer solution (i.e. in the absence of liposomes), and then the CZE data was collected for the same sample. This was found to be the optimum method for creating the same capillary wall conditions (i.e. keeping the wall coated with the liposomes) between LEKC and CZE runs since liposomes have been found to coat the walls of the capillary (55).
Other authors have accounted for differences in CZE and EKC buffers by the addition of high concentrations of NaCl to the CZE buffer to make up for the differences in ionic strength between the two conditions (56). This was necessary in these studies due to the high ionic strength of the sodium dodecyl sulfate (SDS) which was used as the pseudostationary phase (56). However, the situation is different for the LEKC system. The addition of the liposomes has a much smaller contribution to the total ionic strength of the buffer solution since phospholipid concentrations were small (10 mM) and they consist largely of the zwitterionic phospholipid, PC. Therefore this effect was considered negligible.

All solutions used for rinsing were filtered through a 0.45 µm filter disk (Scientific Resources Inc.) prior to use. The capillary was rinsed with the liposome solution for 1 - 2 minutes between LEKC injections. Approximately 0.02 - 0.06 g of each solute was dissolved in around 3 mL of methanol to prepare stock solutions. To prepare a sample for injection, approximately 50 - 200 µL of the stock sample was used, decanophenone dissolved in methanol was added where appropriate, and enough methanol was added to make 1 - 1.5 mL total sample volume. Samples were injected for 1 - 2 seconds by hydrodynamic injection.

Figure 3-1 is a sample electropherogram for 10 mM PG_{20}PC_{80} with 3 mM cholesterol. There is a defined elution window in LEKC which is marked by the retention times of the t_{eo} (MeOH) and t_{lip} (decanophenone) markers, indicated in Figure 3-1 as peaks 2 and 5, respectively. Atenolol, peak 1, has very little interaction with the liposomes and its mobility in LEKC is mostly due to its own mobility in the aqueous phase. Imipramine and amitriptyline, peaks 3 and 4, respectively, have a greater interaction with the liposomes.
Typically, for normal buffer conditions the elution of the final peak (the liposome marker) was between 4 and 7 minutes for liposomes with different charge densities. CZE runs were on the order of 2.5 minutes. The retention factor (and thus, partition coefficient) of one drug can be determined in approximately 30 - 35 minutes (for an average of three measurements). Typically sample mixtures were injected consisting of 2 to 5 drugs. This, of course, greatly increases the number of compounds that could be analyzed simultaneously in a single run.

Since the rinse procedure has not yet been optimized, the conditions used in these experiments were longer than necessary to insure complete equilibration. The rinse procedure as described above could be shortened upon optimization. Additionally, liposomes do not need to be prepared immediately prior to use. In order to avoid the daily preparation time before running experiments, the liposomes can be prepared ahead of time and stored in the refrigerator for later use. Optimizing the rinse procedure and preparing liposomes ahead of use will significantly reduce the time required to prepare for data analysis.

**Determination of Liposome Size**

The average liposome size was determined using Photon Correlation Spectroscopy (PCS) using a Zetasizer 1000HS A (Malvern Instruments Ltd, Malvern UK) with a 5 mW Helium Neon laser at 633 nm. The scattered light was collected at an angle of 90°. All measurements were made at 36°C. The Malvern PCS software algorithm chosen to analyze the data was Contin and the size distribution profiles were analyzed using the method of volume. Average liposome sizes were obtained from at least three repeat measurements of
the mean diameter of the liposome. The average size of the liposomes used in this work is 52.3 (± 0.6) nm.

Results and Discussion

Effect of Buffer Composition

The buffer type, concentration, and ionic strength were varied to investigate the effect of the aqueous buffer on the partitioning into liposomes as determined by the LEKC retention factor. The liposome composition chosen was 10 mM PG\textsubscript{24} PC\textsubscript{46} Chol\textsubscript{30}, where the subscripts represent the molar percentages of the components used. The liposome composition was held constant while the buffer composition was varied. Three different buffers were studied at pH 7.0: HEPES, MES and phosphate with pK\textsubscript{a} values of 7.66, 6.21, and 7.20, respectively (57). Retention data was collected using 10, 25, and 40 mM of each of the three buffers. 40 mM was selected as the highest buffer concentration due to stability issues of the liposomes in LEKC at higher concentrations. At this pH, a set of positively charged drugs including tetracaine and acebutolol as well as the neutral solute phenol were selected as test solutes. For this work, the measurement of the average liposome diameter was used to compare liposome properties with different buffer conditions. The liposome size remained constant over the range of buffers studied.

For the neutral solutes, there is essentially no difference in log k for a given buffer type at different concentrations. For example, phenol has log k values of -1.22 (± 0.01) and -1.20 (± 0.01) for 10 and 40 mM phosphate buffer concentrations. In addition, the type of buffer
does not affect the measured log k value. The log k values of phenol in 10 mM HEPES, MES, and phosphate are -1.21 (± 0.01), -1.20 (± 0.01), and -1.22 (± 0.01) respectively. This is in agreement with previous determinations from this lab, where in a linear solvation energy relationship (LSER) study using LEKC, the type and concentration of buffer had no influence on the LSER coefficients for a series of neutral solutes (58).

The positively charged solutes have a different behavior such that the buffer type and concentration significantly influence log k values for all positive drugs studied. The results of the effect of buffer type on LEKC retention for tetracaine and acebutolol is illustrated in Figure 3-2. Error bars are included however they are smaller than the size of the symbols. For the solutes shown here, in all the three buffer types, log k values decrease with increasing buffer concentrations. HEPES results in the largest log k values followed by MES and then phosphate buffer results in the lowest log k values. This follows along with the ranking of the ionic strength of the three buffers. For the same concentration, the organic buffers HEPES and MES have the lowest ionic strengths, and the inorganic phosphate buffer has the highest ionic strength of the three buffers.

To study the effect of ionic strength on the interaction of charged drugs with liposomes, the buffer concentration was held constant at pH 7.0 using 10 mM HEPES. Sodium chloride was added with concentrations ranging from 0 to 40 mM NaCl. The effect of ionic strength can be seen for several positively charged and neutral solutes in Figure 3-3. Neutral solutes (non-filled symbols) show no change in log k with increasing ionic strength of the buffer solution. Positively charged solutes (filled symbols) show a significant decrease in log k with increasing ionic strength. Since log k is directly proportional to logDlw, the distribution
coefficient would also decrease with increasing ionic strengths. This is true for all basic solutes studied. Lidocaine has a pKₐ value of 7.9 (59), and is about 89 percent ionized at this pH and still follows the same trend as acebutolol and alprenolol, both of which are fully charged with a pKₐ value of 9.2 or greater (59). The trend of a decrease in retention is the same for all positively charged solutes examined, which indicates that ionic strength does not have a selectivity effect on the drug - liposome interactions with the positively charged solutes studied here.

The decrease in partitioning with increase in ionic strength or increase in buffer concentration is consistent with previous determinations (60). This reduced interaction is due to the shielding of the charge on the liposome surface in addition to the shielding of the charge on the drug molecule by the buffer counter ions, thus decreasing the electrostatic interactions. This would also explain the lack of difference in partitioning with the neutral solutes as the buffer is varied since they have no electrostatic interactions. A stronger influence of the ionic strength has been observed when more anionic vesicles are used (60).

To study the effect of buffer concentration at a constant ionic strength, the ionic strength was held constant at 29 mM by the addition of NaCl while different concentrations and types of buffers were investigated. This data is shown in Table 3-1 for 10, 20, 28, and 40 mM HEPES as well as 10 mM MES and two mixed buffers. Mixed buffers consisted of three buffer components: 10 mM each of HEPES, CHES, and CAPS buffers compose the first mixed buffer (Mixbuf), and 10 mM each of MES, TRIS, and CAPS compose the second mixed buffer (Mixbuf-a). It is noted that the zeta potential (calculated according to Equation
3-5) was constant for this series of buffers (average zeta potential for the series = \(-49.2 \pm 0.4\) mV), indicating a constant liposome charge density.

There are no differences in retention between all buffer compositions for the one neutral test solute, benzocaine. For the positively charged solutes, there is very little difference in retention as the HEPES concentration is increased with a constant ionic strength. This is different than the previously discussed data where the buffer concentration was increased with no set ionic strength. There are only slight variations in log \(k\) for the remaining buffer types as well. These studies emphasize the overall buffer ionic strength at a given pH is more important than the buffer type and concentration. This is significant for studies on the pH effect on the interactions of charged drugs with liposomes as discussed in Chapter 4. Mixed buffers are commonly used in studies of the effect of pH on liposome partitioning in order to maintain a constant environment for the liposomes. A mixed buffer of constant ionic strength must be used.

*Effect of Liposome Composition*

Electrostatic interactions between ionized drugs and charged membranes are an important factor influencing drug partitioning. The extent of interaction increases with the charge density of the membrane at a given pH. Cell membranes are often negatively charged due to the presence of lipids with anionic headgroups such as PS and PI. Membrane compositions vary widely in terms of the type and mole fraction of phospholipids, including the anionic lipids that determine the charge density of the membranes. For these reasons the effect of liposome composition was studied in terms of the type and percentage of anionic
phospholipids. To compare the effect of the selection of anionic lipid, PS$_{20}$PC$_{80}$ liposomes were prepared to compare with the PG$_{20}$PC$_{80}$ liposomes. In addition, a liposome composition denoted as *cell-mimic* was prepared to simulate the phospholipids present in Caco-2 cells (61). This composition consisted of PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$, where PS and PI are the negative lipids in this mixture, making the total anionic lipid content 25 percent, leaving 75 percent composed of zwitterionic lipids including PC, PE, and SPH. All liposomes in this study had a total lipid concentration of 10 mM and a constant concentration of cholesterol of 3 mM (30 percent of the total lipid concentration).

Listed in Table 3-2 is a series of drugs having a net positive charge at pH 7.4 which were selected to study the effect of liposome composition. Also included in Table 3-2 are the drug pK$_a$ values (62,63). A constant buffer composition was used and the percentage of anionic phospholipid was varied from 5 to 50 percent while the percent PC (zwitterionic phospholipid) ranged from 95 to 50 percent, respectively. Previous studies have shown that the effect of liposome composition has little effect on the retention of neutral drugs in LEKC. (M.G. Khaledi, unpublished results).

The retention factor increases with increasing percentage of anionic lipids for all basic drugs studied. This can be seen in Figures 3-4 and 3-5 for imipramine, labetalol, nadolol, terbutaline, and metoprolol. A second order polynomial was found to fit the data well. The regression coefficients and R$^2$ values are listed in Table 3-2 for the series of drugs. The lines in Figures 3-4 and 3-5 are second order polynomial fits of the data. For many drugs studied, there is little interaction with liposomes consisting of 5 percent PG; therefore this data is not included in Figures 3-4 and 3-5 or the second order polynomial regression. This little
interaction (with 5 % PG) is likely due to the small total concentration of phospholipids used in this work (10 mM) that results in a low charge density of liposomes.

The increase in retention with increasing PG content is attributed to an increase in the electrostatic interaction between the positively charged drug and the increasing negative charge on the liposome surface. Positively charged drugs have been shown to interact stronger with liposomes containing larger PS content (64). This is consistent with literature reports, for example, greater DMPG content in membranes also resulted in an increase in the degree of binding of quinine to lipid membranes (60). Liposomes consisting of 30 percent PS had an affinity for the positively charged drug, flurazepam, of about 4.8 times that of membranes consisting of PC only, determined using second derivative spectrophotometry (65).

In contrast to the buffer composition, the anionic phospholipid content of the vesicles has a selective effect on the drug - liposome interactions. When examining the electrostatic interactions of drugs, the charge of the drug in addition to the charge on the liposomes will influence the degree of electrostatic interaction. A fully protonated drug will have a greater degree of electrostatic attraction compared to a drug that may only be partially positively charged. Therefore, the increase in retention factor with increasing anionic lipid content may not be the same for all drugs. Figure 3-4 shows three solutes which are all >99 percent ionized. They show similar trends in the increase in log k with increasing PG content. Figure 3-5 shows the selective effect of changing the percent PG on the log k (with a second degree polynomial fit) for 2 solutes which have very little interaction with the liposomes at the lower PG content (12 percent PG) while at higher percentages of PG (50 percent PG) the
difference in interaction is significant. It is possible that the difference in retention with the increasingly negative liposomes is due to differences in the percent ionization (i.e. different charge) of the two drugs. Terbutaline has a pKₐ of 8.8 while metoprolol has a pKₐ of 9.7, (59) thus making metoprolol fully charged while terbutaline is only partially charged. The drug with the greater degree of ionization resulted in a greater increase in retention possibly because of enhanced electrostatic effects. There is only a slight difference in the degree of ionization of the two drugs in this case. Comparing drugs with lower pKₐ values (i.e. lower percentage ionized at the given pH) with the current data set would be beneficial in investigating the pKₐ selectivity effect.

The electrostatic properties in the interface region of the lipid bilayer play a significant role in influencing the partitioning behavior of charged drugs. By changing the fraction of charged lipids in the liposomes, the surface charge density and zeta potential are altered. The zeta potential, ζ of liposomes of varying PG content was calculated from their measured mobility (µₗᵢᵖ) according to the Smoluchowski equation (66):

\[
\zeta = \frac{\mu_{\text{lip}} \eta}{\varepsilon_0 \varepsilon_r}
\]

where \( \eta \) is the aqueous solution viscosity, \( \varepsilon_0 \) is the permittivity of free space and \( \varepsilon_r \) is the relative permittivity of the medium, with values of 7.05 x 10⁻⁴ N s m⁻², 8.854 x 10⁻¹² C² N⁻¹ m⁻² and 75.8 respectively. The effect of the percentage of PG on the calculated zeta potential is seen in Figure 3-6. Many detailed discussions on the effect of anionic lipid content on the zeta potential have been published (67-69). Zeta potentials have been found to have an exponential dependence on the PS content in liposomes (70). Estelrich et al. determined a
zeta potential value of around –40 mV for SUVs composed of 20 percent PS and 80 percent soybean PC prepared by extrusion through 50 nm filters (70). This is on the order of the value of –49.6 (± 0.1) mV for PS20PC80 determined in this work. Differences in ionic strength between the buffers used in this work and by Estelrich may cause the difference in the two values. In addition, soybean PC contains phospholipids with different chain lengths and degrees of unsaturation, which influences the aggregation properties in the liposomes.

Wiedmer et al. calculated the total charge on liposomes consisting of various ratios of anionic lipids. With about 30 percent anionic lipid a flattening of the curve is seen such that the liposomes were almost saturated with negative charges (71). This same flattening after 30 percent PG can be seen with the zeta potential determinations here. The zeta potential only varies from –52.4 (± 0.2) mV to –54.1 (± 1.2) mV between 30 and 50 percent PG.

More negative zeta potentials were obtained with larger PG content in the liposomes. As a result, we see a greater retention of basic drugs with increasing PG content, or a more negative zeta potential. Consistent with the saturation of the charge on the liposome surface, the retention factor starts to plateau after 30 percent PG. McLaughlin et al. found a sigmoidal dependence on the percentage of bound peptide with the mol percent PG in the liposomes. Their data showed a similar trend of a plateau at concentrations of PG greater than 30 percent (72).

To further study the effect of liposome composition on the retention of basic drugs, two anionic phospholipids were chosen to investigate their effect on the retention of the drugs. For almost all drugs studied, there is a slightly greater log k with the PG containing liposomes compared to the PS containing liposomes. However, there is a very good
correlation between the retention factors determined using these compositions. This correlation ($R^2$ is 0.992) is shown in Figure 3-7 for the 26 drugs listed in Table 3-2. Liposomes consisting of 20 percent PG or 20 percent PS also have a similar zeta potential value. Vesicles formed from 5:1 PC:PG or 5:1 PC:PS mixtures have been found to have similar zeta potentials (67,69). This correlation in retention factors is slightly reduced at low percentages (5 percent) of anionic lipid, between liposomes composed of PG and PS, with a $R^2$ value of 0.977 for 17 drugs. This correlation is not as high as with the larger percentages of PG, perhaps due to a greater relative error associated with the measurements of smaller retention factors.

There is a good correlation for log $k$ values determined using the cell-mimic liposomes and the PG$_{20}$PC$_{80}$ or PG$_{30}$PC$_{70}$ liposomes. The zeta potential of the cell-mimic liposomes fits in between the values for 20 and 30 percent negative lipid. Zeta potentials of PI have been found to be less negative than PS and PG (67). Although PE is a zwitterionic lipid, the incorporation of PE into PC vesicles has been found to slightly enhance the negative charge of the phosphate group resulting in a slightly negative zeta potential (70). Despite these differences, the simple composition of liposomes consisting of between 20 and 30 percent PG can be used to simulate interaction of drugs into the more complicated liposomes mimicking the intestinal epithelial cell membranes of Caco-2 cells.

**Correlations With Octanol - Water Partitioning**

The liposome - water partitioning data can be correlated with octanol - water partitioning ($\text{LogD}_{ow}$ or $\text{ClogP}_{ow}$) values obtained from the literature (59,73). The plot of log $k$ cell-mimic liposomes vs. $\text{LogD}_{ow}$ is shown in Figure 3-8. The correlation has an $R^2$ equal to 0.628 for 19
solute. A poor correlation is observed with LogD_{ow} as reported in the literature due to the lack of electrostatic interactions in octanol. A better linear relationship is observed between log k_{cell-mimic} and ClogP_{ow} than between log k_{cell-mimic} and LogD_{ow}. The plot of log k_{cell-mimic} liposomes vs. ClogP_{ow} results in a R^2 of 0.807 for the 26 solutes listed in Table 3-2 and is shown in Figure 3-9.

Charged drugs are able to interact with liposomes by both hydrophobic and electrostatic interactions, while with octanol, only hydrophobic interactions are possible. A great majority of drug molecules have ionizable functional group(s); many of which are partially or fully charged under biological conditions. As discussed above, electrostatic interactions have a significant impact on drug partitioning into liposomes. Clearly, the octanol - water system fails to mimic interfacial properties of biomembranes and the ionic interactions between charged drugs and the phospholipids that constitute cell membranes. Therefore a single scale such as P_{ow} cannot possibly represent drug interactions with various membranes with a wide range of compositions. Ionized drugs typically have low octanol - water distribution coefficients, logD_{ow} due to the absence of electrostatic interactions. On the other hand, basic solutes have an enhanced interaction with membranes due to electrostatic attraction which is not possible with octanol (74). As a result, the correlation between the octanol - water distribution coefficient and the LEKC retention factor (directly related to D_{lw}) is poor.

Conclusions

LEKC is a convenient and powerful method for rapid screening of drug – membrane interactions. Liposomes are more biologically relevant in- vitro models for cell membranes than octanol or HPLC stationary phases (such as IAM columns). LEKC provides a unique
combination of advantages such as small sample size, no sample purity requirement, speed, convenience, automation, flexibility of adjusting the liposome pseudo-phase compositions to mimic natural membranes and high-throughput capability.

It is emphasized that a laboratory built CE system was used in this work, requiring the operator to manually inject every sample. The use of an automated instrument would increase sample throughput allowing for constant data acquisition, since data acquisition could occur without the operator present. Additionally, one could imagine the drastic increase in sample throughput with the use of a 96-capillary CE system which is commercially available. The potential for high-throughput liposome-water distribution coefficient determination is enhanced by the comparison with the pH-metric titration method which can take up to 1 hour per liposome titration (75). This method would also require repeat titrations for an average value, while only analyzing one sample at a time.

In addition, the possibility of determining the LEKC phase ratio would make it possible to create universal scales for drug–membrane interactions, which is not possible using HPLC based systems. Recent reports from this laboratory have demonstrated the possibility of calculating liposome-water partition coefficients from solute structure (76). Such capability will be of great use in drug design and screening as drug candidate affinity for membranes could be predicted prior to their synthesis.
Acknowledgements

A research grant from the U.S. National Institutes of Health (GM 38738) is gratefully acknowledged.
Table 3-1. Log k for various buffers with a constant pH (7.0) and ionic strength (29 mM) for one neutral drug (benzocaine) and 6 positively charged drugs.

<table>
<thead>
<tr>
<th></th>
<th>10 mM HEPES</th>
<th>20 mM HEPES</th>
<th>28 mM HEPES</th>
<th>40 mM HEPES</th>
<th>10 mM MES</th>
<th>Mixbuf&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mixbuf-a&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzocaine</td>
<td>-0.77</td>
<td>-0.72</td>
<td>-0.74</td>
<td>-0.72</td>
<td>-0.75</td>
<td>-0.76</td>
<td>-0.72</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>-1.20</td>
<td>-1.14</td>
<td>-1.14</td>
<td>-1.06</td>
<td>-1.15</td>
<td>-1.12</td>
<td>-1.32</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.03)</td>
<td>(0.02)</td>
<td>(0.03)</td>
<td>(0.04)</td>
<td>(0.03)</td>
<td>(0.04)</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>0.29</td>
<td>0.30</td>
<td>0.19</td>
<td>0.31</td>
<td>0.16</td>
<td>0.23</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>0.92</td>
<td>0.94</td>
<td>0.94</td>
<td>0.89</td>
<td>0.90</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(0.03)</td>
<td>(0.04)</td>
<td>(0.01)</td>
<td>(0.03)</td>
<td>(0.06)</td>
<td>(0.03)</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>-1.00</td>
<td>-1.04</td>
<td>-1.03</td>
<td>-1.01</td>
<td>-0.97</td>
<td>-1.00</td>
<td>-1.01</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.02)</td>
<td>(0.03)</td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>Alprenolol</td>
<td>0.18</td>
<td>0.16</td>
<td>0.15</td>
<td>0.18</td>
<td>0.13</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>Doxepin</td>
<td>0.67</td>
<td>0.69</td>
<td>0.64</td>
<td>0.63</td>
<td>0.66</td>
<td>0.64</td>
<td>0.69</td>
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<tr>
<td></td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.02)</td>
</tr>
</tbody>
</table>

<sup>a</sup> *Mixbuf* consisted of 10 mM each of HEPES, CHES, and CAPS buffers.

<sup>b</sup> *Mixbuf-a* consisted of 10 mM each of MES, TRIS, and CAPS buffers.
Table 3-2. List of basic drugs studied and pKₐ values. pKₐ values shown are basic pKₐ values (BH⁺) unless otherwise noted. Regression results for second-degree polynomial \( y = ax^2 + bx + c \) fit of log k and molar fraction of PG. Regression results are for the fit of the data including 12, 20, 30, 40, and 50 % PG, unless otherwise noted.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Solute Name</th>
<th>pKₐ</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acebutolol</td>
<td>9.20</td>
<td>-1.002</td>
<td>1.516</td>
<td>-0.130</td>
<td>0.957</td>
</tr>
<tr>
<td>2</td>
<td>Alprenolol</td>
<td>9.65</td>
<td>-9.416</td>
<td>8.024</td>
<td>-0.789</td>
<td>0.992</td>
</tr>
<tr>
<td>3</td>
<td>Amitriptyline</td>
<td>9.42</td>
<td>-7.199</td>
<td>6.545</td>
<td>0.119</td>
<td>0.996</td>
</tr>
<tr>
<td>4</td>
<td>Atenolol</td>
<td>9.6</td>
<td>-4.403</td>
<td>4.630</td>
<td>-2.152</td>
<td>0.991</td>
</tr>
<tr>
<td>5</td>
<td>Chlorpheniramine</td>
<td>9.16</td>
<td>-7.042</td>
<td>7.366</td>
<td>-1.038</td>
<td>0.994</td>
</tr>
<tr>
<td>6</td>
<td>Desipramine</td>
<td>10.44</td>
<td>-3.785</td>
<td>4.704</td>
<td>0.404</td>
<td>0.991</td>
</tr>
<tr>
<td>7</td>
<td>Doxepin</td>
<td>9.0</td>
<td>-5.941</td>
<td>6.260</td>
<td>-0.161</td>
<td>0.995</td>
</tr>
<tr>
<td>8</td>
<td>Doxylamine</td>
<td>4.4; 9.2</td>
<td>-3.891</td>
<td>5.147</td>
<td>-1.667</td>
<td>1.000</td>
</tr>
<tr>
<td>9</td>
<td>Imipramine</td>
<td>9.5</td>
<td>-6.159</td>
<td>6.420</td>
<td>-0.125</td>
<td>0.996</td>
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<tr>
<td>10</td>
<td>Labetalol</td>
<td>7.4; 8.7; 2</td>
<td>-5.547</td>
<td>5.960</td>
<td>-0.491</td>
<td>0.978</td>
</tr>
<tr>
<td>11</td>
<td>Lidocaine</td>
<td>7.9</td>
<td>-5.092</td>
<td>5.115</td>
<td>-1.758</td>
<td>0.999</td>
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<tr>
<td>12</td>
<td>Maprotiline</td>
<td>10.2</td>
<td>-1.858</td>
<td>3.880</td>
<td>0.789</td>
<td>0.968</td>
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<tr>
<td>13</td>
<td>Metoprolol</td>
<td>9.7</td>
<td>-5.458</td>
<td>6.080</td>
<td>-1.518</td>
<td>1.000</td>
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<tr>
<td>14</td>
<td>Mianserin</td>
<td>7.1</td>
<td>-4.159</td>
<td>4.640</td>
<td>0.253</td>
<td>0.997</td>
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<td>Nadolol</td>
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<td>-4.058</td>
<td>4.628</td>
<td>-1.858</td>
<td>0.995</td>
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<tr>
<td>16</td>
<td>Nefopam</td>
<td>9.2</td>
<td>-6.136</td>
<td>6.524</td>
<td>-0.995</td>
<td>1.000</td>
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<td>17</td>
<td>Nortriptyline</td>
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<td>-9.732</td>
<td>7.998</td>
<td>0.225</td>
<td>0.997</td>
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<tr>
<td>18</td>
<td>Orphenadrine</td>
<td>8.4</td>
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<td>6.459</td>
<td>-0.680</td>
<td>0.994</td>
</tr>
<tr>
<td>19</td>
<td>Oxprenolol</td>
<td>9.5</td>
<td>-6.224</td>
<td>6.937</td>
<td>-1.496</td>
<td>0.980</td>
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<td>Pheniramine</td>
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<td>-4.098</td>
<td>5.330</td>
<td>-1.629</td>
<td>0.998</td>
</tr>
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<td>Pindolol</td>
<td>8.8</td>
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<td>6.393</td>
<td>-1.462</td>
<td>0.996</td>
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<td>22</td>
<td>Propranolol</td>
<td>9.45</td>
<td>-5.261</td>
<td>5.725</td>
<td>0.066</td>
<td>0.998</td>
</tr>
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<td>23</td>
<td>Terbutaline</td>
<td>8.8; 10.1; 11.2</td>
<td>-5.062</td>
<td>4.510</td>
<td>-1.374</td>
<td>0.995</td>
</tr>
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<td>24</td>
<td>Tetracaine</td>
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<td>4.651</td>
<td>-0.313</td>
<td>0.993</td>
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<td>25</td>
<td>Trimeprazine</td>
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<td>-9.025</td>
<td>8.037</td>
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<td>-4.549</td>
<td>5.224</td>
<td>0.264</td>
<td>0.982</td>
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</tbody>
</table>

2 pKₐ value denotes acidic pKₐ (HA).

3 Regression results do not include data for 50 % PG.
**Figure 3-1.** Electropherogram conditions: 10 mM \( \text{PG}_{20}\text{PC}_{80} \) with 3 mM Chol. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Peak identification: 1, atenolol (sample ID 4); 2, MeOH; 3, imipramine (sample ID 9); 4, amitriptyline (sample ID 3); 5, decanophenone.
Figure 3-2. Log k of tetracaine (filled symbols) and acebutolol (open symbols) as a function of HEPES, MES, and phosphate buffers at various concentrations: 10 mM (●, ○), 25 mM (♦, ◇), and 40 mM (▲, △).
Figure 3-3. Plot of log $k$ versus the concentration of NaCl added to the aqueous buffer (10mM Hepes, pH 7.0): tetracaine (♦); lidocaine (■); acebutolol (●); alprenolol (▲); phenol (□); 4-ethylphenol (○); 2-chlorophenol (△); 3,4-dichlorophenol (◇).
Figure 3-4. Plot of log k versus the percentage of PG in the liposome composition for imipramine (●), labetalol (▲), and nadolol (◆). Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Symbols are the measured data points and lines are a second order polynomial fit of the data.
Figure 3-5. Plot of log k versus the percentage of PG in the liposome composition for terbutaline (♦) and metoprolol (▪). Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Symbols are the measured data points and lines are a second order polynomial fit of the data.
Figure 3-6. Zeta potential as a function of percent PG in the liposomes. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.
Figure 3-7. Correlation between log k PS$_{20}$PC$_{80}$ and log k PG$_{20}$PC$_{80}$. The equation of the line is $y = 0.900x - 0.194$ with a correlation of 0.992 for 26 points as determined by linear regression. Points are labeled according to Table 3-2. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.
Figure 3-8. Correlation between log k _cell-mimic_ and log D<sub>ow</sub>. The equation of the line is y = 0.505 x - 0.056 with a correlation of 0.628 as determined by linear regression. Points are labeled according to Table 3-2. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.
**Figure 3-9.** Correlation between log \( k_{cell-mimic} \) and Clog\( P_{ow} \). The equation of the line is \( y = 0.533 \, x - 1.022 \) with a correlation of 0.807 as determined by linear regression. Points are labeled according to Table 3-2. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36\(^\circ\)C.
References


Chapter 4

pH Effect on the Interactions of Basic Drugs With Liposomes
Abstract

Liposome Electrokinetic Chromatography (LEKC) provides convenient and rapid methods for studying drug interactions with lipid bilayers using liposomes as a pseudostationary phase. LEKC was used to determine the effect of pH on the partitioning of basic drugs into liposomes composed of zwitterionic phosphatidylcholine (PC), anionic phosphatidylglycerol (PG), and cholesterol, which mimic the composition of natural cell membranes. An increase in pH results in a smaller degree of ionization of the basic drugs and consequently leads to a lower degree of interaction with the negatively charged membranes. From the LEKC retention data, the fractions of drugs distributed in the bulk aqueous and the liposome phase were determined at various pH values. Finally, lipid mediated shifts in the ionization constants of drugs were examined.
Introduction

The interaction of drugs with membranes is an important field of study due to the significant and useful applications in Quantitative Structure Activity Relationship (QSAR) studies (1-8). Liposomes are suitable models for biomembranes. Partition coefficients of drugs between a bulk aqueous and liposome phase ($K_{LW}$) can be used as a measure of the extent of drugs affinities toward lipid bilayers of cell membranes, or drugs lipophilicity (9). Partitioning of uncharged solutes into liposomes is controlled by a combination of hydrophobic, dipolarity/polarizability, and hydrogen bonding interactions (10). A great majority of drug molecules have ionizable functional groups, thus electrostatic interactions also play a major role in their partitioning behavior, as measured by the liposome - water distribution coefficient ($D_{LW}$) which is dependent on the extent of solute ionization as determined by the pH.

Partitioning into a cell membrane is generally considered the first step in the passage of drugs across biological membranes (11,12). Transport of drugs through biological membranes (membrane permeability) is primarily by passive diffusion for large numbers of drugs. Passive transport through a cell membrane involves an initial partitioning into the lipid bilayer of the membrane, followed by diffusion through the bilayer, and finally a partitioning out of the membrane. This initial partitioning into liposomes is measured by $K_{LW}$ or $D_{LW}$.

The liposome - water distribution coefficient depends on a number of variables, such as solute lipophilicity, composition of the membrane, temperature, and among others, pH. At a physiological pH (5.5 - 7.5), many drugs are partially or fully charged and are
electrostatically attracted or repelled by many biological membranes that are composed of acidic lipids, and carry a net negative charge. Basic functional groups are ubiquitous among drugs that impart a net positive charge on the molecule and interact favorably with the charged membrane.

For ionizable compounds, partitioning into liposomes is influenced by protropic equilibria as illustrated for a basic drug in Figure 4-1. The extent of drug ionization is determined by their $pK_a$ and solution pH, which in turn influence a drug’s ability to interact with (or partition into) cell membranes, and is of great importance to the behavior, activity, and usefulness of the drug. The observed distribution coefficient of a charged drug takes into account the partitioning of both the charged and neutral forms of the drug. Each of these forms will partition into the liposomes as illustrated in Figure 4-1; $K_B$ is the partition coefficient of the neutral form and $K_{BH^+}$ is the distribution coefficient of the charged form of a basic drug.

Much work has been done studying the pH - dependent drug - membrane binding and lipid - mediated $pK_a$ shifts of drugs, especially anesthetics, including tetracaine (13-15), which is used in this work. pH - dependent partitioning of charged solutes into liposomes has been determined using pH-metric, ultrafiltration, and equilibrium dialysis methods (16,17,32,33). Additionally, authors have examined the differences between liposome - water and octanol - water partitioning as a function of pH, where the octanol - water partition coefficient is the standard model for drug lipophilicity (17). The use of LEKC to study electrostatic interactions influencing charged drug partitioning into liposomes is discussed in Chapter 3. This work included studies on the effect of membrane and buffer compositions on
the retention of basic drugs. Additionally, LEKC has been used in QSAR correlations with membrane permeability (Caco-2, MDCK, human jejunal) and intestinal absorption of a series of charged and neutral drugs. These influence of pH on these QSARs is discussed in Chapter 5.

LEKC is a capillary electrophoresis (CE) technique that uses phospholipid vesicles as a pseudostationary phase in CE. In LEKC, solutes interact with certain sites on the pseudostationary phase by hydrophobic and/or electrostatic interactions and are separated based on their differential partitioning into the liposome phase. The retention factor, k, represents the fraction of the solute in the liposome versus the aqueous phase, which is used to describe the degree of interaction with the liposome.

Determining distribution coefficients for the binding of charged solutes to liposomes can be accomplished via LEKC much in the same manner as it is done for micelles via Micellar Electrokinetic Chromatography (MEKC) (18,19). The exception is that liposomes constitute the pseudostationary phase instead of micelles. LEKC is a simple approach to determining liposome-water partition coefficients for organic solutes (10) and drugs (20).

In this work the pH-dependent affinity for net negatively charged liposomes was determined for a series of basic drugs using LEKC/CZE methods. Quantitative models were applied to examine the mobility and retention as a function of drug ionization. Traditional titration methods were used to determine the pK_a of tetracaine in aqueous and liposome solutions. The shift in pK_a between the aqueous and liposome phase was determined as a function of the mole fraction of the negatively charged lipids in the liposome.
Materials and Methods

Reagents

2-[N-Morpholino]ethanesulfonic acid (MES), 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), and Tris(hydroxymethyl)-aminopropane (TRIS) were purchased from Sigma. Tetracaine, nefopam, and lidocaine were purchased from Aldrich. 1,2-Dihexanoyl-sn-Glycero-3-Phosphocholine (DHPC), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), and 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DPPG), were obtained from Avanti Polar Lipids, Inc.

Liposome Preparation

A mixed buffer was used for the CE studies in order to maintain a high buffer capacity and constant ionic strength across the pH range 6 to 11.5. The importance of using a constant ionic strength buffer in LEKC studies involving charged drugs is discussed in Chapter 3. Buffers containing multiple components and constant ionic strengths were prepared according to the software program developed by Okamoto, which is used to determine the quantity of various buffer components required to achieve a certain pH at given ionic strength and temperature conditions (21). A description and application of the buffer program is found in reference 21 by Okamoto. Dr. Okamoto has graciously donated a copy of the software. The desired buffer conditions (buffer type, concentration, total solution ionic strength, pH, and temperature) were entered into the program, and the software program determined the quantities of buffer components (buffer amounts and NaCl) as well as acid/base (HCl or NaOH) required to prepare the buffer at the specified conditions. All
buffers consisted of 10 mM MES, 10 mM TRIS, and 10 mM CAPS. The total ionic strength of all buffers was held constant at 29 mM by the addition of NaCl; the quantity of NaCl added was determined by the buffer program. All buffers were prepared and the pH was measured at 36°C, the temperature for CE studies. Buffers were prepared to cover the pH range of 6 to 11.5.

Liposomes for use in CE experiments were prepared using a mixture of the short and long chain phosphatidylcholines, DHPC and DPPC, respectively, (both zwitterionic lipids) and the long chain anionic phospholipid, DPPG. DPPG was added to provide a net negative charge to the liposomes. Phospholipid structures are found in Appendix A-1. The composition consisted of DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$, where the subscripts represent molar percentages. The total lipid concentration was 10 mM. Similarly, mixtures of the short chain DHPC and long chain dimyristoylphosphatidylcholine (DMPC) have been used as a pseudostationary phase in electrokinetic chromatography (22). Mixtures of short and long chain lipids are known to form bicelles, or biomimetic vesicles (23,24). The lipid solution consisting of DPPG, DPPC, and DHPC in this work is referred to as "liposomes" even though it has not been verified whether it is actually mixed micelles, bicelles, or liposomes in solution. Bicelles form under very specific of conditions including concentration, molar ratio of lipids, buffer, temperature, etc. (25).

The appropriate amounts of phospholipids were dissolved in a 9:1 volume mixture of chloroform and methanol (respectively). The organic solvent was removed under reduced pressure using a rotary evaporator in a water bath maintained at 70°C. The thin lipid film was
hydrated with the buffer solution. The solution was then vortex mixed for one minute and
sonicated in a bath sonicator for approximately 5 minutes before use.

Liposomes for use in titration experiments included various molar ratios of DPPG and
DPPC; the compositions consisted of DPPC_{100}, DPPG_{10}DPPC_{90}, DPPG_{20}DPPC_{80}, and
DPPG_{30}DPPC_{70}, where the subscripts represent molar percentages. The appropriate amounts
of phospholipids were dissolved in a 9:1 volume mixture of chloroform and methanol
(respectively). The organic solvent was removed under reduced pressure using a rotary
evaporator in a water bath maintained at 70°C. For the titration of tetracaine in the presence
of liposomes, the appropriate concentration of drug was first dissolved in the NaCl solution.
The drug - salt solution was used to hydrate the lipids. Following hydration, the lipids were
subjected to a freeze - thaw cycle where the hydrated liposomes were first frozen in liquid
nitrogen and then thawed in a water bath maintained at 70°C. A brief (~ 30 seconds) vortex
mixing was performed in between freeze - thaw cycles. This process was repeated for a total
of five freeze - thaw cycles. Westman et al. suggested that a true equilibrium between lipid,
water and anesthetic is only obtained after freeze - thawing because of the difficult diffusion
of the charged drug across a negatively charged membrane (13). The authors found a total of
five freeze - thaw cycles sufficient to equilibrate the drug and stabilize the concentrations
associated with the membrane, as a result of the breaking and reforming of the liposomes.

After the freeze - thaw procedure, the multilamellar vesicles (MLVs) were processed to
small unilamellar vesicles (SUVs) using the extrusion method to achieve a uniform size
distribution and smaller size liposomes. Extrusion was performed through polycarbonate
membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada) maintained at
a temperature of 60ºC by a circulating water bath. Extrusion was performed a total of five times through 200 nm pores size filters and ten times through filters with a pore size of 100 nm.

**CE Methods**

CZE and LEKC experiments were carried out on a laboratory built CE instrument. A Spellman SL30 high voltage power supply was used to apply a positive voltage over the length of the fused silica capillary (Polymicro Technologies, Phoenix, AZ), with an inner diameter of 50 µm and an outer diameter of 375 µm. The temperature of the system was maintained at 36°C using a circulating oil bath. The absorbance was measured at 214 nm using a SSI 500 variable - wavelength UV detector.

The retention factor, k, for a neutral drug was calculated from the LEKC data using Equation 4-1, where \( t_R \) is the retention time of the drug of interest, \( t_{eo} \) is the retention time of the electroosmotic flow marker, methanol, and \( t_{lip} \) is the retention time of decanophenone, the marker of the liposomes.

\[
\text{Equation 4-1} \quad k = \frac{(t_R - t_{eo})}{t_{eo}\left(1 - \frac{t_R}{t_{lip}}\right)}
\]

Charged solutes will possess their own electrophoretic mobility in the aqueous phase in addition to partitioning into the liposomes and migrating at the liposome mobility. As a result, the migration of the solutes in the bulk aqueous (\( t_o \), measured in CZE), needs to be
included in the calculation of retention factor. Equation 4-2 is used to calculate the retention factors of charged solutes using CZE and LEKC measured retention times.

\[
\text{Equation 4-2 \quad k} = \frac{(t_R - t_o)}{t_o \left(1 - \left(\frac{t_R}{t_{lip}}\right)\right)}
\]

The capillary was conditioned in the following manner: 10 minutes with Milli-Q water; 20 minutes with 1.0 M NaOH; 10 minutes with Milli-Q water; 10 minutes with methanol; 10 minutes with Milli-Q water. For LEKC experiments, the capillary was rinsed for 30 minutes with the liposome solution following the capillary conditioning. Following this rinse procedure, a voltage was applied for approximately 30 minutes to further equilibrate the column with the liposomes before sample injections were performed. At the end of the day, the capillary was rinsed for 10 minutes with Milli-Q water.

Since the rinse procedure has not yet been optimized, the conditions used in these experiments were longer than necessary to insure complete equilibration. The rinse procedure as described above could be shortened upon optimization. Additionally, liposomes do not need to be prepared immediately prior to use. In order to avoid the daily preparation time before running experiments, the liposomes can be prepared ahead of time and stored in the refrigerator for later use. Optimizing the rinse procedure and preparing liposomes ahead of time will significantly reduce the time required to prepare for data analysis.

As mentioned above and described by Equation 4-2, in order to determine retention factor, \(k\), for charged solutes in LEKC, the migration times, \(t_R\), in the presence of liposome (LEKC condition) and in the absence of liposome, \(t_o\), (CZE condition) have to be determined.
All CZE and LEKC solute mobility and retention factor values are the average of 4 measurements. The CZE data for each sample was collected immediately following the LEKC data after rinsing the capillary for 2 minutes with the buffer solution (i.e. in the absence of liposomes).

All buffers, liposomes, and solutions used for rinsing were filtered through a 0.45 µm filter disk (Scientific Resources Inc.) prior to use. The capillary was rinsed with the liposome solution for 1 - 2 minutes between LEKC injections. Approximately 0.02 - 0.06 g of each solute was dissolved in 3 mL of methanol to prepare stock solutions. Drugs for LEKC experiments are tetracaine, nefopam, and lidocaine. Drug structures are found in Appendix A-2. To prepare a sample for injection, approximately 50 - 200 µL of the stock sample was used, decanophenone dissolved in methanol was added where appropriate, and enough methanol to make 1 - 1.5 mL total sample volume. Generally mixtures of samples were injected for 1 - 2 seconds by hydrodynamic injection.

Retention factor in LEKC is directly related to the liposome - water partition coefficient, $K_{LW}$ as in Equation 4-3. Similarly, the liposome - water distribution coefficient ($D_{LW}$) can be substituted for $K_{LW}$ in Equation 4-3.

$$\text{Equation 4-3} \quad K_{LW} = \frac{k}{\phi_{LEKC}}$$

The LEKC phase ratio, $\Phi_{LEKC}$, is defined as the ratio of the volume of the liposome pseudo - phase ($V_{lip}$) over that of the aqueous phase ($V_{aq}$) and can be determined from the intrinsic properties of the phospholipids such as molar volume ($\nu$), critical aggregation
concentration (CAC), and phospholipid concentration (CPL) using the following relationship (Equation 4-4):

$$\phi_{LEKC} = \frac{V_{lip}}{V_{aq}} = \frac{v(C_{PL} - CAC)}{1 - v(C_{PL} - CAC)}$$

0.554 L mol$^{-1}$ was used as an estimate of the partial specific molar volume of the lipid solution ($v$), which was obtained as the weighted sum of the partial specific volumes of the individual lipids. This is similar to the estimation of partial specific molar volume in reference 20. Values of $v_{DPPG}$, $v_{DPPC}$, and $v_{DHPC}$ were taken as 1.01, 0.954, and 0.851 mL g$^{-1}$, for DPPG, DPPC, and DHPC, respectively (26). A value of zero was used for the CAC.

Titration Methods

Titrations were performed on the drug in the presence and absence of liposomes. For aqueous titrations, 10 mL of a 0.80 mM solution of tetracaine was used. For the liposome titrations, tetracaine was dissolved in the NaCl solution used to hydrate the lipids. Following extrusion, the liposomes were equilibrated with stirring at 36°C for 30 minutes prior to titration. The total phospholipid concentration was 5.0 mM and the tetracaine concentration was approximately 3.0 mM such that the molar ratio of lipid to drug was held constant at approximately 2:1. The concentration of NaCl in all titrations was 0.10 M. The pH meter was calibrated and all titrations were performed at 36°C and the temperature was maintained constant by a circulating oil bath. The total starting volume for all titrations was 10 mL. Standardized 0.5 M HCl was used to lower the starting pH to approximately 3 - 4. The titrant, standardized 0.1 M NaOH, was added in 0.010 or 0.020 mL increments to a final pH.
of at least 10.0. Following one minute of equilibration after an addition of NaOH, the pH was recorded when the change in pH was less than 0.01 per minute. pKₐ values were calculated according to the procedure outlined in reference 27 using the Henderson-Hasselbach equation. Under conditions of partial neutralization, the molar ratios of [BH⁺] and [B] are known from the degree of neutralization and calculated based on the known added amounts of standardized acid/base. The pKₐ is calculated at each addition of NaOH and the average of these pKₐ values is reported. For one titration, the pKₐ value is the average. The reported pKₐ values are the average of 3 or 4 titrations. It was assumed the ionic strength was nearly constant due to the added NaCl so that the activities of the ions were not taken into account.

Results and Discussion

LEKC Experiments

LEKC is a method that allows the rapid determination of liposome - water partition and distribution coefficients of neutral or charged drugs. This approach can be applied to determine the pH - dependent partitioning behavior of ionizable drugs where the migration parameters (retention factor or mobility) are measured as a function of varying aqueous buffer pH values. Applying quantitative models to the LEKC data allows very specific assessment of drug - membrane interactions as a function of drug ionization and pH. Models have previously been developed in this laboratory to quantitatively describe the migration (retention factor and mobility) of ionizable compounds in MEKC (18,19). These universal methods can be applied to the description of retention in LEKC.
The retention factor (k) of a basic drug is the weighted average of the retention factors of the charged and neutral forms, described by Equation 4-5. \( \alpha_{BH^+}^{aq} \) and \( \alpha_{B}^{aq} \) are the fractions of the charged and neutral drug forms in the aqueous phase, respectively. \( k_{BH^+} \) and \( k_B \) are the limiting retention factors of the fully charged and neutral forms, respectively.

Equation 4-5
\[
k = \alpha_{BH^+}^{aq} k_{BH^+} + \alpha_{B}^{aq} k_B
\]

Using Equation 4-5, one can derive the following equation (Equation 4-6), which is used to model the retention factor of a basic drug as a function of pH.

Equation 4-6
\[
k = \frac{k_{BH^+} + k_B (K_{a,q}^{[H^+]})}{1 + (K_{a,q}^{[H^+]})}
\]

\( K_{a,q} \) is the aqueous ionization constant of the drug in the absence of the liposomes, as noted in Figure 4-1. The sigmoidal retention profile (versus pH) reaches a plateau at low pH where the limiting retention factor is determined (i.e. this value of \( k_{BH^+} \) is the retention factor of the fully ionized drug). Likewise, there is a plateau region at high pH where \( k_B \) is the limiting retention factor for the fully dissociated form of the drug.

Similar to Equation 4-6, the sigmoidal relationship between distribution coefficient and pH is given by Equation 4-7, where \( D_{L,W} \) is the observed liposome - water distribution coefficient; \( K_{BH^+} \) and \( K_B \) are the limiting distribution coefficients of the fully protonated and neutral drug forms, respectively.

Equation 4-7
\[
D_{L,W} = \frac{K_{BH^+} + K_B (K_{a,q}^{[H^+]})}{1 + (K_{a,q}^{[H^+]})}
\]
Figure 4-2 illustrates the effect of the buffer pH on the distribution coefficients of the three basic drugs tetracaine (▲), nefopam (●), and lidocaine (♦). The symbols are the measured $K_{LW}$ values and the lines are the calculated values according to Equation 4-7. The regression results for the fit of the data in Figure 4-2 (using Equation 4-7) are given in Table 4-1.

The differences in partitioning of the three drugs can be explained by examining a combination of drug mobility in the presence and absence of the liposomes, along with the fractions of B and BH$^+$ associated with the lipid and aqueous phases. These fractions of association ($f$) were calculated using Equations 4-8 through 4-12, derived much like those in reference 18 for acidic solutes in MEKC. The derivation is described briefly below.

The fraction of the neutral drug (B) in the lipid phase is given by the concentration of the neutral drug in the lipid phase ([B]$_{lip}$) over the total concentration of drug. The total concentration of the drug includes the concentration of the charged and neutral drug in the lipid phase ([BH$^+$]$_{lip}$ and [B]$_{lip}$, respectively), and the concentration of the charged and neutral drug in the aqueous phase ([BH$^+$]$_{aq}$ and [B]$_{aq}$, respectively). This relationship is given in Equation 4-8.

\[
\frac{[B]_{lip}}{[B]_{lip} + [B]_{aq} + [BH^+]_{lip} + [BH^+]_{aq}} = f_B
\]

Substituting $K_{b,B} [B]_{aq} C_{PL}$ for [B]$_{lip}$, $K_{b,BH^+} [BH^+]_{aq} C_{PL}$ for [BH$^+$]$_{lip}$, and $[B]_{aq} [H^+] / K_{a,aq}$ for [BH$^+$]$_{aq}$, results in Equation 4-9, which is used to calculated the fraction of B in the lipid phase ($F_B$) as a function of aqueous pH, where values of $K_{b,B}$ and $K_{b,BH^+}$ are the limiting binding constant values (Table 4-2), described below; $K_{a,aq}$ is from Table 4-1; $C_{PL}$ is 10 mM.
Likewise, the fraction of the charged drug (BH\textsuperscript{+}) in the lipid phase is given by the concentration of the charged drug in the lipid phase over the total concentration of drug. After the same substitutions, Equation 4-10 is derived which is used to calculate the fraction of BH\textsuperscript{+} in the lipid phase ($f_{BH^+\text{lip}}$) as a function of pH.

**Equation 4-10**

\[
 f_{BH^+\text{lip}} = \frac{K_{b,BH^+C_{PL}}}{(1 + K_{b,B}C_{PL}) + ([H^+] / K_{a,aq}) (1 + K_{b,BH^+}C_{PL})}
\]

Similarly, the fraction of charged drug (BH\textsuperscript{+}) in the aqueous phase is given by the concentration of the charged drug in the aqueous phase over the total drug concentration. Using Equation 4-11, the fraction of the charged drug, BH\textsuperscript{+}, in the aqueous phase ($f_{BH^+\text{aq}}$) as a function of pH can be determined.

**Equation 4-11**

\[
 f_{BH^+\text{aq}} = \frac{([H^+] / K_{a,aq})}{(1 + K_{b,B}C_{PL}) + ([H^+] / K_{a,aq}) (1 + K_{b,BH^+}C_{PL})}
\]

The fraction of the neutral drug, B in the aqueous phase ($f_{B\text{aq}}$) is given by Equation 4-12.

**Equation 4-12**

\[
 f_{B\text{aq}} = 1 - f_{B\text{lip}} - f_{BH^+\text{lip}} - f_{BH^+\text{aq}}
\]

According to reference 28, the binding constant of a solute to liposomes ($K_b$) is directly related to the liposome - water partition coefficient ($K_{LW}$) as in Equation 4-13 (28); $v$ is the molar volume of the lipids. Converting $K_{BH^+}$ and $K_B$ (Table 4-1), to their respective binding constants (according to Equation 4-13) results in the values of $K_{b,BH^+}$ (binding constant of the
charged form) and $K_{b,B}$ (binding constant of the neutral form) listed in Table 4-2. Values of $K_{b,BH^+}$ and $K_{b,B}$ were used in the above equations (Equations 4-9 through 4-12) to calculate the fraction of association.

Equation 4-13

$$K_b = K_{LW}v$$

Figures 4-3, 4-4, and 4-5 show the various fractions of the neutral and charged form of tetracaine, nefopam, and lidocaine, respectively, in the lipid and aqueous phases. Solid lines represent the fraction of the drug in the lipid phase, and dashed lines represent the fraction in the aqueous phase. The neutral form (B) is represented by circles (●), and the ionized form ($BH^+$) is represented by triangles (▲).

Equations can also be used to describe the mobility behavior of charged drugs in the presence and absence of the lipids (18). In a CZE system (in the absence of the liposomes), the mobility of a charged solute in the aqueous phase is given by Equation 4-14, where $\alpha_{BH^+}$ is the fraction of protonation and $\mu_{BH^+}$ is the mobility of $BH^+$.

Equation 4-14

$$\mu = \alpha_{BH^+}\mu_{BH^+}$$

From Equation 4-14, one can derive Equation 4-15, which is used to model the drug's aqueous mobility as a function of solution pH. $\mu_0$ is the observed mobility of the solute, $\mu_{BH^+}$ is the mobility of the fully protonated cation, while $[H^+]$ is from the aqueous buffer pH, and $K_{a,aq}$ is the dissociation constant of the solute in the aqueous phase. All mobilities are calculated from the measured retention times, capillary length and voltage used in the CE experiments. Regression results of the CZE mobility data using Equation 4-15 are given in Table 4-3.
Equation 4-15
\[ \mu_0 = \frac{\mu_{BH+}([H^+]/K_{a,aq})}{1 + ([H^+]/K_{a,aq})} \]

The net mobility of the drug in LEKC is a weighted average of the mobility of the charged (BH\(^+\)) and neutral (B) drug in the aqueous (aq) and the lipid (lip) phase and is described by Equation 4-16.

Equation 4-16
\[ \mu = f_{BH+}^{aq} \mu_{BH+}^{aq} + f_{BH+}^{lip} \mu_{lip} + f_{BH}^{lip} \mu_{lip} \]

\( \mu \) is the observed mobility, \( f_{BH+}^{aq} \) and \( f_{BH+}^{lip} \) are the fractions of the charged drug (BH\(^+\)) associated with the aqueous (aq) and lipid (lip) phases, respectively. \( f_{BH}^{lip} \) is the fraction of the neutral drug form in the lipid phase. \( \mu_{BH+}^{aq} \) is the aqueous mobility of the drug and \( \mu_{lip} \) is the mobility of the liposomes. In the neutral form, the drug mobility is a result of its interaction with the lipids. The neutral form has zero mobility in the aqueous phase. The liposomes used in this work (DPPG\(_{20}\)DPPC\(_{30}\)DHPC\(_{50}\)) have a net negative charge, resulting in a negative mobility in LEKC. In the charged form, the drug mobility is a function of its interaction with the liposomes as well as its own aqueous mobility.

Using Equation 4-16, one could derive the following equation (Equation 4-17) to model the LEKC mobility of a basic drug as a function of pH.

Equation 4-17
\[ \mu = \frac{\mu_B + \mu_{BH+}([H^+]/K_{a,app})}{1 + ([H^+]/K_{a,app})} \]

In this equation, \( \mu \) is the observed LEKC mobility of a basic drug at a given [H\(^+\)]. \( \mu_B \) and \( \mu_{BH+} \) are the limiting mobilities of the neutral and protonated forms of the drug, respectively. \( K_{a,app} \) is the apparent ionization constant of the drug in the presence of the lipids. Regression
results for the LEKC mobility as a function of pH (using Equation 4-17) are given in Table 4-4.

The mobility of tetracaine as a function of pH in the presence (●) and absence (♦) of DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ (subscripts represent molar percentage) is shown in Figure 4-6. The structure of tetracaine is included in the figure as well. The corresponding plots for nefopam and lidocaine are given in Figures 4-7 and 4-8, respectively. The symbols are the measured data points and the lines are the calculated mobility values in the presence and absence of DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ lipids, according to Equations 4-17 and 4-15, respectively. Error bars are included for all data points, however due to the scaling, the bars are generally not seen since they are smaller than size of the symbols.

Simultaneously examining the three types of plots discussed above (retention vs. pH, fraction of association vs. pH, mobility vs. pH) provides a better understanding of the partitioning behavior of the three drugs. For the lipid composition used in these studies, DPPG$_{20}$DPPC$_{30}$DPHC$_{50}$ (i.e. possessing a net negative charge), the basic drugs exhibit a sigmoidal relationship between $K_{LW}$ and pH (Figure 4-2), such that they have a larger distribution coefficient at low pH, which decreases as the pH is increased (i.e. $K_{BH^+} > K_B$). At low pH when the drugs are protonated, they have a greater interaction with the lipids compared to the interaction of the drug in the neutral form. The enhanced retention at low pH values is due to the electrostatic attraction of the positively charged drug to the net negatively charged lipid bilayer membrane. Electrostatics plays a significant role in the interactions of charged drugs with membranes which is discussed further in Chapter 3.
Out of the three drugs illustrated in Figure 4-2, the distribution coefficients (at all pH values) decrease in the order tetracaine > nefopam > lidocaine. The largest difference in distribution coefficient between the three drugs is at the low pH values, when the drugs are in the completely protonated form. According to Equation 4-7, the values for the limiting distribution coefficients (listed in Table 4-1) of the charged form ($K_{BH^+}$), are 1406 (± 47), 549 (± 16), and 46 (± 2) for tetracaine, nefopam, and lidocaine, respectively. As seen in the fraction plots for tetracaine and nefopam (Figures 4-3 and 4-4), at the lower pH values there is a significant fraction of the charged drug associated with the lipids. There is only a very small fraction of the drug associated with the aqueous phase at low pH (pH 6). Lidocaine is quite different due to its very small interaction with the liposomes. The lower retention of lidocaine (seen in Figure 4-2) can be explained by the larger fraction of charged lidocaine residing in the aqueous as opposed to the lipid phase (Figure 4-5). There is only a small fraction of $BH^+$ for lidocaine associated with the lipids at pH 6.

The difference in distribution coefficients (among the three drugs) is much smaller at high pH values (pH 11) when the drug is completely in the non-protonated (neutral) form. Values of $K_B$ are 360 (± 29), 129 (± 13), and 20 (± 1) for tetracaine, nefopam, and lidocaine, respectively (Table 4-1). For nefopam, the fractions of the neutral form of the drug in the aqueous and lipid phase are fairly close at high pH. For tetracaine, there is a significant difference, with a much greater fraction of the neutral drug (B) associated with the lipids. Tetracaine also has a larger distribution coefficient than nefopam at high pH (Figure 4-2). For the high pH values, the neutral lidocaine is almost exclusively in the aqueous. There is
only a very small fraction of neutral lidocaine associated with the liposomes. As a result, this drug has the least amount of retention in the liposomes.

The mobility of tetracaine in the absence of the liposomes (CZE) is positive at low pH values and sigmoidally decreases to zero as the pH increases and the equilibrium shifts to the neutral drug form, which has zero mobility in CZE (Figure 4-6). This is the typical migration profile for a basic drug in CE. The mobility of tetracaine in the presence of the liposomes (LEKC) is negative both at low and high pH due to a large interaction with the lipids (see Figure 4-3). The liposomes have a large negative mobility (-19.51 cm²/kVmin at pH 7.0), and therefore once the drug partitions into the liposomes, it will travel at this negative liposome mobility. The mobility sigmoidally decreases (becomes less negative) as the pH increases. The limiting mobilities of tetracaine in LEKC, \( \mu_B \) and \( \mu_{BH^+} \) are -12.2 (± 0.2) and -15.8 (± 0.3) cm²/kV min, respectively, calculated according to Equation 4-17. In this case, there is only a small difference in mobility between the charged and neutral forms of the drug because both forms interact with the lipids significantly, as seen in the corresponding fraction plot, Figure 4-3. In the low pH range, the first two terms in Equation 4-16 are the dominant ones, while the third term dominates at high pH values. For tetracaine, \( f_{BH^+}^{aq} \) is small (see Figure 4-3), therefore, \( f_{BH^+}^{lip} \) is the dominant term, and the mobility at low pH is primarily due to the interaction with the liposomes (and hence, the mobility of the liposome).

The mobility of nefopam as a function of pH in the presence (●) and absence (♦) of the liposomes, and the structure of nefopam is seen in Figure 4-7. Nefopam has a similar profile to lidocaine (Figure 4-6). Nefopam has a slightly reduced negative mobility in the presence of the liposomes compared with tetracaine. The limiting mobilities of nefopam in LEKC, \( \mu_B \)
and $\mu_{BH^+}$ are -7.9 (± 0.2) and -12.3 (± 0.2) cm$^2$/kV min, respectively (Equation 4-17, Table 4-4). Again, $f_{BH^+\text{lip}}$ is very important at low pH.

The mobility profile of lidocaine as a function of pH in the presence ($\bullet$) and absence ($\bullet$) of liposomes is different than tetracaine and nefopam and is displayed in Figure 4-8. Lidocaine has a very small interaction with the lipids (see Figure 4-2). The overall mobility observed in Figure 4-8 is mostly due to the drug mobility in the aqueous phase (as seen in Figure 4-5). In this case (contrary to tetracaine and nefopam), $f_{BH^+\text{aq}}$ is the dominant term in Equation 4-16 at low pH. The limiting mobility of lidocaine in LEKC, is -2.2 (± 0.1) and 6.1 (± 0.1) cm$^2$/kV min, for $\mu_B$ and $\mu_{BH^+}$, respectively (Equation 4-17). The difference in mobility (between CZE and LEKC) is greater at the lower pH values when the drug is in the protonated form. At high pH, there is only a small fraction of B associated with the lipids ($f_B\text{lip}$ is small), therefore the time the drug is traveling at the mobility of the liposome ($\mu_{\text{lip}}$) is very small (see Equation 4-16). Thus the mobility of lidocaine in LEKC at high pH is close to zero, because the neutral form has zero mobility in CE.

Austin et al. studied the distribution coefficients of charged drugs into the zwitterionic DMPC as a function of pH using ultrafiltration methods (17). They found a sigmoidal relationship between distribution coefficient and pH, however they found larger partitioning of the neutral form of the drug into the net neutral phospholipid liposomes. Similarly, Avdeef et al. determined membrane - water distribution coefficients for basic drugs (including lidocaine and tetracaine) into zwitterionic liposomes of dioleylphosphatidylcholine (DOPC) using the pH - metric technique (32). A sigmoidal partitioning - pH profile is observed, with the charged drug partitioning to a lesser extent into the zwitterionic membranes than the
neutral drug form. The authors rationalized these findings reporting that an uncharged amphiphilic species will be favorably bound to the membrane if the hydrophobic portion is embedded in the interior of the lipid bilayer, while the polar headgroup is oriented towards the membrane surface (32). An electrostatic pairing of the positively charged drug and the negatively charged phosphate group in the headgroup region of the lipid bilayer would require a movement away from the bilayer interior and cause the drug to reposition in the bilayer, moving to a less bound position (32). This is not surprising since the protonated form ($BH^+$) has an overall smaller polarity than the unprotonated form (B); thus has smaller affinity towards the neutrally charged medium of DOPC bilayers. In fact, a similar behavior is observed for partitioning into octanol; that is drugs ionization leads to smaller partitioning into octanol. The situation, however is different in natural membranes that possess a net negative charge due to the presence of anionic lipids.

The partitioning profiles obtained for PC membranes discussed above are quite different than those presented in this work for the net negatively charged DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ membranes (membranes containing PG). In this work, the electrostatic pairing of the charged drug with the net negatively charged membrane is stronger than the partitioning of the neutral drug form. The result is a greater retention factor at low pH (Figure 4-2). Kramer et al. reported on the pH dependent interactions of the basic drug propranolol in membranes composed of phosphatidylcholine (PC) and mixtures of PC and the anionic lipid, phosphatidylinositol (PI) using equilibrium dialysis (29). The authors reported the neutral propranolol is more strongly attracted to the PC membranes (net zero charge) than the protonated drug. In contrast, the protonated propranolol has a larger affinity for the PI
containing membranes than the neutral form of the drug (29). The results of Kramer for PC/PI mixed membranes is in agreement with the results presented here for mixed DPPC/DPPG membranes.

Much like the influence of pH on the retention and partitioning of charged drugs, pH will also significantly impact the membrane permeability of acidic and basic drugs. The relationship between permeability and pH is also sigmoidal in shape. Permeability coefficients of basic drugs through Caco-2 monolayers are small at low pH, and sigmoidally increase as the pH is increased, with the neutral form having a greater permeability coefficient. Palm et al. reported this for the cationic drugs cimetidine and alfentanil in the pH - dependent permeation through Caco-2 monolayers (30).

In general, the neutral form of drugs is thought to permeate through membranes to a greater extent than the charged form. Traditionally, the pH - partition theory has been applied to the transport of drugs across cell membranes. This idea relies on the assumption that only the unionized form of an ionizable drug is able to diffuse across the membrane. However, studies have shown that the contribution of the ionized form of the drug to membrane transport is significant when the drug has a fraction unionized less than 0.1 (30). This is significant because many drugs will be fully ionized over the entire physiological pH range. Similar to the transport of the drug across membranes, the partitioning of the ionized form of the drug into charged membranes is significant.

*Lipid - Mediated pKₐ Shifts*
The interactions of drugs with lipid bilayers alter their acid-base properties, thus shifting their ionization constants. The apparent ionization constants in lipid solutions are different from those in a purely aqueous phase. The magnitude of the pKₐ shift (ΔpKₐ = pKₐ,app - pKₐ,aq) depends on the difference of binding (i.e. partitioning) of the charged and uncharged forms of the drugs with the membranes. The pKₐ of the drug in the lipid (pKₐ,app) and in the aqueous phase (pKₐ,aq) are labeled on Figure 4-1. The magnitude of the shift in pKₐ (ΔpKₐ) is a function of various properties of the solute and properties of the lipid bilayer microenvironment where the solute resides, including the dielectric constant and surface potential. Drugs that reside deeper in the lipid bilayer will experience a lower dielectric constant and as a result, a greater shift in pKₐ value. Additionally, the charged surface of liposomes influences the shift in pKₐ.

When a drug interacts with a lipid bilayer, it experiences a significantly different microenvironment from the bulk aqueous. For example, estimates for the dielectric constant in the region of the phospholipid headgroups is about 32, compared with 78 in the bulk aqueous (31). On the other end, the dielectric constant deep in the hydrocarbon core of the lipid bilayer is reported to be around 2 (31). Therefore a drug residing in this headgroup region will be in a very different dipolarity region than a drug residing the aqueous or one embedded in the bilayer.

The pH-metric titration method uses these principles of a shift in ionization constant to determine the distribution coefficients of charged drugs (32,33). This pH-metric method involves a two-phase potentiometric titration where the drug substance is titrated both in the presence and absence of liposomes. The apparent pKₐ in the presence of liposomes may
deviate from the pKa in the absence of liposomes based on the differential partitioning of the charged and neutral form into liposomes. This shift in ionization constant is used to calculate the distribution coefficient of the charged drug.

From the LEKC retention data, it is possible to examine the lipid-induced shift in ionization constant. MEKC has previously been used to determine the micellar-mediated shifts in ionization constants upon binding of amino acids and peptides (34). Equation 4-18 is used to determine the $\Delta pK_a$ from the binding constants (18). $K_{b,BH^+}$ and $K_{b,B}$ are from Table 4-2 and $C_\text{PL}$ is 10 mM. $\Delta pK_a$ values calculated with Equation 4-18 are included in Table 4-5.

Equation 4-18

$$\Delta pK_a = \log \frac{1 + K_{b,BH^+}C_\text{PL}}{1 + K_{b,B}C_\text{PL}}$$

The $\Delta pK_a$ values obtained (from Equation 4-18) are 0.47, 0.37, and 0.05 for tetracaine, nefopam, and lidocaine, respectively.

Tetracaine has the largest retention with the liposomes, and the greatest $\Delta pK_a$ (from Equation 4-18). On the other hand, lidocaine has a much smaller overall partitioning and therefore has a $\Delta pK_a$ close to zero. The positive shift in pK_a as observed here is obtained when the charged form of the drug has a stronger binding than the neutral form. This is seen in Figure 4-2 comparing the retention factors at low and high pH, as well as by examining the binding constants in Table 4-2.

Shifts in pK_a values ($\Delta pK_a = pK_{a,\text{app}} - pK_{a,aq}$) can be examined using $pK_{a,\text{app}}$ as the apparent pK_a in the liposomes, from the regression data included in Table 4-4. $pK_{a,aq}$ is the aqueous ionization constant determined from the nonlinear regression fit of the CZE data (Table 4-3). $\Delta pK_a$ values determined this way for tetracaine, nefopam, and lidocaine are
0.12, 0.33, and 0.22, respectively. These values are also positive, indicating a stronger binding of the charged drug form as previously discussed. However, the $\Delta pK_a$ values are smaller than those determined by Equation 4-18. In this case, nefopam has a larger $\Delta pK_a$ value, due to a larger differential partitioning of the charged and neutral drug forms. The difference in $\log K_{BH^+}$ and $\log K_B$ for nefopam is greater than for tetracaine. Also, the difference between the fraction of charged and neutral nefopam in the liposomes is greater than for tetracaine.

There are good fits of the CZE mobility data, however the fits are not as good for the LEKC mobility data. This is likely due to the small difference in mobility of the associated and dissociated forms especially of the more hydrophobic drugs, similar to the work reported in reference 18. On the other hand, the fits of the retention data is better. In this case, the fit of $k$ versus $pH$ is better for the more hydrophobic drugs due to the greater difference in partitioning of the two forms, and lidocaine with the smallest interaction has a small differential partitioning and the worst fit out of the three. The $\Delta pK_a$ should be the same as calculated with Equation 4-18, however it is likely the error associated with fitting the plots which causes the differences.

In addition, potentiometric titration was used to investigate the effect of liposome composition on the shift in drug ionization constant. This was accomplished by varying the surface charge on the liposomes through the incorporation of 0 to 30 % DPPG (with 100 to 70 % DPPC, respectively). The lipid composition used in the CE studies (DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$) was unstable upon titration, resulting in a precipitation. Therefore liposomes composed of only the long chain DPPG and DPPC lipids were used in the titration.
studies. Tetracaine was selected for additional studies on the lipid-mediated shift in ionization constant. There was no evidence of precipitation of the drug or the lipids using these liposome compositions. Additionally, a higher ionic strength was used in the titration studies compared with the CE studies. Generally high salt concentrations are used in titration studies to reduce the repulsion of the positively charged drug molecules due to positively charged surface-bound drugs. These higher ionic strengths are not practical in CE experiments due to the negative effects of high current and joule heating.

The titration of 0.80 mM tetracaine in the absence of liposomes (0.10 M NaCl) resulted in a $pK_{a,aq}$ value of 8.28 ($\pm$ 0.01). This value is lower than the value of 8.48 ($\pm$ 0.04) that was determined by CE according to Equation 4-15. Again note different ionic strengths were used in the CE and titration studies. The ionization constant of tetracaine was also measured in the presence of liposomes (prepared using 0.10 M NaCl). According to the titration experiments, tetracaine has a lower $pK_{a,app}$ in the presence of liposomes. There is a shift in $pK_a$ ($\Delta pK_a = pK_{a,app} - pK_{a,aq}$), which varies based on the composition of the liposomes. $pK_{a,app}$ values measured using titration for DPPC$_{100}$, DPPG$_{10}$DPPC$_{90}$, DPPG$_{20}$DPPC$_{80}$, and DPPG$_{30}$DPPC$_{70}$ and their corresponding $\Delta pK_a$ values are included in Table 4-5.

With an increasing content of the anionic phospholipid, DPPG, the $pK_a$ of tetracaine becomes closer to that of the aqueous $pK_a$. Liposomes consisting of 100% DPPC (DPPC$_{100}$), the zwitterionic lipid, has the largest shift in $pK_a$, and the $\Delta pK_a$ decreases with an increasing DPPG content in the liposomes. However, the difference in $pK_a$ between the various compositions is very small. There is a trend of $\Delta pK_a > 0$ when comparing DPPC versus liposomes composed of DPPC and DPPG. The partitioning of basic drugs into liposomes
consisting of 5 to 50 % DPPG was examined in Chapter 3. Retention of the positively charged drugs increased with increasing percentage of the anionic lipid, DPPG.

When a drug partitions into a liposome, it primarily experiences the headgroup region. The outer headgroup area of a liposome is very complex with an interfacial region spanning the bulk aqueous to the hydrocarbon interior of the lipid bilayer. In this interfacial region there is a significant change of physical and chemical properties with location in the bilayer. Therefore a charged drug that electrostatically binds to a charged lipid headgroup might reside in a different location than the neutral form of the same drug which might find a position in the bilayer interior. In this case, the charged and neutral drug forms will experience different microenvironments. Therefore drugs that penetrate the lipid bilayer headgroup region to different depths will consequently experience varied dielectric constants.

As the percentage of DPPG in the liposomes increases, there is an increased water penetration in the liposomes due to a more open headgroup configuration caused by an increased electrostatic repulsion of the negatively charged headgroups. The dielectric constant and dipolarity will be influenced by the selection phospholipid, and importantly by the percentage of negative lipid in the liposome. The microenvironment in the lipid bilayer a drug will experience upon partitioning will therefore depend on the composition of lipids comprising the liposomes. The dipolarity of the liposome headgroup environment was probed as a function of lipid composition in Chapter 2.

The negative $\Delta pK_a$ for PC liposomes (Table 4-5) determined using titration is in agreement with the work of Schreier et al. who found a decrease in the apparent $pK_{a,app}$ of
tetracaine in the presence of egg phosphatidylcholine (egg PC) membranes (13). The decrease seen by Schreier was also a function of membrane concentration, such that larger membrane concentrations (at a constant lipid to drug ratio) resulted in a greater pK\textsubscript{a} shift (13). It is important to note that the liposomes for titration were prepared to maintain a constant lipid to drug ratio to avoid this dependence on concentration as discussed by Schreier. Additionally, Marsh et al. reported negative interfacial pK\textsubscript{a} shifts using EPR spectroscopy for different tertiary amine local anesthetics (including a tetracaine analogue) for the binding to dimyristoylphosphatidylcholine (DMPC) bilayers (15). These authors both report greater shifts in interfacial pK\textsubscript{a} values (on the order of $\Delta$pK\textsubscript{a} = -0.95) than reported here.

Westman et al. determined the pH - dependent partitioning of tetracaine into lipid bilayers and reported $\Delta$pK\textsubscript{a} values on the order of -0.77 for bilayers prepared using egg PC. Additionally, the authors report a positive $\Delta$pK\textsubscript{a} for tetracaine when studying the partitioning into PC/PS bilayers (13). The lower apparent pK\textsubscript{a} in the PC system (compared to the aqueous) could be due to the positive surface charge of bound (charged) tetracaine and the higher pK\textsubscript{a} values in PC/PS systems (compared to the aqueous) is due to the large negative surface charge caused by the anionic PS lipid (13).

$^2$H-NMR studies have indicated that the weakly bound charged tetracaine resides in the electrical double layer at the membrane - water interface with the charged dimethylammonium moiety of the anesthetic being located in the headgroup region (35). On the other hand, the uncharged form of tetracaine at high pH penetrates more deeply into the PC bilayer to a region of lower order (35). The authors also concluded that the positions of
charged and neutral tetracaine in the lipid bilayer membrane were the same for both PC and PC-PS containing membranes (35). If the same holds true in this work (i.e. tetracaine resides in a similar location for PC and PC-PG membranes), this could explain why larger differences in pK_a were not determined between the different compositions for titration.

The negative ΔpK_a results obtained via titration mean that the neutral tetracaine has a greater binding to the membranes than the charged form. This makes sense for the liposomes composed solely of PC, since the neutral form has been shown to have a stronger binding to the zwitterionic liposomes than the protonated form. However this is in contrast to the ΔpK_a results obtained using a slightly different vesicle composition (both have 20 % DPPG) using LEKC. The shift in pK_a is influenced by the dielectric constant in the interfacial zone of lipid bilayer membranes, and an additional (electrostatic) shift occurs when the surface of the lipid vesicles is charged (31). It is possible that the anesthetic concentration in the titration experiments was high enough (3.0 mM) to cause a buildup of positive charge on the membrane surface due to bound tetracaine. A positive surface charge would cause a negative shift in pK_a. For example, Fernández et al. found a shift in pK_a for tetracaine from 8.26 in the aqueous to 6.88 in the presence of cationic micelles due to the lower interfacial polarity and positive surface potential (35). This highlights one of the disadvantages of the potentiometric method, being the need for higher solute concentration. Additionally, impure substances will influence the quality of titration data. In the CE experiments, much smaller drug concentrations are used, and impurities are not as much of a problem.

Conclusions
The effect of the aqueous pH on the partitioning of basic drugs can easily be determined by LEKC methods. Basic drugs partitioning into net negatively charged liposomes have a sigmoidal decrease in retention with increasing pH. Applying quantitative models allowed an investigation of the contributions of the charged and neutral forms of the drug to partitioning by examining the fractions of each of these forms of the drug associated with the liposomes. The shift in the pK\textsubscript{a} value of the basic drug, tetracaine was examined using titration methods. The pK\textsubscript{a} shifts to a lower pK\textsubscript{a} value in the presence of liposomes, and the extent of the pK\textsubscript{a} shift depends on the membrane composition.

**Acknowledgement**

A research grant from the U.S. National Institutes of Health (GM 38738) is gratefully acknowledged.
Table 4-1. Estimates of $K_{BH+}$, $K_B$, and $pK_{a,aq}$ (± standard deviation) for tetracaine, nefopam, and lidocaine and the $R^2$ value from the nonlinear regression determined using Equation 4-7. Distribution coefficients were measured using 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>$K_{BH+}$ (±)</th>
<th>$K_B$ (±)</th>
<th>$pK_{a,aq}$ (±)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracaine</td>
<td>1406 (47)</td>
<td>360 (29)</td>
<td>7.9 (0.1)</td>
<td>0.990</td>
</tr>
<tr>
<td>Nefopam</td>
<td>549 (16)</td>
<td>129 (13)</td>
<td>8.0 (0.1)</td>
<td>0.986</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>46 (2)</td>
<td>20 (1)</td>
<td>7.2 (0.2)</td>
<td>0.982</td>
</tr>
</tbody>
</table>
Table 4-2. Values of $K_{b,BH^+}$ and $K_{b,B}$ for tetracaine, nefopam, and lidocaine determined using Equation 4-13, using the limiting distribution coefficient values in Table 4-1. $\Delta pK_a$ values are calculated according to Equation 4-18. Distribution coefficients were measured using 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36ºC in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>$K_{b,BH^+}$ (±)</th>
<th>$K_{b,B}$ (±)</th>
<th>$\Delta pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracaine</td>
<td>778 (± 26)</td>
<td>199 (± 16)</td>
<td>0.47</td>
</tr>
<tr>
<td>Nefopam</td>
<td>304 (± 9)</td>
<td>71 (± 7)</td>
<td>0.37</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>26 (± 1)</td>
<td>11 (± 1)</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 4-3. Estimates of $\mu_{BH+}$ (± standard deviation) and $pK_{a,aq}$ for tetracaine, nefopam, and lidocaine, and the $R^2$ value from the nonlinear regression of the CZE mobility data using Equation 4-15. Drug mobility values were measured at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>$\mu_{BH+}$ (cm²/kVmin)</th>
<th>$pK_{a,aq}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracaine</td>
<td>12.7 (± 0.2)</td>
<td>8.48 (± 0.04)</td>
<td>0.995</td>
</tr>
<tr>
<td>Nefopam</td>
<td>14.2 (± 0.3)</td>
<td>8.17 (± 0.05)</td>
<td>0.993</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>13.3 (± 0.3)</td>
<td>7.80 (± 0.05)</td>
<td>0.994</td>
</tr>
</tbody>
</table>
Table 4-4. Estimates of $\mu_{BH^+}$, $\mu_B$, ($\pm$ standard deviation) and $pK_{a,app}$ of tetracaine, nefopam, and lidocaine and the $R^2$ value from the nonlinear regression of the LEKC mobility data using Equation 4-17. LEKC drug mobility values were measured using 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36ºC in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>$\mu_{BH^+}$ (cm$^2$/kVmin)</th>
<th>$\mu_B$ (cm$^2$/kVmin)</th>
<th>$pK_{a,app}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracaine</td>
<td>-15.8 ($\pm$ 0.3)</td>
<td>-12.2 ($\pm$0.2)</td>
<td>8.6 ($\pm$ 0.2)</td>
<td>0.961</td>
</tr>
<tr>
<td>Nefopam</td>
<td>-12.3 ($\pm$ 0.2)</td>
<td>-7.9 ($\pm$0.2)</td>
<td>8.5 ($\pm$ 0.2)</td>
<td>0.978</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>6.1 ($\pm$ 0.1)</td>
<td>-2.2 ($\pm$ 0.1)</td>
<td>8.02 ($\pm$ 0.03)</td>
<td>0.999</td>
</tr>
</tbody>
</table>
Table 4-5. Values of pK_{a,app} and ΔpK_{a} (ΔpK_{a} = pK_{a,app} - pK_{a,aq}) for tetracaine in the presence of DPPC_{100}, DPPG_{10}DPPC_{90}, DPPG_{20}DPPC_{80}, and DPPG_{30}DPPC_{70}. All titrations were performed in 0.10 M NaCl at 36ºC; drug concentration was 0.80 mM for the aqueous titration and 3.0 mM for liposome titrations; lipid concentration was 5 mM. The aqueous pK_{a} of tetracaine (pK_{a,aq}) by titration is 8.28 (± 0.01).

<table>
<thead>
<tr>
<th>Composition</th>
<th>pK_{a,app}</th>
<th>ΔpK_{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC_{100}</td>
<td>7.85 (± 0.01)</td>
<td>-0.43</td>
</tr>
<tr>
<td>DPPG_{10}DPPC_{90}</td>
<td>7.90 (± 0.01)</td>
<td>-0.39</td>
</tr>
<tr>
<td>DPPG_{20}DPPC_{80}</td>
<td>7.97 (± 0.03)</td>
<td>-0.32</td>
</tr>
<tr>
<td>DPPG_{30}DPPC_{70}</td>
<td>8.04 (± 0.01)</td>
<td>-0.24</td>
</tr>
</tbody>
</table>
**Figure 4-1.** Ionization equilibrium and partitioning of a basic drug into a lipid bilayer. $K_B$ and $K_{BH^+}$ are the partition and distribution coefficients for the neutral and charged forms, respectively. $K_{a,aq}$ and $K_{a,app}$ are the ionization constants in the aqueous and lipid bilayer phases, respectively.
Figure 4-2. Distribution coefficient of tetracaine (▲), nefopam (●), and lidocaine (♦) as a function of pH. Symbols represent measured $K_{LW}$ values and lines are the fit of the data using Equation 4-7. Distribution coefficients were measured using 10 mM DPPG$_{20}$DPCC$_{30}$DHPC$_{50}$ at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.
Figure 4-3. Fractions of the ionized (▲) and neutral (●) forms of tetracaine in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Equations 4-9 and 4-10), and dashed lines represent the fraction in the aqueous phase (Equations 4-11 and 4-12). Distribution coefficients were measured with 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and $K_{b,BH^+}$ used to calculate the curves are from Table 4-2; $K_{a,aq}$ is from Table 4-1; $C_{PL}$ is 10 mM.
Figure 4-4. Fractions of the ionized (▲) and neutral (●) forms of nefopam in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Equations 4-9 and 4-10), and dashed lines represent the fraction in the aqueous phase (Equations 4-11 and 4-12). Distribution coefficients were measured with 10 mM DPPG₂₀DPPC₃₀DHPC₅₀ at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and $K_{b,BH^+}$ used to calculate the curves are from Table 4-2; $K_{a,aq}$ is from Table 4-1; $C_{PL}$ is 10 mM.
Figure 4-5. Fractions of the ionized (▲) and neutral (●) forms of lidocaine in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Equations 4-9 and 4-10), and dashed lines represent the fraction in the aqueous phase (Equations 4-11 and 4-12). Distribution coefficients were measured with 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36ºC in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and $K_{b,BH^+}$ used to calculate the curves are from Table 4-2; $K_{a,aq}$ is from Table 4-1; $C_{PL}$ is 10 mM.
Figure 4-6. Mobility of tetracaine as a function of pH in the presence (●) and absence (♦) of DPPG_{20} DPPC_{30} DHPC_{50}. Symbols are the measured mobilities and solid lines are the fit of the data according to Equations 4-15 and 4-17. Drug mobility values were measured in the presence and absence of 10 mM DPPG_{20} DPPC_{30} DHPC_{50} at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.
Figure 4-7. Mobility of nefopam as a function of pH in the presence (●) and absence (♦) of DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$. Symbols are the measured mobilities and solid lines are the fit of the data according to Equations 4-15 and 4-17. Drug mobility values were measured in the presence and absence of 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36°C in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.
Figure 4-8. Mobility of lidocaine as a function of pH in the presence (●) and absence (♦) of DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$. Symbols are the measured mobilities and solid lines are the fit of the data according to Equations 4-15 and 4-17. Drug mobility values were measured in the presence and absence of 10 mM DPPG$_{20}$DPPC$_{30}$DHPC$_{50}$ at 36ºC in a buffer consisting of 10 mM each of MES, TRIS, and CAPS with a total ionic strength of 29 mM.
References


Chapter 5

Drug - Liposome Partitioning Using LEKC for the Evaluation of Permeability and Intestinal Absorption
Abstract

Liposome Electrokinetic Chromatography (LEKC) is a convenient and powerful method for rapid screening of drug - membrane interactions. Liposomes were prepared using a composition of phospholipids to simulate Caco-2 cells. Retention factors were measured using LEKC for a series of charged and neutral drugs at pH 6.5 and 7.4. LEKC retention factor data was correlated with human intestinal oral absorption in comparison with other methods such as octanol - water partitioning, total number of hydrogen bonding groups, and polar surface area. LEKC retention data was also related to Caco-2, MDCK, and human jejunal permeability in comparison with the standard partitioning model, octanol - water.
Introduction

A great majority of drugs are administered orally due to convenience. Thus, transmembrane transport across the intestinal epithelia plays a predominant role in the gastrointestinal absorption of many orally administered drugs (1-3). Properties such as hydrophobicity, charge, hydrogen bonding, size, rigidity, and perhaps shape are the most important molecular and structural parameters that influence initial interactions and subsequent permeation of drugs through cell membranes that define the overall process of passive transmembrane transport.

With the widespread use of combinatorial synthesis of drug compounds there is a need for the development of High - Throughput Screening (HTS) of physico - chemical properties for characterization of pharmacokinetic properties that involve Absorption - Distribution - Metabolism - Excretion (ADME) in the early stages of drug discovery (4-6). The in- vitro methods for assessment of the absorption properties of drug candidates involve two key areas including membrane permeability assays and determination of compound affinities toward lipid bilayers of cell membranes. The permeability assays in cell cultures, such as the widely popular Caco-2 cells, are quite effective in predicting intestinal absorption behavior; however, they are time consuming and suffer from poor reproducibility. Alternatively, Parallel Artificial Membrane Permeability (PAMPA) assay has been introduced by Kansy et al. (7), for the permeability screening of drug compounds.

For assessment of drug - membrane interactions, the partition coefficient between water and octanol (logP_{ow}) has extensively been used as the scale for drug lipophilicity. However, it is well documented that the partitioning into isotropic bulk solvents is quite inadequate in
modeling solute partitioning into anisotropic, heterogeneous, and organized environments of lipid bilayers in cell membranes (8-11). Large numbers of drug molecules have ionizable functional groups and are either partially or fully charged under biological conditions. Octanol - water partitioning is unable to model these electrostatic interactions, and therefore is a serious misrepresentation of the actual drug affinity for membranes. As a result, a scale such as logP<sub>ow</sub> cannot possibly represent drug interactions with various membranes in absorption and distribution studies. Moreover, direct measurement of logP<sub>ow</sub> is time consuming, cumbersome, and requires pure samples in sufficient quantity.

Subsequently, much work has been done to use retention factor in HPLC systems with aqueous based mobile phases and stationary phases that mimic the lipid bilayer environments of cell membranes; examples are Immobilized Artificial Membrane (IAM-HPLC) (12-14), Immobilized Liposome Chromatography (ILC) (15,16), immobilized biomembrane chromatography (16), and biopartitioning micellar chromatography (17).

The key advantages of the HPLC methods are speed, small sample size, and lack of sample purity requirement. However, the most important shortcoming of HPLC based methods in Quantitative Structure Activity Relationship (QSAR) studies is the dependence of retention factor on column phase ratio, defined as the ratio of the volume of the stationary phase over the volume of the mobile phase. This phase ratio varies significantly between columns (of the same chemical composition) and with time, therefore it is not possible to establish a uniform, continuous scale for solute lipophilicity based on HPLC retention factors. On the contrary, a key advantage of lipophilicity scales that are based on the octanol - water partition coefficient is the continuity (or uniformity) that does not depend on extrinsic
parameters such as column phase ratio; although the variance in the logP<sub>ow</sub> databases can be quite large.

The main focus of an ongoing research in this laboratory is to better understand solute interactions with lipid bilayers and to define more biologically relevant measures of lipophilicity for use in QSAR research using artificial bilayer membranes such as liposomes. Liposome Electrokinetic Chromatography (LEKC) is the method of choice in such studies. LEKC is a capillary electrophoresis (CE) technique that uses liposomes as a pseudostationary phase and can be used in the separations of both uncharged and charged molecules. In LEKC, the liposomes are part of the solution in the capillary. Through the selection of appropriate lipids (i.e. lipid type and molar ratio) and “additives” such as cholesterol and proteins, the composition of the lipid bilayer “artificial membrane” can be controlled to mimic the properties of natural membranes that are important in trans-cellular (passive) diffusion.

LEKC provides clear advantages over both logP<sub>ow</sub> and HPLC techniques for true assessment of drug affinity for cell membrane lipid bilayers, or solute lipophilicity in pharmacokinetic and QSAR studies. Liposomes are more suitable models for biological membranes (than octanol or HPLC stationary phases) because they are composed of phospholipids and cholesterol and organize as lipid bilayers in aqueous solution (18). LEKC offers significant advantages over other existing methods such as speed, convenience, small sample size, and lack of sample purity requirement (19-22). Multiplexed 96-capillary CE systems with both absorbance and fluorescence detection are now commercially available, which would allow the rapid and high-throughput screening of large compound libraries.
Contrary to HPLC methods, the phase ratio in LEKC can be determined for a given pseudo-phase, and more importantly, phase ratio does not vary between instruments, capillaries, or with use (as with HPLC). Thus, it is possible to establish universal and consistent liposome-water partition coefficient scales for drug–membrane interaction studies. Retention factor in LEKC, \( k \), is directly related to the liposome-water partition coefficient, \( K_{LW} \), and phase ratio, \( \phi^{LEKC} \), as:

\[
Equation 5-1 \quad k = K_{LW} \phi^{LEKC}
\]

The LEKC phase ratio \( (\phi^{LEKC}) \) is defined as the ratio of the volume of the liposome pseudo-phase \( (V_{lip}) \) over the volume of the aqueous phase \( (V_{aq}) \) and can be determined from the intrinsic properties of the phospholipids such as molar volume \( (v) \), critical aggregation concentration \( (CAC) \), and phospholipid concentration \( (C_{PL}) \) using the following relationship (Equation 5-2):

\[
Equation 5-2 \quad \phi^{LEKC} = \frac{V_{lip}}{V_{aq}} = \frac{v(C_{PL} - CAC)}{1 - v(C_{PL} - CAC)}
\]

Previous reports from this laboratory discussed the use of LEKC for the determination of liposome–water partition coefficients and assessment of uncharged and charged solute interactions with lipid bilayers using LEKC \((23,24)\). Quantitative structure–activity relationships (QSARs) are mathematical models that can be used to relate the biological properties of a drug with its physicochemical properties. Other groups have reported the successful use of liposome–water partition coefficients in QSAR applications ranging from correlations with intestinal absorption to pharmacokinetic parameters like binding to plasma proteins \((25-29)\). The main focus of this chapter is to report the correlations between the
measured retention factor (which is directly related to the liposome – water partition coefficient) of a series of drugs in LEKC and intestinal absorption as well as relationships with several in-vitro and in-vivo measures of membrane permeability.

Materials and Methods

Reagents

3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS buffer), 2-(Cyclohexylamino)ethanesulfonic acid (CHES buffer), N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], and N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], sodium salt (HEPES buffer), were purchased from Sigma. Cholesterol (Chol), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (PC), 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-L-Serine] (Sodium Salt) (PS), 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine (PE), L-α-Phosphatidylinositol (Soy -Sodium Salt) (PI), and Sphingomyelin, (Egg, Chicken) (SPH) were obtained from Avanti Polar Lipids, Inc. All drug samples were purchased from Sigma.

Liposome Preparation

The buffer used was a three-component mixture consisting of 10 mM of each of the following buffers: HEPES, CHES, and CAPS. The total buffer ionic strength was held constant at 11 mM by the addition of NaCl and buffers were prepared at pH 6.5 and 7.4. All buffers were prepared and the pH was measured at the running temperature of 36°C. Buffers containing multiple components and constant ionic strengths were prepared according to the
software program developed by Okamoto which is used to determine the quantity of various buffer components to achieve a certain pH at given ionic strength and temperature conditions (30). A description and application of the buffer program is found in reference 30 by Okamoto. Dr Okamoto has graciously donated a copy of the software.

A complex liposome composition denoted as cell-mimic was prepared to simulate the phospholipids present in Caco-2 cells. This composition consisted of PC\textsubscript{52.6} PE\textsubscript{19.3} PS\textsubscript{16.6} PI\textsubscript{8.4} SPH\textsubscript{3.1}, where the subscripts represent the molar percentages of the components used. PS and PI are negative lipids in this mixture, making the total anionic lipid content 25 percent, leaving 75 percent composed of the zwitterionic lipids including PC, PE, and SPH. All liposomes in this study had a total lipid concentration of 10 mM and a constant cholesterol concentration of 3 mM (30 percent of the total lipid concentration).

The appropriate amounts of phospholipids and cholesterol were dissolved in a 9:1 volume mixture of chloroform and methanol (respectively). The organic solvent was removed under reduced pressure using a rotary evaporator in a water bath maintained at 70°C. The thin lipid film was hydrated with the appropriate buffer. Liposomes were prepared according to the extrusion method where the multilamellar vesicles (MLVs) were processed to small unilamellar vesicles (SUVs) to achieve a uniform size distribution and smaller size liposomes. Extrusion was performed through polycarbonate membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada) maintained at a temperature of 60°C by a circulating water bath. Extrusion was performed a total of five times through 200 nm pores size filters, five times through filters with a pore size of 100 nm, and ten times through the smallest pore size filter, 50 nm.
**CE Methods**

The CZE and LEKC experiments were carried out on a laboratory built CE instrument. A Spellman SL30 high voltage power supply was used to apply a positive voltage over the length of the fused silica capillary (Polymicro Technologies, Phoenix, AZ), with an inner diameter of 50 µm and an outer diameter of 375 µm. The temperature of the system was maintained at 36°C using a circulating oil bath. The absorbance was measured at 214 nm using a SSI 500 variable-wavelength UV detector.

The retention factor, k, for a neutral drug was calculated from the LEKC data using Equation 5-3, where $t_R$ is the retention time of the drug of interest, $t_{eo}$ is the retention time of the electroosmotic flow marker, methanol, and $t_{lip}$ is the retention time of decanophenone, the marker of the liposomes.

Equation 5-3  

$$k = \frac{(t_R - t_{eo})}{t_{eo} \left[1 - \left(\frac{t_R}{t_{lip}}\right)\right]}$$

Charged solutes will possess their own electrophoretic mobility in the aqueous phase in addition to partitioning into the liposomes and migrating at the liposome mobility. As a result, the migration of the solute in the bulk aqueous ($t_o$) needs to be included in the calculation of retention factor. Equation 5-4 was used to calculate the retention factor of a charged solute.
Equation 5-4

\[ k = \frac{(t_R - t_o)}{t_o \left(1 - \frac{t_R}{t_{lip}} \right)} \]

The capillary was conditioned in the following manner: 10 minutes with Milli-Q water; 20 minutes with 1.0 M NaOH; 10 minutes with Milli-Q water; 10 minutes with methanol; 10 minutes with Milli-Q water. For LEKC experiments, the capillary was then rinsed for 30 minutes with the liposome solution. Following this rinse procedure, a voltage (25 kV) was applied for approximately 30 minutes to further equilibrate the column with the liposomes before sample injections were performed. Typically liposome solutions were prepared simultaneously while the initial capillary rinse procedure was carried out. At the end of the day, the capillary was rinsed for 10 minutes with Milli-Q water.

Since the rinse procedure has not been optimized, the conditions used in these experiments were longer than necessary to ensure complete equilibration. The rinse procedure as described above could be shortened upon optimization. Additionally, liposomes do not need to be prepared immediately prior to use. In order to avoid the daily preparation time before running experiments, the liposomes can be prepared ahead of time and stored in the refrigerator for use at a later date. Optimizing the rinse procedure and preparing liposomes in advance will significantly reduce the time required to prepare for data acquisition.

As mentioned above and described by Equation 5-4, in order to determine the retention factor, \( k \), for charged solutes in LEKC, the migration times, \( t_R \), in the presence of liposomes (LEKC condition) and in the absence of liposomes, \( t_o \) (CZE condition) have to be determined. All CZE and LEKC solute mobilities are the average of 4 repeated
measurements. The CZE data for each sample was collected immediately following the LEKC data after rinsing the capillary for 1 minute with the buffer solution (i.e. in the absence of liposomes).

All solutions used for rinsing were filtered through a 0.45 µm filter disk (Scientific Resources Inc.) prior to use. The capillary was rinsed with the liposome solution for 1 - 2 minutes between LEKC injections. Approximately 0.02 - 0.06 g of each solute was dissolved in 3 mL of methanol to prepare stock solutions. To prepare a sample for injection, approximately 50 - 200 µL of the stock sample was used, decanophenone dissolved in methanol was added where appropriate, and enough methanol to make 1 - 1.5 mL total sample volume. Generally mixtures of samples were injected for 1 - 2 seconds by hydrodynamic injection.

**Determination of Liposome Size**

The average liposome size was determined using Photon Correlation Spectroscopy (PCS) using a Zetasizer 1000HS\textsubscript{A} (Malvern Instruments Ltd, Malvern UK) with a 5 mW Helium Neon laser at 633 nm. The scattered light was collected at an angle of 90°. All measurements were made at 36°C. The Malvern PCS software algorithm chosen to analyze the data was Contin and the size distribution profiles were analyzed using the method of volume. Average liposome sizes were obtained from at least three repeat measurements of the mean diameter of the liposomes. The average size of the liposomes used in this work is 52.3 (± 0.6) nm.
Results and Discussion

Correlations of LEKC Retention Data With Percent Oral Absorption

Table 5-1 includes log k values (with standard deviations) for 29 charged and neutral drugs. The distribution of drugs is 18 net positively charged, 6 net negatively charged, and 5 with a net neutral charge. Log k values were calculated according to Equations 5-3 and 5-4 for neutral and charged drugs, respectively. Note the phase ratio for the liposomes in this study has not yet been determined; however, LEKC retention factors are directly related to and thus represents liposome - water partition and distribution coefficients (K_{LW} and D_{LW}).

Table 5-2 includes literature values for human percentage oral absorption (17,31-41), calculated logP_{ow} (ClogP_{ow}) (42), Polar Surface Area (PSA) (38,43) and total number of hydrogen bonding groups (HB) (43). Drugs are listed by Sample ID, according to Table 5-1. When possible, oral absorption values were selected from reference 36. Other values are averages of absorption data taken from other sources. To the best of our knowledge the absorption behavior of the selected drugs is predominantly controlled by a transmembrane passive transport mechanism. Note the absorption data found in the literature contains large variance because drug absorption is strongly dependent on the experimental conditions used in the determination. Zhao et al. estimated the measurement error to be on the order of 14 % for a large set of absorption data compiled from the literature (36). This of course limits the predictability of any methods.

For drugs possessing ionizable functional groups, partitioning (represented by the retention factor), is pH - dependent. Therefore, retention factors were measured at two pH
values: 6.5 and 7.4. The pH value of 6.5 is used as an estimate of the average pH of the small intestine and 7.4 is the value for the plasma pH. For most drugs the retention at pH 6.5 was greater than the retention at pH 7.4, as expected. Basic drugs with high pKₐ values (i.e. pKₐ of maprotiline and nortriptyline both greater than 9.4) (42) show no change in retention factors (between the two pH values) due to complete protonation at both pH values. The retention of other basic drugs with lower pKₐ values (i.e. orphenadrine, pKₐ = 8.4) (42) decreases with an increase in pH from 6.5 to 7.4. This is due to a lower degree of ionization of the positively charged drug and hence a decrease in electrostatic attractions with the net negatively charged cell-mimic liposomes. Acidic solutes have a decreased interaction with the liposomes at high pH values due to the increased electrostatic repulsion of the negatively charged drug with the negatively charged lipid bilayer. As a result of the selective effect of pH on partitioning and the pH gradient in the small intestine, two pH values were examined. A more detailed study on the effect of pH on the partitioning of basic drugs is found in Chapter 4.

To assess the usefulness of LEKC data in the evaluation of important drug properties, the retention factor (k) or logarithm of the retention factor (log k) was correlated with absorption and permeability. Correlations of human fraction absorbed (%) with PAMPA data exhibit a steep slope region at low permeability values and a plateau region at higher permeability values (7,38). As a result, the PAMPA method is able to classify drugs into a poor or high absorption category. Interestingly, the relationship between % oral absorption and LEKC retention factor (at both pH 6.5 and 7.4) follows the same sigmoidal trend. This is illustrated for k measured at pH 6.5 in Figure 5-1 for the complete series of drugs (29 drugs)
listed in Table 5-1. The relationship of % oral absorption with LEKC retention factor (or liposome - water partition coefficient) indicates that initially drugs with higher affinity for lipid bilayers absorb better until the trend levels off. The existence of the plateau shows a minimum (or critical value) of lipophilicity that is necessary to reach a high level of absorption.

Sigmoidal relationships have been observed for the correlation of % oral absorption with octanol - water partitioning (36). To compare the LEKC retention data with ClogP_{ow}, the retention factor data was also examined in terms of the log k value. A sigmoidal relationship (Equation 5-5) is observed between percent oral absorption (%Abs) and liposome - water partitioning (log k), where x is either log k or ClogP_{ow}. A similar sigmoidal relationship was reported by Zhao et al. (reference 36) for logP_{ow}.

\[
\text{Equation 5-5} \quad \frac{\text{%Abs}}{100} = \frac{100}{1 + 10^{-a+bx}}
\]

This sigmoidal correlation is illustrated in Figure 5-2 for the complete series of drugs listed in Table 5-1 (log k, pH 6.5). Figure 5-3 is the corresponding plot for the retention factors (log k) measured at pH 7.4. The $R^2$ for the sigmoidal fit was 0.72 ($R = 0.85$) for pH 6.5, which is significantly better than the correlation at pH 7.4 ($R^2 = 0.25$, $R = 0.50$). At the higher pH value (pH 7.4), there is a significant scattering of points in the upper left corner of the plot (i.e. high absorption and low log k) for solutes with a log k less than zero. The drugs in this area all have a negative or net neutral charge. Many acidic solutes are known to be transported through a pathway other than passive transcellular diffusion, for example by carrier mediated active transport (i.e. salicylic acid, warfarin) (44), or paracellular transport.
Salicylic acid and warfarin were intentionally excluded from these experiments for this reason.

The corresponding plot for ClogP_{ow} is shown in Figure 5-4 with a R^2 of 0.48 (R = 0.69). The low correlation is due to one serious outlier, sulfasalazine. This drug has a high ClogP_{ow} value and a low % oral absorption; however sulfasalazine does not appear to be an outlier in LEKC determinations. Removing this outlier significantly improves the correlation to R^2 = 0.82 (R = 0.91). This plot shows much less scatter of the data points at the lower partition coefficient values.

Note that log P_{ow} values represent partitioning of solutes in their uncharged state, which is not the case for all of the compounds in this data set, the majority of which are either partially or fully charged. The correlation with log D_{ow} would then be a more accurate representation of the actual charge state of the compounds in the data set. Unfortunately, this is more difficult to examine due to the lack of availability of log D_{ow} data as well as a reduced solute set due to log D_{ow} only applying to charged drugs. The corresponding plot for log D_{ow} is essentially a scatter plot with very poor correlation.

The sigmoidal correlation of percent oral absorption with log k is similar in shape to the results reported in the literature for fraction absorbed correlated with partition coefficients into immobilized liposome membranes (35). In other work an R-value of 0.50 was reported for the sigmoidal correlation of liposome retention of 16 drugs with human fraction absorbed of orally administered drugs (determined by CE) (29). This R-value increased to 0.69 when the drugs sulfasalazine and antipyrine were excluded from the regression (29). Notably, these
authors observed better correlations for liposomes compared with micellar or microemulsion systems (29).

Comparing Figures 5-2 and 5-3 (i.e. pH 6.5 and 7.4), a better fit results from the lower pH data. Most of the positively charged drugs have a greater interaction with the liposomes at the lower pH values due to an enhanced electrostatic attraction with the negatively charged model membrane. The effect of liposome composition and pH on the liposome - water partitioning of positively charged drugs using LEKC is discussed in Chapter 4. Most of the negatively charged drugs have very little interaction with the liposomes due to the electrostatic repulsion with the negatively charged liposome. However, many cells have a large percentage of anionic lipids, and therefore this cell-mimic liposome composition was selected to mimic the composition of intestinal epithelial cells. Generally, an increased retention with the liposomes is observed at the lower pH values for the negatively charged drugs where a smaller fraction of the acidic drug is ionized.

The gastrointestinal tract exhibits a significant pH gradient, which suggests that the absorption of ionizable drugs is dependent on pH (and hence, location in the gastrointestinal tract). In this case, weak acids might be better absorbed in the lower pH region of the jejunum (e.g. the pH in the proximal jejunum can drop as low as 4.5 after food intake) and weak bases in the higher pH region of the ileum (45). The inherent flexibility of adjusting the composition of the pseudo-phase solution in LEKC greatly facilitates studying the effects of factors such as pH and membrane composition.

The steep slope in the % oral absorption vs. retention factor plot limits the predictive power of the relationship. Therefore to develop a linear QSAR relationship, the % oral
absorption data was represented by the logarithm of the ratio of % absorbed (A) over the % non-absorbed (NA): \[ \log \left( \frac{A}{NA} \right) \]. This ratio better represents the solute partitioning process since \( \log k \) is related to the ratio of the fraction of the drug associated with the liposome over the unassociated fraction in the bulk aqueous phase. This allows a linear correlation between \( \log k \) and \[ \log \left( \frac{A}{NA} \right) \]. An earlier report from this laboratory showed high correlations between retention factor in MEKC using bile salts micelles and small intestinal absorption in the rat (represented by \( \log \left[ \frac{A}{NA} \right] \)) for a series of 8 steroids (46). For drugs with 100% absorption, a value of 99% was used for the ratio correlations since the uncertainty in the % absorption is far greater than 1%. This allows all solutes to be used in the correlation, instead of removing the drugs with 100% absorption.

The linear relationship between \[ \log \left( \frac{A}{NA} \right) \] and \( \log k \) using the pH 6.5 data is shown in Figure 5-5 with three outliers removed (corticosterone, naproxen, and prednisolone). These three drugs all have higher absorption than estimated by the fit of the rest of the data. This data has a good correlation with a \( R^2 \) value of 0.71 (\( R = 0.84 \)) for 26 drugs. Additionally, according to this data, \( \log k \) determined at pH 6.5 results in a better correlation than pH 7.4. The corresponding correlation for the same set of solutes at pH 7.4, has a \( R^2 \) value equal to 0.55 (\( R = 0.74 \)). A significantly better correlation is obtained using LEKC retention data compared to the standard octanol-water partitioning model (ClogP_{ow}), which has a \( R^2 \) of 0.40 (\( R = 0.63 \)). Once again, if sulfasalazine is removed from the correlation as an outlier, there is a significant improvement in correlation to \( R^2 = 0.71 \) (\( R = 0.84 \)) which is similar to the correlation using the liposomes at pH 6.5. Again, the correlation with logD_{ow} is poor.
A long-standing pH-partitioning theory states that only the uncharged form of ionized drugs permeate through membranes (47). This theory is mainly based on the solubility-diffusion model that considers the lipid bilayer environments as isotropic solvents such as octanol, where partitioning of ionized drugs is substantially reduced. Thus, in QSAR models some workers simply ignore the solute ionization effects and use logP\text{ow}, which has provided improved correlations, however, one has to wonder if such models are chemically meaningful.

Octanol-water can only interact with drugs by hydrophobic, hydrogen-bonding, and dipolar interactions. Thus it fails to mimic ionic interactions between anionic phospholipids in the cell membranes and charged drugs in the interfacial region of the lipid bilayers. Liposomes provide both hydrophobic and charged sites for interaction, resulting in important differences in the interactions of the ionized forms of drugs with phospholipids compared with octanol. Additionally, liposomes can be composed of acidic phospholipids, much as in a cell membrane, resulting in differences in partitioning compared to net neutral systems. Liposome-water partitioning should be a more accurate representation of drug-membrane interactions than the octanol-water system regardless of the results of correlation analysis that could vary greatly depending on the set of test solutes. Nonetheless, the QSAR models show better correlations between LEKC retention with membrane permeability than the octanol-water system as discussed in the following section.

*Correlations Between LEKC Retention Data and Permeability*
The QSAR models were extended to the relationships of LEKC retention with permeabilities in two in-vitro cell culture systems of Caco-2 and MDCK as well as in the in-vivo human jejunal. Effective intestinal permeability is a fundamental parameter in describing the rate and amount of drug intestinal absorption, however such permeability measurements are often time consuming and are not amenable to rapid and / or high-throughput screening of large compound libraries. MDCK and Caco-2 cell permeabilities have approximately sigmoidal relationships with percent human absorption (32,48). Permeability measurements through a jejunal perfusion system give a direct in-vivo estimation of the absorption rate across the intestinal barrier (49).

Table 5-3 includes literature values for the logarithm of the permeability values for Caco-2 (38), MDCK (32), and human jejunal (HJ) (49) systems. There is a considerable variability in measurements of permeability with Caco-2 cells. Artursson et al. discussed these problems of inter- and intra-laboratory variability with Caco-2 data (50). Egan et al. found a variation ranging from 5.6 to 28.3 % when comparing average percent relative standard deviations from five different sets of published Caco-2 permeability data (51). Therefore, the Caco-2 data used in this study was selected from only one reference (reference 38) instead of combining Caco-2 permeabilities from various sources.

A sigmoidal relationship between octanol-water partitioning and Caco-2 permeability has previously been reported (41). Over a wide range of lipophilicity, the relation of logD<sub>ow</sub> to the logarithm of the permeability coefficient is sigmoidal in shape, and only linear in the mid range. This sigmoidal relationship between permeability in Caco-2 or MDCK cells and lipophilicity (expressed as log k or ClogP<sub>ow</sub>) is given in Figures 5-6 through 5-11 where the
sigmoidal curves are the fit of the experimental data using Equation 5-6. In Equation 5-6, \( x \) represents either \( \log k \) or \( \text{ClogPow} \).

\[
\text{Equation 5-6} \quad \text{LogPermeability} = \frac{a + b \left( \frac{c}{10^{-x}} \right)}{1 + \left( \frac{c}{10^{-x}} \right)}
\]

Past a certain retention factor, there is a plateau region where increasing the lipophilicity does not result in increased permeability. Figures 5-6 and 5-7 are the sigmoidal correlations of \( \log k \) with Caco-2 cell permeability (\( \log P_{\text{app}, \text{Caco-2}} \)) for pH 6.5 and 7.4, respectively. For the 15 drugs shown in these plots, correlations of \( R^2 \) equal to 0.81 (\( R = 0.90 \)) and 0.72 (\( R = 0.85 \)) are found for pH 6.5 and 7.4, respectively. This is better than the linear correlation for the same set of drugs (including those in the plateau region), which have \( R^2 \) values equal to 0.72 (\( R = 0.85 \)) and 0.65 (\( R = 0.80 \)) for pH 6.5 and 7.4, respectively. In these plots, three drugs (corticosterone, hydrocortisone, and ibuprofen) are removed as outliers. The outliers all fall above the line, having small \( \log k \) values and high permeabilities.

The corresponding sigmoidal plot for \( \text{ClogPow} \) with \( \log P_{\text{app}, \text{Caco-2}} \) is not as good, with an \( R^2 \) value of 0.41 (\( R = 0.64 \)) as seen in Figure 5-8. Again, removing the same point that was the outlier for \( \text{ClogPow} \) in the absorption studies (sulfasalazine), the correlation is significantly improved to \( R^2 = 0.59 \) (\( R = 0.77 \)). However, the correlation using LEKC retention factors determined at both pH values are better than this correlation. \( \text{LogD}_{\text{ow}} \) has a similar sigmoidal correlation to \( \text{ClogPow} \) with an \( R^2 \) value of 0.58 (\( R = 0.76 \)).

Other investigators have found an \( R \)-value of 0.70 for the linear correlation of Caco-2 cell permeability for 22 drugs with their respective liposome retention factor determined.
using CE (29). Pidgeon et al. found a good correlation (R-value of 0.762) for the correlation of the logarithm of permeability coefficients of eleven drugs through Caco-2 cells with the logarithm of the capacity factor in IAM (similar to the retention factor in LEKC) (12). In the work presented in this Chapter, sigmoidal correlations provided better fits (better R²) than linear ones.

Comparable to the Caco-2 correlations, a similar relationship between permeability and log k is observed for MDCK cells (Figure 5-9 and 5-10, for pH 6.5 and 7.4, respectively). The sigmoidal correlations with MDCK permeability are very good for 14 drugs with R² of 0.85 (R = 0.92) and R² of 0.79 (R = 0.89) for pH 6.5 and 7.4, respectively. This is better than the corresponding linear correlation for the same set of drugs of MDCK permeability with log k which has R² values of 0.77 (R = 0.88) and 0.74 (R = 0.86) for pH 6.5 and pH 7.4, respectively. This is for 14 solutes with two outliers removed (corticosterone, hydrocortisone). The linear correlation for the same set of drugs of MDCK permeability with ClogPow is very poor, with a R² value of 0.22 (R = 0.47); there is a significantly better correlation using the sigmoidal fit as shown in Figure 5-11 (R² = 0.47, R = 0.69). Again, removing the same ClogPow outlier, sulfasalazine, the sigmoidal correlation is significantly improved to R² = 0.71 (R = 0.84). The sigmoidal correlation of logDow with MDCK permeability is R² = 0.58 (R = 0.76). Once again, the better correlation is observed with log k compared to octanol - water partitioning.

The sigmoidal correlation of the solutes listed in Table 5-3 with human jejunal permeability is very good with R² values of 0.94 (R = 0.97) and 0.92 (R = 0.96) for pH 6.5 and 7.4, respectively. The linear correlation of the solutes listed in Table 5-3 with human
jejunal permeability is also very good with $R^2$ values of 0.86 ($R = 0.93$) for both pH 6.5 and 7.4. In this case, for such a small set of drugs, the sigmoidal correlation using ClogP$_{ow}$ is very similar, with a $R^2$ value of 0.92 ($R = 0.96$). The correlation with logD$_{ow}$ is not as good, with a $R^2$ value of 0.73 ($R = 0.85$).

Similar to the comparison of LEKC retention factor with percentage oral absorption, better correlations are seen in the correlation with permeability for the liposome - water interactions measured at the lower pH value of 6.5. Additionally, the correlations using LEKC retention data are better than the correlations using octanol - water partition or distribution coefficients. Generally, the negatively charged drugs are the points removed as outliers. Similarly, Ren et al. found much better correlations with Caco-2 permeability for various physicochemical parameters (including log D$_{ow}$) when the compounds were divided into three subgroups for neutral, cationic, and anionic compounds (52).

**PSA and HB Correlations With % Oral Absorption and Log k**

Many theoretical or computational methods have been developed for use in QSAR applications based on the calculation of molecular descriptors (53) including polar surface area (PSA) (33,54), number of hydrogen bonding groups (HB) (55), and Linear Solvation Energy Relationships (LSERs) developed by Abraham et al. using descriptors such as hydrogen bond accepting/donating ability, dipolarity, and size (44).

PSA and hydrogen bonding have been shown to influence in- vivo absorption (33,54,55). Sigmoidal relationships between PSA or HB and oral drug absorption in humans have been observed. Luthman et al. found strong sigmoidal relationships for the correlation
of 20 drugs with fraction absorbed in humans, with an $R^2$ value of 0.94 for polar surface area, and an $R^2$ value of 0.87 for the total number of hydrogen bonds (54).

Figures 5-12 and 5-13 show the relationship between % oral absorption and the two descriptors PSA and HB, respectively for the drugs listed in Table 5-1. The data was fit according to Equation 5-5. These correlations show a sigmoidal shape with low PSA ($\leq 60 \text{ A}^2$) relating to high absorption, and high PSA with low oral absorption. On the other hand, a smaller number of hydrogen bonding groups correlates with high absorption, and as the HB count increases, there is a sigmoidal decrease in absorption. The correlations of percent oral absorption with PSA or HB have been shown to be not as good when large sets of drugs are used.

Abraham et al. found the drug descriptors of hydrogen bond acidity, hydrogen bond basicity, and McGowan characteristic volume to be significant in their multilinear regression fit of the Abraham descriptors with oral absorption of a large database of drugs (44). Similarly, linear solvation energy relationship (LSER) models were used to unravel the contributions of various types of interactions to the partitioning into lipid bilayers as determined by LEKC. In these studies, size and hydrogen bond acceptor strength of solutes were the main factors that determined partitioning into liposomes (23).

PSA and HB are also factors that influence partitioning into liposomes. The log $k$ as a function of PSA correlation for all solutes has an $R^2$ of 0.59 ($R = 0.77$) for the complete solute set at pH 7.4 and a $R^2$ of 0.78 ($R = 0.88$) at pH 6.5. Removing three outliers (flurbiprofen, ibuprofen, and naproxen) significantly improves the correlations to $R^2 = 0.87$ ($R = 0.93$) and $R^2 = 0.91$ ($R = 0.95$) for pH 7.4 and 6.5, respectively. This linear correlation
is shown in Figure 5-14 for log k measured at pH 6.5 for 23 drugs. A higher PSA generally results in a lower log k value. For instance, dexamethasone has a high PSA (94.8 Å²) with a low retention in the liposomes (log k = -0.69 ± 0.02) and nortriptyline has a much lower PSA (12.0 Å²) and a significantly greater retention in the liposomes (log k = 1.59 ± 0.07). The higher PSA and lower retention relates to the lower absorption values seen in Figures 5-2 and 5-12.

The linear correlation of log k as a function of HB for all solutes has an R² equal to 0.51 (R = 0.71) for the complete solute set at pH 7.4 and an R² of 0.67 (R = 0.82) at pH 6.5. Removing the same three outliers (flurbiprofen, ibuprofen, and naproxen) significantly improves the correlations to R² equal to 0.86 (R = 0.93) and 0.87 (R = 0.93) for pH 7.4 and 6.5, respectively. This correlation is illustrated in Figure 5-15 for log k measured at pH 6.5 for 26 solutes. In general, a greater total number of hydrogen bonds results in a low degree of interaction with the liposomes. For example, at pH 6.5, atenolol, with 8 total hydrogen bonds, has a log k of -1.07 (± 0.03), while amitriptyline, with a hydrogen bond count of 1 has a log k of 1.55 (± 0.01) with the same liposome composition. The lower HB count (and hence higher retention) relates to the greater absorption values seen in Figures 5-2 and 5-15.

The correlations between log k and PSA or HB are slightly improved using a QSAR involving both PSA and HB. For all solutes (n = 26), there is a R² of 0.63 (R = 0.80) and R² of 0.81 (R = 0.90) for pH 7.4 and 6.5, respectively for the multilinear regression of log k with PSA and HB. The equations are as follows: log k = 1.32 - 0.04 PSA + 0.21 HB for pH 6.5, and log k = 0.80 - 0.04 PSA + 0.23 HB for pH 7.4. Once again, removing the three outliers (flurbiprofen, ibuprofen, and naproxen) significantly improves the correlations to R² equal to

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0.88 (R = 0.94) for pH 7.4 and R² equal to 0.91 (R = 0.96) for pH 6.5. The equations are as follows: log k = 1.89 - 0.03 PSA - 0.04 HB for pH 6.5, and log k = 1.59 - 0.01 PSA - 0.12 HB for pH 7.4. For both PSA and HB, the solutes determined as outliers all have a lower log k value than expected based on the correlation with the rest of the solutes. Notably, these are all negatively charged solutes. The relationship between log k and PSA and HB is not surprising. Previous LSER studies from this laboratory clearly indicated that increasing hydrogen bonding and dipolarity of solutes leads to smaller interactions with liposomes and vesicles (23).

**Conclusions**

LEKC was demonstrated as a valid method for the assessment of solute lipophilicity for evaluating membrane permeability and passive drug absorption. Better correlations with absorption and permeability were found for LEKC retention factors determined at pH 6.5 (compared to pH 7.4). LEKC retention data offers better correlations in these QSAR studies than the traditional octanol - water partitioning model. LEKC offers a number of advantages in studies of intestinal absorption and membrane permeability due to a small sample size with no sample purity requirement, in addition to the speed, convenience, automation, flexibility of adjusting the liposome pseudo - phase compositions to mimic natural membranes, and importantly the high - throughput capability. In addition, the possibility of determining the liposome phase ratio would make it possible to create universal scales for drug - membrane interactions based on liposome - water partition coefficients.
One of the advantages of the octanol - water system in QSAR research is the possibility of calculating solutes partition coefficients from their structure. Recent work in this laboratory has developed methodologies for calculation of liposome - water partition coefficients from molecular composition and structure (56). The models are based on additivity of constituent functional groups and fragments that have been originally developed for calculation of octanol - water partition coefficients. The combination of capabilities for calculation of partition coefficients and QSAR absorption - partitioning models could be quite useful for predicting partition coefficients (or lipophilicity) and subsequently absorption or permeability behavior of drug candidates prior to their synthesis.

**Acknowledgement**

A research grant from the U.S. National Institutes of Health (GM 38738) is gratefully acknowledged.
Table 5-1. Log k for 10 mM cell-mimic (10 mM PC_{52.6} PE_{19.3} PS_{16.6} PI_{8.4} SPH_{3.1} + 3 mM Chol) liposomes (measured at pH 6.5 and 7.4). Retention factors were measured at 36°C in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Drug Name</th>
<th>Log k (pH 6.5)</th>
<th>Log k (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acebutolol</td>
<td>-0.64 (0.01)</td>
<td>-0.62 (0.02)</td>
</tr>
<tr>
<td>2</td>
<td>Alprenolol</td>
<td>0.78 (0.03)</td>
<td>0.62 (0.01)</td>
</tr>
<tr>
<td>3</td>
<td>Amitriptyline</td>
<td>1.55 (0.01)</td>
<td>1.23 (0.07)</td>
</tr>
<tr>
<td>4</td>
<td>Atenolol</td>
<td>-1.07 (0.03)</td>
<td>-1.07 (0.03)</td>
</tr>
<tr>
<td>5</td>
<td>Chlorothiazide</td>
<td>-2.3 (0.6)</td>
<td>-1.09 (0.09)</td>
</tr>
<tr>
<td>6</td>
<td>Corticosterone</td>
<td>-0.545 (0.005)</td>
<td>-0.69 (0.01)</td>
</tr>
<tr>
<td>7</td>
<td>Desipramine</td>
<td>1.6 (0.2)</td>
<td>1.29 (0.09)</td>
</tr>
<tr>
<td>8</td>
<td>Dexamethasone</td>
<td>-0.55 (0.01)</td>
<td>-0.69 (0.02)</td>
</tr>
<tr>
<td>9</td>
<td>Doxepin</td>
<td>1.28 (0.07)</td>
<td>1.12 (0.12)</td>
</tr>
<tr>
<td>10</td>
<td>Flurbiprofen</td>
<td>-0.69 (0.03)</td>
<td>-1.08 (0.08)</td>
</tr>
<tr>
<td>11</td>
<td>Hydrochlorothiazide</td>
<td>-1.29 (0.01)</td>
<td>-1.43 (0.02)</td>
</tr>
<tr>
<td>12</td>
<td>Hydrocortisone</td>
<td>-0.90 (0.03)</td>
<td>-0.98 (0.01)</td>
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<td>13</td>
<td>Ibuprofen</td>
<td>-0.88 (0.09)</td>
<td>-1.4 (0.3)</td>
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<td>Imipramine</td>
<td>1.40 (0.08)</td>
<td>1.14 (0.02)</td>
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<td>Maprotiline</td>
<td>1.8 (0.1)</td>
<td>1.8 (0.5)</td>
</tr>
<tr>
<td>16</td>
<td>Metoprolol</td>
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<td>-0.29 (0.02)</td>
</tr>
<tr>
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<td>Nadolol</td>
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<td>Naproxen</td>
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<td>-1.7 (0.2)</td>
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<td>1.59 (0.07)</td>
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<td>Orphenadrine</td>
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<td>Oxprenolol</td>
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<td>Pindolol</td>
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<td>-0.10 (0.01)</td>
</tr>
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<td>23</td>
<td>Prednisolone</td>
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<td>-0.98 (0.01)</td>
</tr>
<tr>
<td>24</td>
<td>Progesterone</td>
<td>0.776 (0.004)</td>
<td>0.70 (0.01)</td>
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<td>Propranolol</td>
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<td>Sulfasalazine</td>
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<td>-1.49 (0.09)</td>
</tr>
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<td>Terbutaline</td>
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</tr>
<tr>
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<td>Trimipramine</td>
<td>1.54 (0.1)</td>
<td>1.2 (0.1)</td>
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<tr>
<td>29</td>
<td>Verapamil</td>
<td>1.01 (0.04)</td>
<td>0.64 (0.01)</td>
</tr>
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Table 5-2. Literature values of % oral absorption (17,31-41) ClogP$_{ow}$ (42), HB (43), and PSA (38,43).

<table>
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<tr>
<th>Sample ID</th>
<th>% Oral Absorption</th>
<th>ClogP$_{ow}$</th>
<th>HB</th>
<th>PSA ($A^2$)</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>1.61</td>
<td>9</td>
<td>92.8</td>
</tr>
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<td>93</td>
<td>2.59</td>
<td>5</td>
<td>43.7</td>
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<tr>
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<td>95</td>
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</tr>
<tr>
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<td>84.6</td>
</tr>
<tr>
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<td>76</td>
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<td>94.8</td>
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<td>3.53</td>
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Table 5-3. Literature values of the logarithm of the permeability for Caco-2 (38), MDCK (32), and human jejunal (HJ) (49) systems.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Log $P_{app}$ Caco-2 (cm/s, x 10^6)</th>
<th>Log $P_{app}$ MDCK (nm/s)</th>
<th>Log $P_{eff}$ HJ ($10^{-4}$ cm/s)</th>
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</thead>
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<tr>
<td>1</td>
<td>-0.29</td>
<td>1.23</td>
<td>-</td>
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<td>2</td>
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<td>-0.70</td>
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<td>5</td>
<td>-0.82</td>
<td>0.48</td>
<td>-</td>
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<tr>
<td>6</td>
<td>1.74</td>
<td>3.15</td>
<td>-</td>
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<tr>
<td>7</td>
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<td>-</td>
<td>0.64</td>
</tr>
<tr>
<td>8</td>
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<td>2.30</td>
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<td>-1.40</td>
</tr>
<tr>
<td>12</td>
<td>1.15</td>
<td>2.49</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
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</table>
Figure 5-1. % Oral absorption vs. k (pH 6.5) for 29 neutral and charged drugs, labeled according to Table 5-1. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC_{52.6} PE_{19.3} PS_{16.6} PI_{8.4} SPH_{3.1} with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-2. % Oral absorption vs. log k (pH 6.5), labeled according to Table 5-1. Regression results according to Equation 5-5: $R^2 = 0.72$, $R = 0.85$, $a = 1.26$, $b = -0.76$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-3. % Oral absorption vs. log k (pH 7.4), labeled according to Table 5-1. Regression results according to Equation 5-5: $R^2 = 0.25$, $R = 0.50$, $a = 0.85$, $b = -0.37$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-4. % Oral absorption vs. ClogP_{ow}, labeled according to Table 5-1. Regression results according to Equation 5-5: $R^2 = 0.48$, $R = 0.69$, $a = 0.00$, $b = -0.53$. 
Figure 5-5. Log (A / NA) vs. Log k (pH 6.5), labeled according to Table 5-1. Log (A / NA) is the logarithm of the ratio of % absorbed (A) over the % non-absorbed (NA). Linear regression results: $R^2 = 0.71$, $R = 0.84$; regression equation: $y = 0.51x + 0.92$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC_{52.6} PE_{19.3} PS_{16.6} PI_{8.4} SPH_{3.1} with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-6. Log Caco-2 permeability vs. log k (pH 6.5), labeled according to Table 5-1. Regression results according to Equation 5-6: \( R^2 = 0.81 \), \( R = 0.90 \), \( a = -0.96 \), \( b = 1.48 \), \( c = 4.51 \). Retention factors were measured at 36°C in liposomes consisting of 10 mM PC\textsubscript{52.6} PE\textsubscript{19.3} PS\textsubscript{16.6} PI\textsubscript{8.4} SPH\textsubscript{3.1} with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-7. Log Caco-2 permeability vs. log k (pH 7.4), labeled according to Table 5-1. Regression results according to Equation 5-6: $R^2 = 0.72$, $R = 0.85$, $a = -1.17$, $b = 1.46$, $c = 5.52$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-8. Log Caco-2 permeability vs. ClogP_{ow}, labeled according to Table 5-1. Regression results according to Equation 5-6: $R^2 = 0.41$, $R = 0.64$, $a = 1.17 \times 10^{14}$, $b = 0.87$, $c = -1.27 \times 10^{14}$. 
Figure 5-9. Log MDCK permeability vs. log k (pH 6.5), labeled according to Table 5-1.

Regression results according to Equation 5-6: $R^2 = 0.85$, $R = 0.92$, $a = 0.50$, $b = 3.47$, $c = 2.78$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-10. Log MDCK permeability vs. log $k$ (pH 7.4), labeled according to Table 5-1. Regression results according to Equation 5-6: $R^2 = 0.79$, $R = 0.89$, $a = 0.36$, $b = 3.48$, $c = 3.20$. Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-11. Log MDCK permeability vs. ClogP<sub>ow</sub>, labeled according to Table 5-1.

Regression results according to Equation 5-6: \( R^2 = 0.47 \), \( R = 0.69 \), \( a = 0.41 \), \( b = 2.59 \), \( c = 0.36 \).
Figure 5-12. % Oral absorption vs. polar surface area (PSA), labeled according to Table 5-1.

Regression results according to Equation 5-5: $R^2 = 0.66$, $R = 0.81$, $a = 2.10$, $b = 0.02$. 
Figure 5-13. % Oral absorption vs. HB, labeled according to Table 5-1. Regression results according to Equation 5-5: $R^2 = 0.72$, $R = 0.85$, $a = 2.73$, $b = 0.28$. 
Figure 5-14. Log k (pH 6.5) as a function of PSA, labeled according to Table 5-1. (Regression results: $R^2 = 0.91$, $R = 0.95$; regression equation: $y = -0.03 x + 1.83$) Retention factors were measured at 36°C in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
Figure 5-15. Log k (pH 6.5) as a function of HB, labeled according to Table 5-1. (Regression results: $R^2 = 0.87$, $R = 0.93$; regression equation: $y = -0.36 x + 2.22$) Retention factors were measured at 36ºC in liposomes consisting of 10 mM PC$_{52.6}$ PE$_{19.3}$ PS$_{16.6}$ PI$_{8.4}$ SPH$_{3.1}$ with 3 mM Chol in a buffer consisting of 10 mM each of HEPES, CHES, and CAPS, with a total ionic strength of 11 mM.
References


Chapter 6

Future Trends
Much of this work had focused on developing the basic methods for determining the distribution coefficients of charged drugs into model cell membranes, or liposomes. All of the factors studied in this work including varying buffers, dipolarity, membrane composition, pH, etc. are important factors influencing drug partitioning. The goal of exploring the fundamental factors influencing the liposome-water partitioning will aid in the development of LEKC as a rapid and high-throughput method for determining liposome-water distribution coefficients, and importantly the use of LEKC in Quantitative Structure Activity Relationship (QSAR) studies.

The use of LEKC in QSAR studies in evaluating parameters essential to drug development including intestinal absorption and membrane permeability was explored in Chapter 5. Good correlations are observed between the LEKC retention factor and intestinal oral absorption. The correlations are better than the corresponding correlations for ClogP_\text{ow} (calculated octanol-water partition coefficient) and logD_\text{ow} (octanol-water distribution coefficient). Likewise, the LEKC retention factor was correlated with intestinal permeability data, including the Caco-2 system. Again, this resulted in good correlations, and better results were obtained with the LEKC system compared with ClogP_\text{ow}.

The application of LEKC in a combinatorial chemistry setting for the high-throughput profiling of new drug compounds is discussed in the introduction and Chapter 5. While the preliminary data for using LEKC in these QSAR studies looks promising, before this can be applied as a high-throughput screening method, more work needs to be done. The number of solutes (n) in the correlations was fairly small in this work (n = 29 for intestinal absorption studies; n = 15 for Caco-2 permeability correlations; n = 14 for MDCK permeability
correlations; n = 7 for human jejunal permeability correlations) due to a small number of drug compounds available, and the high cost associated with purchasing a large set of drugs. Most importantly, the set of solutes needs to be significantly increased to provide a more meaningful correlation.

While large numbers of commercially available drugs are positively charged, the expanded set should also include more neutral and negatively charged drugs. A large number of the drugs included in this work have high oral absorption. Ideally, for studies relating LEKC retention data and intestinal absorption, the expanded data set should include more drugs with low to intermediate percent absorption values (0 - 80 % absorption). However, the problem here is due to the actual number of drugs available in these categories. Obviously, the drugs that are commercially available have favorable absorption properties in order to have passed through all stages of drug development. Therefore the number of drugs available with poor absorption properties is very small compared with the significant quantity of highly absorbing drugs. This, of course limits the selection of drugs.

An additional reason for the small number of solutes in the permeability studies is a result of the availability of permeability data. There is a large amount of variability between permeability measurements in different labs (1,2), therefore the permeability was selected from only one reference, and not combined from multiple sources. Therefore having a larger set of permeability data (i.e. all measured under the same conditions) would be beneficial. A set of permeability data measured in - lab would be good, or a collaboration with a laboratory measuring a common set of data would also be an advantage.
An alternative to measuring cell permeability (i.e. Caco-2 or MDCK), is using Parallel Artificial Membrane Permeability (PAMPA). The PAMPA method is used in studies of drug transport (3). Measuring drug permeability using the PAPMA method could be accomplished in the lab. Using a combination of PAMPA and LEKC would be beneficial for further exploring the relationship between the liposome - water partition/distribution coefficient (LEKC) and effective permeability (PAMPA).

There has been work in this lab to predict partition coefficients into liposomes and micelles based on solute structure (4). This allows the prediction of a liposome - water partition coefficient without actually measuring the retention by LEKC. The liposome - water partitioning behavior could be predicted and the values used in QSAR correlations to estimate properties such as absorption and permeability without having to run experiments. Expanding on the work for predicting partition coefficients and applying it in combination with the QSARs discussed in this work would make a powerful combination of techniques if applied in combinatorial chemistry laboratories.

As discussed in this work, there are great advantages to using chromatographic methods for the prediction of transport across biological membranes. In other examples, drug partitioning into Immobilized Artificial Membranes (IAMs) has been applied in the prediction of drug permeability across membranes such as human skin, rat and mice intestinal epithelium, and Caco-2 cells (5). Likewise, the use of LEKC to evaluate membrane permeability could be expanded and applied to different systems. Each biological membrane will have its own typical biochemical and physicochemical characteristics.
A very important parameter for central nervous system research is blood-brain distribution. The blood-brain barrier (BBB) must be permeated for drug uptake in the brain (6). The BBB is situated at the brain capillary endothelial wall, which segregates blood from interstitial fluid (6). Similar to intestinal permeation, measuring blood-brain (BB) distribution is a time-consuming and complicated task with a large amount of experimental error. Much as Caco-2 is used as a model for gastrointestinal absorption, BBEC (brain bovine endothelial cells) or BAEC (bovine aortic endothelial cells) are used to establish a model for the BBB (7).

IAM chromatography has been used in the study of BB distribution (8-10). The uptake of compounds across the in-vivo BBB expressed as the brain uptake index (BUI) has been correlated with IAM capacity factors (log kIAM) (10). Log kIAM was superior to the octanol-water system (logDow or ClogPow) for modeling brain uptake of lipophilic, polar, and ionizable compounds (10). LEKC could be applied to this same system, with the significant advantage over the IAM system being the flexibility of adjusting the lipid pseudostationary phase, as well as the issue of phase ratio (discussed in Chapter 3).

It is noted that the use of LEKC retention data to model permeation across cell membranes, whether they are intestinal epithelial cells or brain capillary endothelial cells, etc. relies on the assumption that the rate limiting step for the permeation across the membrane is the initial partitioning of the drug into the membrane. Therefore these methods may fail when other processes are involved, such as metabolism, protein binding, or carrier mediated transport.
References


APPENDICES
Appendix A-1

Phospholipid Structures

1,2-Dipalmitoyl-\textit{sn}-Glycero-3-Phosphocholine (DPPC)

1,2-Dipalmitoyl-\textit{sn}-Glycero-3-[Phospho-\textit{rac}-(1-glycerol)] (Sodium Salt) (DPPG)

1,2-Dipalmitoyl-\textit{sn}-Glycero-3-[Phospho-L-Serine) (Sodium Salt) (DPPS)
1,2-Dipalmitoyl-\textit{sn}-Glycero-3-Phosphoethanolamine (DPPE)

1,2-Dipalmitoyl-\textit{sn}-Glycero-3-Phosphate (Monosodium Salt) (DPPA)

L-\textit{α}-Phosphatidylinositol (Soy-Sodium Salt) (PI)

Sphingomyelin (Egg, Chicken) (SPH)
1,2-Dihexanoyl-sn-Glycero-3-Phosphocholine (DHPC)
### Solute Structures

**Table A-1.** Drug structures.

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Appendix A-3

Data Tables

Table A-2. log k (± standard deviation) values as a function of liposome composition for the effect of the percentage of anionic lipid. Data in Table A-1 was used in the second order polynomial regressions for the results listed in Table 3-2. Liposomes consisted of 10 mM lipids (compositions listed in table) and 3 mM Chol. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at pH 7.4 and 36°C.

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Table A-2 (continued)

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