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Elucidation of Chemically-Induced Transdermal Transport Processes

by

Betty Yu

Submitted to the Department of Chemical Engineering
in partial fulfillment of the requirements for the degree of

Doctor of Science

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2002

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Abstract

In this thesis, chemically-induced transdermal transport enhancement has been examined as one of the many techniques currently used to increase the skin permeability to a broader range of pharmaceutically relevant drugs. By taking advantage of the current developments in instrumentation technology, the mechanisms underlying the well-known chemical enhancer action of oleic acid have been examined using novel applications of Two-Photon Microscopy (TPM).

For the first time, TPM was used to visualize and quantify the oleic acid-induced three-dimensional spatial distributions of rhodamine B hexyl ester (RBHE), a model hydrophobic fluorescent probe, and of sulforhodamine B (SRB), a model hydrophilic fluorescent probe, based on a sampling of 4 to 6 different skin sites per skin sample. The fluorescent probe intensity profiles, that capture the fluorescent probe concentrations as a function of skin depth, were further evaluated using fundamental transport equations to quantify the oleic acid-induced changes in the vehicle to skin partition coefficient, the concentration gradient, the skin diffusion coefficient, and the skin barrier diffusion length. The application of the quantification methodology revealed that oleic acid-induced increases in the probe vehicle to skin partitioning was the primary effect for both the hydrophobic and the hydrophilic model fluorescent probes. The validity of the transport property enhancement values calculated based on the sample sizes examined (4-6 skin sites), was then addressed, in light of the inherent heterogeneity of the skin morphology.

The increased sampling efficiency provided by High-Speed Two-Photon Microscopy (HTPM) enables the imaging of clinically more relevant skin areas over shorter times. Using HTPM, the fluorescent probe spatial distributions in 400 consecutive skin sites, comprising a total skin area of 2mm by 2mm, were quantified for the control (no oleic acid exposure) and the enhancer (oleic acid exposure) cases of RBHE and SRB. Following the application of a randomized skin site sampling subroutine, the optimum number of skin sites needed to accurately represent the globally-induced changes in transdermal transport properties was determined. For the hydrophobic probe, a limited sampling of 4-6 skin sites was found to be sufficient, whereas for the hydrophilic probe, 12-24 skin sites was recommended. Furthermore, the oleic acid-induced variations in the wide-area spatial distributions of two transdermal transport parameters— the probe surface intensity and the probe intensity gradient— were evaluated to determine the rate-limiting
steps in transdermal transport for each fluorescent probe examined. Lateral diffusion through the lipid multilamellae, for the hydrophobic fluorescent probe, and probe partitioning from the vehicle into the skin, for the hydrophilic fluorescent probe, were determined to be the rate-limiting steps in transdermal transport.

In the final application of TPM presented in this thesis, the oleic acid-induced changes in the fluorescent probe spatial distributions with respect to the skin structural features were examined, for the first time, utilizing dual-channel HTPM, where the skin autofluorescence intensity and the probe intensity spatial distributions are simultaneously visualized. Using the skin autofluorescence image as a fingerprint that defines the spatial coordinates of the skin structural features, the relative spatial distribution of the exogenous fluorescent probe was characterized using image analysis techniques that included the application of the autocorrelation function and of the linear image intensity deconstruction methodology introduced to determine the extent of fluorescent probe penetration into the corneocytes. Oleic acid was observed to increase the hydrophobic probe localization to the intercellular region, while, for the hydrophilic probe, oleic acid-induced increases in the intracorneocyte probe penetration. In these studies, the corneocytes are proposed to act as diffusion sinks that contribute to the transdermal transport lag-time. In this light, oleic acid acts to minimize the lag-time effects by (1) circumventing the diffusion sinks altogether, for the hydrophobic probe, and (2) by 'filling-up' the diffusion sinks, for the hydrophilic probe.

The HTPM analyses of the oleic acid-induced changes in transdermal transport properties, presented in this thesis, demonstrate the universal applicability of the techniques introduced to elucidate the mechanisms underlying a broad spectrum of chemical enhancer formulations. The permeability enhancing effects of additional chemical enhancement techniques, including skin pretreatment with chemical enhancer formulations and protein-surfactant complexation, were also examined using diffusion cell experiments, and require the future application of the TPM analyses presented in this thesis to further elucidate their different mechanisms of action. Therefore, the high-throughput imaging capability of HTPM, coupled with the TPM imaging methodologies introduced in this thesis, serve as valuable tools in expediting the discovery of effective chemical enhancer formulations through the mechanistic insight extracted from the efficient evaluation of a wide array of chemical enhancer formulations.

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He who stands on tiptoe doesn't stand firm.
He who rushes ahead doesn't go far.
He who tries to shine dims his own light.

He who defines himself can't know who he really is.
He who has power over others can't empower himself.
He who clings to his work will create nothing that endures.

If you want to accord with the Tao, just do your job, then let go.

-LAO TZE

FOR MOM AND DAD,
TO DEAR FRIENDS AND COLLEAGUES.
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Chapter 1

Background: The Skin Barrier and Transdermal Drug Delivery

1.1. Introduction

Transdermal drug administration provides a noninvasive route of drug delivery with benefits including patient compliance, as well as the opportunity for continuous intervention in cases when the transdermal delivery device requires repositioning, removal, or replacement (Naik et al., 2000). In addition to the accessibility of large skin areas, the potential for sustained drug release and controlled input kinetics associated with transdermal drug delivery devices enables the unsupervised administration of drugs requiring frequent dosages and of drugs with very specific therapeutic windows (Guy, 1996).

Nevertheless, the US$2 billion transdermal market consists of only a handful of drugs. Among these drugs available to commercial transdermal drug delivery devices are eight FDA approved active agents: scopolamine, nitroglycerin, clonidine, oestradiol, fentanyl,
nicotine, testosterone, and norethisterone (Naik, et al., 2000). Despite the advantages offered by transdermal drug administration, the challenges presented by overcoming the skin permeability barrier has limited the availability of pharmaceutically relevant compounds via this route of delivery. Sonophoresis, iontophoresis, electroporation, and chemical enhancer formulations have been examined as potential methods of enhancing transdermal drug transport and are well described in the literature (Chen et al., 1998, Johnson et al., 1996, Mitragotri et al., 1995, Prausnitz et al., 1993). The focus of the work presented in this thesis is on the elucidation of the mechanisms of chemical enhancer action to expedite the discovery of effective chemical enhancer formulations that are currently subjected to trial and error experimentation.

1.2. The Skin

The primary functions of the skin include reducing water loss, protecting the body against the external environment, and providing a permeability barrier to toxic chemicals. The

![Schematic illustration of the brick and mortar model of the skin.](image)

Figure 1-1: Schematic illustration of the brick and mortar model of the skin.
skin is composed of three main layers— the stratum corneum (SC), the viable epidermis (VE), and the dermis (D). Figure 1-1 shows a schematic illustration of the SC and the VE, for which the SC, the uppermost 10 to 20 μm of the skin, is described by the brick and mortar model (Schaefer and Redelmeier, 1996). The stratum corneum provides the major resistance to transdermal drug delivery and consists of flat, polyhedral shaped cells termed corneocytes in a continuous domain of lipid multilamellae. In Figure 1-1, the corneocytes are labeled by the letter C and the SC lipid multilamellae are labeled by the letters LB. Corneocytes are approximately 40 μm in diameter and 0.5 μm thick (Johnson et al., 1997). The intercellular lipids surrounding the corneocytes reflect the mortar surrounding the bricks in Figure 1-1, and are required for proper barrier function of the skin (Norlén, 2001). In Figure 1-1, the keratinocytes, the undifferentiated corneocytes in the viable epidermal layer, are depicted by the letter K.

1.2.1. Skin Barrier Function

The skin barrier function is well illustrated when the SC is removed. Experiments involving tape-stripping of the stratum corneum indicated that Trans-Epidermal Water Loss (TEWL) increased with subsequent strippings (Scheuplein and Blank, 1971). Water and solute permeabilities are increased 10 to 20 fold following the removal of the stratum corneum.

The viable epidermis (100 μm) beneath the stratum corneum receives nutrients from interstitial fluid. Approximately 15% of the viable epidermis, by volume, is comprised of interstitial fluid which drains back to the lymphatic system (Halprin et al., 1967) The
dermal-epidermal junction, formed by papillae which increase the surface contact between the two layers, facilitates transport of nutrients to the epidermis by increasing the surface area through which passive diffusion occurs. A network of interconnecting proteins consisting of collagen, sulfated glycoproteins, and laminin, reduces the flux of large molecules (>40 kDa) across the epidermal-dermal junction (Mihara et al., 1992).

The dermis (1 mm) contributes primarily to the skin thickness, and provides the physical support for extensive nerve and vascular networks. Capillary beds reach the upper layer of the dermis such that capillary loops line the papillae at the dermal-epidermal junction (Ryan, 1983). The presence of the capillary networks at the dermal-epidermal junction exposes the permeant to the blood supply once it diffuses across the epidermis. In transdermal transport experiments, the epidermis (SC and VE) has been used as a skin model (Li et al., 1998), because of its relevance to the targeted site for systemic drug delivery— the capillary network at the dermal-epidermal junction. Furthermore, the dermal permeability barrier is insignificant relative to that of the SC (Schaefer and Redelmeier, 1996), such that skin permeabilities are well captured by the permeabilities of the SC and of the viable epidermal skin layers alone.

1.2.2. Transdermal Drug Transport Pathways Revealed by the Morphology of the Skin

Proposed drug penetration pathways include the intercellular and the transcellular routes, where molecules traverse the tortuous path of the SC lipid domain in the intercellular route, and molecules diffuse directly through the corneocytes and the lipid bilayers in the transcellular route. Despite these two physically available routes of penetration, the
intercellular route has been cited as the primary pathway of transdermal drug transport (Suhonen et al., 1999), because the rigid cornified envelopes surrounding the corneocytes exhibit a high resistance to drug penetration (Matoltsy, 1976). Moreover, the intercellular lipids provide the only continuous domain in the SC, through which permeants may diffuse. Hence, transdermal transport must involve traversal of the lipid multilamellae region.

![Image of transmission electronmicrograph](image)

**Figure 1-2: Transmission electronmicrogram of ruthenium tetroxide stained normal porcine skin illustrating intact lipid multilamellae (inset) bound to corneocytes (Warner et al., 1999).**

The structure of these well-ordered lipid multilamellae is illustrated in Figure 1-2, in which transmission electron microscopy was used to visualize ruthenium tetroxide stained normal porcine skin (Warner et al., 1999). As reflected by the “brick in mortar skin structure” described in Section 1.1, the corneocytes (the lighter gray domains surrounded by the darker gray lipid multilamellae in Figure 1-2) are tightly embedded in the continuous phase of lipid multilamellae. The inset of Figure 1-2 (upper left-hand
corner) is a higher magnification of the porcine skin sample, where the ordered lipid multilamellar intercellular region is now visible. Although the images shown in Figure 1-2 depict cohesive, ordered lipid multilamellae formations in the SC, imperfections have also been observed in normal murine skin using electron microscopy (Hou et al., 1991).

The presence of defects in the lipid lamellae, however, suggests interfacial transport of molecules between neighboring lipid bilayers as another possible pathway which may be less resistant than the interfacial transport of molecules across perfect lamellae. Evidence of the possible presence of gaps in the lipid bilayer head region would allow transfer of a molecule from a bilayer to the neighboring bilayer without the resistance presented by an interface in which no gaps are present. The presence of gaps in the SC has also been described as lacunae, and provides morphological evidence for hydrophilic permeant transport across the skin (Menon and Elias, 1997). For the hydrophobic permeants, lipoidal pathways paved by the intercellular multilamellar region provide a favorable environment for transdermal transport (Potts and Guy, 1995).

The lipid multilamellar spacing in human SC has been determined using small angle X-ray diffraction studies. Two lamellar phases were detected with periodicities of 13.4 nm and 6.4 nm. Furthermore, the lateral lipid spacing dimensions of 0.417 nm and 0.412 nm have been attributed to gel and crystalline lipid lattices, respectively (Bouwstra et al., 1992). The significance of the SC lipids in maintaining proper skin barrier function is illustrated in skin samples exhibiting Essential Fatty Acid Deficiency (EFAD), where increased TEWL was attributed to the presence of defects in the lipid depleted regions
(Hou, et al., 1991). Nevertheless, the region devoid of lipids in EFAD murine skin suggests that barrier properties observed in normal murine skin can be attributed to the presence of continuous lipid lamella throughout the interstices of the stratum corneum.

Figure 1-3: Transmission electron microgram of ruthenium tetroxide stained porcine SC exposed to the surfactant sodium dodecyl sulfate reveals chemical enhancer induced structural alterations of the lipid multilamellar structure (Warner et al. 1999).

Figure 1-3 depicts the alterations in porcine skin structure resulting from exposure to the surfactant sodium dodecyl sulfate, a known skin penetration enhancer (Warner, et al., 1999). In contrast to the well knit arrangement of the corneocytes within the lipid multilamellae matrix that is observed in Figure 1-2, skin exposure to a chemical enhancer such as SDS results in compromised barrier function. The structural features in Figure 1-3 (Warner, et al., 1999) indicate a deterioration of the barrier structure, with the corneocytes no longer tightly embedded within the SC lipid multilamellae. Note that, in Figure 1-3(a) (left image), the intercellular regions appear curled and detached from the corneocytes. Furthermore, Figure 1-3(b) (right image) illustrates the increased disorder in the lipid multilamellae that results from the chemical enhancer action. Hence,
understanding the effects of chemical enhancer induced perturbations of the SC structure on transdermal transport enhancement emerges as one aspect of this thesis.

1.2.3. Physicochemical Properties of the Stratum Corneum

The stratum corneum contains approximately 15% water, 70% protein, and 15% lipid by weight. Eighty-five percent of the dry mass of the stratum corneum is associated with protein, most of which can be attributed to the corneocytes. The core of the corneocytes contains densely-packed keratin and some filaggrin (Lopez et al., 2000). Keratin is insoluble in aqueous solutions and high ionic strength buffers containing nonionic detergents, but can be solubilized with denaturants such as the ionic surfactant sodium dodecyl sulfate. The lipid composition consists of 50% ceramide 1, a 34 carbon omega-hydroxy acid amide linked to sphingosine, 25% omega-hydroxy acid containing ceramide (more polar), 13% fatty acids, and 11% omega-hydroxy acids (Schaefer and Redelmeier, 1996).

Lipids covalently bound to the cornified envelopes correspond to 0.5 to 2.0 % of the dry weight of the stratum corneum, which has been calculated to sufficiently cover the surface of the corneocytes (Wertz et al., 1989). Covalently bound lipids have been hypothesized to play a role in limiting barrier discontinuities, organizing intercellular lipids, and restricting water from permeating into the corneocytes (Wertz, et al., 1989). Covalently bound lipids form an envelope around the corneocytes. Recently, the lipids bound to the corneocytes have been implicated in maintaining SC barrier integrity (Behne et al., 2000, Elias et al., 2000), where octyl glucoside induced disruption of the
corneocyte envelope reduces SC barrier function (Lopez, et al., 2000, Lopez et al., 2000). By volume, intercellular lipids take up 20% of the total stratum corneum volume (Elias and Leventhal, 1979). Lipid bilayers are formed by the alignment of the lipid hydrophilic head groups and the hydrophobic attraction of the lipid tail regions, such that the polar head groups interface the aqueous regions. A schematic representation of three different lipid bilayer conformations is shown in Figure 1-4 (Pilgram et al., 2001).

![Figure 1-4: Schematic illustration of lipid bilayer packing in liquid, hexagonal, and orthorhombic phases of SC lipid multilamellae (Pilgram et al., 2001).](image)

The liquid crystalline phase is illustrated by the wavy alkyl chain groups of the first lipid bilayer shown in Figure 1-4, and corresponds to a lipid separation distance of 0.46 nm. The next set of bilayers corresponds to the more ordered gel phase, which displays lipid spacings of 0.41nm. The final set of bilayers shown in Figure 1-4 illustrates the increased order of the solid crystalline phase which exhibits lipid spacings of 0.41nm and 0.37nm. Note that increased bilayer order corresponds with a decrease in the lipid packing
distances.

The ordered nature of the lipid bilayers, and the role of the covalently bound lipids, deter penetration of hydrophilic molecules, because of the unfavorable interaction between the hydrophobic tail region and the hydrophilic permeant. Although the high-octanol-to-water partition coefficients that hydrophobic permeants possess promotes favorable partitioning into the hydrophobic bilayer tail group region, the well-packed lipid head group region, as well as the ordered alkyl chain tails, can considerably restrict molecular partitioning due to hydrophobic permeant size.

Estimates of lateral diffusion for the SC lipid solid crystalline, gel, and liquid crystalline phases yield approximate values of $10^{-14}$ cm$^2$/s, $10^{11}$ cm$^2$/s, and $10^{-8}$ cm$^2$/s, respectively (Schaefer and Redelmeier, 1996). The packing structure and the degree of trans-gauche isomerizations of the lipids can be used to distinguish the three phases mentioned above. Orthorhombic packing structures are characteristic of the solid crystalline phase, and hexagonal packing structures are characteristic of the gel phase. Whereas the lipids in the crystalline phase all exhibit the trans conformation, the gel phase exhibits some gauche conformations, which decreases the interactions between the lipid chains and thus decreases the packing density of the lipid bilayers. Recently, the intercellular lipids of the lower SC lipid multilamellae have been described to exist in one single and coherent gel phase, devoid of any phase boundaries (Norlén, 2001). The existence of crystalline separation and phase separation in the lipid region at the SC surface has been attributed to the desquamation process in which keratinocytes differentiate into the final corneocyte
form (Norlén, 2001).

The liquid crystalline phase possesses no specific packing structure and is comprised of a mixture of trans and gauche isomers. Recall that in the gauche conformation, a kink is formed in the alkyl chain, decreasing chain-chain interactions (Schaefer and Redelmeier, 1996). Changes in the lipid phase more significantly affect the mobility of a permeant than do changes in lipid composition (1000-fold versus 10-fold). In the more fluidized states, more gauche isomers appear, which increases the area occupied by a lipid molecule in the bilayer (Knutson et al., 1987). The fluidity of the SC lipid multilamellae influences the permeant mobility across the skin, as the rate of diffusion is inversely related to the viscosity of the environment (Schaefer and Redelmeier, 1996).

Infrared (IR) spectroscopy and Differential Scanning Calorimetry (DSC) of oleic acid treated skin samples suggested that the chemical enhancer (oleic acid) increased drug permeation by fluidizing the stratum corneum (Francoeur et al., 1990, Suhonen, et al., 1999). Molecules with the capability of fluidizing lipid bilayers present themselves as possible chemical enhancers. The acidic pH of the stratum corneum at 5.0 may also affect the permeability of weak acids or bases (Knutson, et al., 1987). For example, the permeability coefficient of ibuprofen, which has a pKₐ of 5.2, is strongly dependent on pH, with higher permeabilities observed in the low pH range where the bulk of the compound is not ionized. For weak bases, such as narcotic analgesics, high pH values have yielded maximum permeabilities (Roy and Flynn, 1989). The pH of the skin may influence the ionization state of the permeant which, in turn, may influence its
The skin preference for hydrophobic compounds has limited the commercial availability of transdermally administered drugs of pharmaceutical relevance such as large or hydrophilic compounds. The ordered lipid multilamellar structure of the SC bilayers further inhibit penetration of molecules. While hydrophilic permeants possess inherently low SC permeabilities, the hydrophobic permeant transport may eventually be limited by its molecular size due to the highly ordered conformation of the lipids comprising the lipid bilayers. The hydrophobic environment of the SC intercellular region, coupled with the ordered lipid bilayer conformations that comprise this region, presents considerable challenges to transdermal transport of macromolecular compounds such as proteins, in the absence of external, mechanical SC barrier perturbation, such as ultrasound (Guy, 1996, Mitragotri, et al., 1995).

1.3. Mechanisms of Transdermal Transport

The mechanisms of transdermal transport include lateral diffusion, transbilayer transport,

![Diagram of transdermal transport mechanisms]

Figure 1-5: Schematic illustration of transdermal transport mechanisms.
and interfacial transbilayer transport, as illustrated in Figure 1-5. Referring to the schematic illustration of SC lipid multilamellae shown in Figure 1-5, lateral diffusion is depicted by the arrows labeled by \( D_{lat} \), where the permeant moves through the lipid tail region of the intercellular region. Transbilayer transport refers to permeant diffusion from one bilayer to the next, crossing the lipid head groups in the process (see arrows in Figure 1-5 corresponding to \( k' \)). Interfacial bilayer transport corresponds to the partitioning of the permeant into and out of the skin and is depicted by the arrows labeled by \( k_1 \) in Figure 1-5. The relevance of \( D_{lat} \), \( k' \), and \( k_1 \) to permeant transdermal transport will be explained below with respect to the expression for permeability shown in Eq.(1.1).

Johnson et al. employed the FRAP technique to measure the lateral diffusion of hydrophobic fluorescent probes, which spanned the molecular weight range of 223 to 854 Da, through lipid bilayers, and concluded that overall diffusion is rate limited by lateral diffusion for the molecules examined (Johnson, et al., 1997). The lateral diffusion coefficient characterizing the tortuous path traveled by a molecule in crossing the stratum corneum is represented by \( D_{lat} \), where for hydrophobic molecules, \( D_{lat} \) decreases with increasing molecular weight, until \( D_{lat} \) plateaus at approximately 1000 Da. Johnson et al. presented the following steady-state model of stratum corneum permeability (Johnson, et al., 1997):

\[
\frac{1}{P} = \frac{h \tau}{K_m D_{lat}} + \frac{2}{k_1} + \frac{n}{K_m k'} \tag{1.1}
\]

where

\[
D_{lat} = \frac{P}{K \frac{0.76}{0.76}} (3.6) \tag{1.2}
\]

26
where $P$ is the permeability, $h$ is the stratum corneum thickness, $\tau$ is the effective tortuosity, $K_m$ is the permeant lipid bilayer/water partition coefficient, $k_1$ is the mass transfer coefficient for entering the bilayer, and $k'$ is the intramembrane transbilayer transport coefficient. From Johnson et al. (Johnson, et al., 1997), good agreement exists between the calculated value of $D_{lat}$ from human skin permeabilities and those obtained experimentally using video FRAP on stratum corneum extracted lipids. Considering the three contributions to resistance in transdermal transport, Eq.(1.1) describes the permeability, $P$, of a drug across the skin.

Note that, in Eq.(1.2), the lipid bilayer/water partition coefficient, $K_m$, has been replaced by the octanol/water partition coefficient, $K_{o/w}$, and that the $ht$ term has been accounted for by its estimated value of 3.6 (Johnson, et al., 1997). Note that, if interfacial transbilayer transport were accounted for in calculating the $D_{lat}$ values, those values calculated from permeability data using Eq.(1.1) would be higher than those obtained from the FRAP measurements. The applicability of the lateral diffusion mechanism to larger probes, or to hydrophilic probes, requires further examination.

Correlations between the physicochemical properties of specific classes of permeants and skin permeation have implicated the intercellular route as the predominant pathway in transdermal transport. Tayar et al. used the parameter $\Delta \log K_{oh}$ ($\log K_{o/w}$ minus $\log K_{h/w}$, the log of the heptane-water partition coefficient) as a measure of the hydrogen bonding capacity of the permeant, and the lipophilicity of the permeant was expressed as $\log K_{o/w}$ (Tayar et al., 1991). Note that $K_{o/w}$ and $K_{h/w}$ are the octanol-water and the heptane-water
partition coefficients, respectively. The good correlation established between $\Delta \log K_{o/h}$ and $\log P$, the log of the permeability coefficient, suggested that an intercellular route took precedence over the transcellular route because of the abundance of hydrogen bond acceptor groups in the lipid phase arising from ester linkages, phosphate groups, and the like. Because of the hydrogen-bond interactions in the lipid bilayers, diffusion is much more hindered in the intercellular route than in the transcellular route in which the permeant crosses a series of aqueous and lipid phases. The alcohols and steroids investigated showed good correlation between the hydrogen-bond parameter and the permeability coefficient, thus implicating the intercellular pathway for those classes of compounds.

The question of structure-permeability relationships was also addressed by Guy and Potts (Guy and Potts, 1992). The log of the permeability coefficient, $P$, of 59 molecules was related to $\log K_{o/w}$ and to the molecular weight of the permeant. The derived value of $D_{o/h}$, which represents the diffusion coefficient of a hypothetical molecule having zero molecular weight divided by the diffusion path length, is related to the diffusion coefficient, $D$, in Eq.(1.3) below (Potts and Guy, 1992):

$$\frac{D}{h} = \frac{D_0}{h} \left[ \exp (-\gamma \cdot MW) \right] \quad (1.3)$$

where $MW$ is the molecular weight of the permeant, and $\gamma$ is a constant. As $\gamma$ approaches zero, the diffusion coefficient of a permeant through the SC approaches that of a molecule with zero molecular volume. $D$ can then be related to the partition coefficient of
the permeant between the stratum corneum and water, $K_m$, as follows (Potts and Guy, 1992):

$$\frac{D}{h} = \frac{K_m}{P}$$ \hspace{1cm} (1.4)

$K_m$ can then be linearly related to $K_{o/w}$ to take advantage of the values available in the literature. Using Eqs.(1.3) and (1.4), Potts and Guy concluded that the derived value of $D_o/h$, based upon fitting to experimental data, was orders of magnitude smaller than that corresponding to a simple biomembrane. That inconsistency led to the conclusion that the permeant does traverse a tortuous path, and that the value of $D_o/h$ is so small because of the large $h$ encountered in the stratum corneum, which is suggestive of the intercellular route of transdermal transport.

### 1.4. Empirical Relationships Describing Permeant Transport across the Stratum Corneum

Currently, many empirical models exist which relate permeability to the physicochemical parameters of relevant chemical compounds. Among such relationships are two separate models which are shown in Eqs.(1.5) and (1.6) below (Wilschut et al., 1995):

Guy and Potts (1993):

$$\log P = b_1 + b_2 \log K_{o/w} + b_3 MW^{0.5}$$ \hspace{1cm} (1.5)
Robinson (1993):

\[
P = \frac{1}{\frac{1}{P_{pcc}} + \frac{1}{P_{pol}} + \frac{1}{P_{aq}}}
\]  \hspace{1cm} (1.6)

In Eq.(1.5), \( P \) is the permeability coefficient, \( K_{o/w} \) is the octanol-water partition coefficient, \( MW \) is the permeant molecular weight, and the various \( b_n \)'s are regression coefficients fit to data in the literature. In Eq.(1.6) \( P_{pcc}, \ P_{pol}, \) and \( P_{aq} \) represent the permeability coefficient of the lipid, protein, and watery layer fractions of the stratum corneum, respectively. The Robinson model predicted the skin permeation of both highly hydrophilic and hydrophobic compounds with more accuracy than the Guy and Potts model (Wilschut, et al., 1995). The Guy and Potts model assumes that the stratum corneum is a lipophilic barrier to transport. Robinson also takes into account the presence of a watery layer in the epidermis, which affects the prediction of permeability for highly lipophilic chemicals. The hypothesis of the existence of a watery layer in the SC allows inclusion of a term in the overall permeability equation which describes the pathway of hydrophilic compounds across the SC. Moreover, Robinson considers the effect of molecular weight on three regions of the stratum corneum separately. Each of the three permeation pathways considered by Robinson are described in Eqs. (1.7), (1.8), and (1.9) below (Wilschut, et al., 1995):

\[
\log P_{pcc} = -1.326 + 0.6097 \log K_{o/w} - 0.1786 MW^{0.5} \]  \hspace{1cm} (1.7)

\[
P_{pol} = \frac{0.0001519}{MW^{1/2}} \]  \hspace{1cm} (1.8)
\[ P_{aq} = \frac{2.5}{MW^{1/2}} \]  

where \( P_{psc}, P_{pol}, P_{aq} \) are given in cm/hr.

The main difference between the models lies in the breakdown of the stratum corneum into separate components in the model presented by Robinson. The resistances caused by the lipid and protein fractions are considered to be in parallel, while the contribution to resistance of the watery layer acts in series with these two paths. The fact that the watery layer is represented as acting in series with the protein and lipid fractions of the stratum corneum suggests that in addition to diffusion through the "brick in mortar" structure of the stratum corneum, permeants may encounter a watery layer below the SC. The ability of Eq.(1.6) to yield more accurate predictions of permeability at the extreme hydrophobic and hydrophilic ranges indicates the importance of the assumptions about the pathway of diffusion made in formulating the model. While Robinson has assumed different mechanisms of operation for molecules of varying hydrophobicities by considering both a lipid and an aqueous pathway, Potts and Guy have assumed that transport across the skin occurs primarily through the SC lipid region. Good agreement between calculated and experimental permeability values will further reinforce the path by which diffusion of molecules occurs, depending on the assumptions of the model. Despite the availability of these empirical models, few models exist which predict transport based upon the physical structure of the lipid bilayers. Potts and Guy presented an algorithm to predict skin
permeability, which depends on the permeant molecular size and hydrogen-bond activity. Specifically,

$$\log P = (a_1 - \beta)MV + a_2\pi + a_3H_a + a_4H_d + a_5R_2 + \log \left( \frac{D_0}{\delta} \right) \quad (1.10)$$

where $R_2$ and $\pi$ reflect the polarity of the solute, $H_a$ and $H_d$ reflect its hydrogen-bond activity, and $MV$ is the molecular volume of the solute. The $a_n$'s in Eq.(1.10) are regression coefficients for multiple linear regressions of published permeability data, $\beta$ is a constant, and $\delta$ is the diffusion path length of the molecule. The molecules examined had molecular volumes between 10.6 and 114 cm$^3$/mol, and the log K_{ow} values ranged from -1.4 to 4.24. For such molecules, Eq.(1.10) suggests that epidermal transport decreases exponentially with increasing permeant volume, which is consistent with a lamellae based route of penetration (Potts and Guy, 1992). The ordered structure of the lipid lamellae suggests that solute diffusion through this barrier involves the creation of voids. As the energy to create a void increases with the size of the void, the probability of the event occurring in the lipid bilayer decreases. The effects of packing density on solute partitioning was investigated by DeYoung and Dill, who measured the membrane/water partition coefficients of benzene as a function of the surface density of the phospholipid chains (DeYoung and Dill, 1988). Benzene partitioning is decreased by an order of magnitude as surface density is increased from 50% to 90% of its maximal value. Furthermore, DeYoung and Dill concluded that benzene partitioning depends on the packing density of the lipids, regardless of the method used to order the lipids, including temperature, lipid chain length, and cholesterol addition.
1.5. Mechanisms of Chemical Enhancer Action

The pharmaceutical relevance of many molecules, such as peptides, proteins, and other hydrophilic molecules, has stimulated the investigation of permeability enhancement methods, including the application of chemical compounds to the skin to reversibly alter its resistance to drug transport. The limited understanding of the mechanisms of chemical enhancer action has required the development of transdermal drug-chemical enhancer formulations on a trial-and-error basis, a time-consuming and costly process. A description and understanding of the processes involved in chemically-enhanced transdermal drug transport would enable the rational design of drug-chemical enhancer formulations.

Experimental techniques utilized in mechanistic studies of chemically-induced transdermal drug permeability enhancement have included Fourier transform infrared spectroscopy, differential scanning calorimetry, and nuclear magnetic resonance spectroscopy (Schaefer and Redelmeier, 1996). For oleic acid, a well-known chemical enhancer, these techniques have been helpful in identifying the mode of chemical enhancer action as a fluidization of the lipid bilayers which form the continuous phase of the SC, which provides the primary resistance to transdermal drug transport (Francoeur, et al., 1990, Schaefer and Redelmeier, 1996). The correlation between increased transdermal drug flux and increased lipid fluidity strongly suggests that oleic acid alters the packing structure of the stratum corneum lipids to increase lipid fluidity and reduce skin resistance to drug transport (Golden et al., 1987). Moreover, oleic acid has been
hypothesized to increase the size of the aqueous pores and thus facilitate the transdermal transport of hydrophilic permeants (Yamashita et al., 1995). While the above-mentioned studies have clearly elucidated the role of oleic acid in SC lipid fluidization, the changes in the micro-scale transport properties of the permeant, including the vehicle to skin partition coefficient, skin diffusion coefficient, and skin barrier diffusion length, that underlie the experimentally measured increases in the transdermal permeability of the permeant, remain to be clarified.

These changes in transdermal transport properties provide details pertaining to the effects of chemical enhancers on the permeant transport microenvironment for both hydrophobic and hydrophilic permeants. Transdermal transport of hydrophobic and hydrophilic permeants has been described by lateral diffusion through the lipid bilayers of the SC and by diffusion through an ‘aqueous’ pore pathway, respectively (Johnson, et al., 1997, Peck et al., 1994). Johnson et al. have demonstrated that for hydrophobic molecules of molecular weight less than 800 Da, the primary transport pathway consists of lateral diffusion in the lipid bilayers which form the continuous phase of the SC (Johnson, et al., 1997). Furthermore, for 90 compounds of molecular weights ranging from 18 Da to 750 Da and log $K_{o/w}$ (where $K_{o/w}$ is the octanol-water partition coefficient) values ranging from −3 to 6, Potts and Guy concluded that the stratum corneum lipids alone characterize mammalian epidermal barrier properties (Guy and Potts, 1992), suggesting that aqueous pores lie within the intercellular matrix of the lipid bilayers present in the SC. Despite the abundance of literature data referring to these two pathways (Li et al., 1999, Li, et al., 1998, Potts and Guy, 1992, Yoneto et al., 1995) a detailed description of the pathway
parameters, including the morphology and microtransport environment, remain important issues requiring resolution. A plethora of literature exists which suggests that lipid disorder is the prime mechanism in chemical enhancement of transdermal transport. Barry proposed several modes of penetration enhancement, and these are summarized below (Barry, 1987):

(1) Differential Scanning Calorimetry (DSC) shows that all penetration enhancers examined disrupted the lipid organization of the lipid structure, allowing drugs to permeate through a less rigid environment. The solubility of the drug may also be affected by the fluidization of the lipids.

(2) Interaction of the enhancer with intracellular proteins was shown by alpha-keratin denaturation observed in the DSC graphs.

(3) In fully-hydrated stratum corneum, the diffusional barrier is lowered because the water interacts with the free hydrogen-bonding groups whose interactions with permeants in less hydrated environments usually hinder their movement through the stratum corneum.

(4) Small penetration enhancers, such as, DMSO, pyrrolidones, and polyethylene glycol, accumulate in both the intercellular and protein regions, which then increases drug partitioning into the skin.

(5) The octanol/water partition coefficient of the chemical enhancer determines if the molecule will disorder the lipid region or interact with the corneocyte region. Polar compounds more likely interact with the protein-rich regions, whereas nonpolar materials interact with the lipid region in disordering the structure and reducing the barrier properties.
In addition to DSC, spectroscopic techniques also include Nuclear Magnetic Resonance (NMR) and Infrared (IR). Golden, McKie and Potts have shown a correspondence between flux and fluidity measurements performed on porcine stratum corneum (Golden, et al., 1987). Using DSC and IR analyses, they showed that cis isomers of monounsaturated fatty acids have a greater effect on disturbing the membrane structure than their trans counterparts. Because cis monounsaturated and saturated fatty acids differ in packing properties, the increased fluidity was attributed to the insertion of the "kinked" molecule into a relatively packed domain. Oleic acid-induced enhancement was explained by Naik et al. in terms of disorder in the lipid bilayers as exhibited by the spectral shifts indicated by FTIR in tape-stripped stratum corneum (Naik et al., 1995). Naik et al. went on to show that oleic acid disorders the stratum corneum in the upper layers, but that the oleic acid detected in the lower layers of the stratum corneum exists in a separate domain. This finding suggests that the upper layers incorporate more oleic acid compared to the lower layers, although oleic acid is present in the lower layers as well. The existence of a separate domain in the lower layers of the SC may suggest the creation of an alternate pathway, through which molecules are preferentially channeled. Enhancement resulting from oleic acid may result from the formation of channels which circumvent the tortuous route of the SC lipid bilayers. While the investigation of the mechanisms of chemical enhancer action has remained relevant to the field of transdermal drug delivery, knowledge of the permeant transport pathway alterations that result from chemical enhancer induced skin barrier perturbation remains limited. In Section 1.5 below, the scope of this thesis is outlined, where questions pertinent to elucidating the mechanisms of chemical enhancer action will be addressed.
1.6. Scope of Thesis

The mechanisms of chemical enhancer action are evaluated using the well studied model chemical enhancer, oleic acid, in conjunction with a series of model permeants that possess a range of physicochemical properties, including permeant size (MW) and hydrophobicity (as reflected in log $K_{ow}$). The current understanding of the mechanisms of chemical enhancer action have relied on the evaluation of model systems like the lipid multilamellar liposome system examined by Johnson et al. to determine the rate-limiting step in transdermal drug transport for hydrophobic permeants (Johnson, et al., 1997). Furthermore, experimental methodologies applied to visualize chemically induced transdermal transport have involved the use of invasive techniques such as electron scanning microscopy, transmission electron microscopy, and histology, all of which require chemical or mechanical treatment of the skin specimen prior to imaging. In addition to the possible introduction of artifacts that result from these techniques, the inherent skin heterogeneity and its impact on the accuracy of drawing conclusions based on a limited sample of skin sites are rarely considered. Following a description of the experimental methodology used in this thesis, presented in Chapter 2, Chapters 3, 4, 5, 6, and 7 address the current challenges impeding the efforts to expedite our understanding of the mechanisms underlying chemical enhancer action. The major findings that these following chapters reveal are summarized below, and are followed by Chapter 8, in which the thesis is summarized and future work is proposed.
1.6.1. Chapter 3: Visualization and Quantification of Chemically-Induced Transdermal Transport

In response to the often intrusive skin visualization techniques mentioned above, two-photon scanning fluorescence microscopy (TPM) was introduced as a noninvasive method to evaluate three-dimensional fluorescent probe distributions throughout the skin. Three-dimensional spatial distributions of the hydrophilic and the hydrophobic fluorescent probes, sulforhodamine B (SRB) and rhodamine B hexyl ester (RBHE), in excised full-thickness human cadaver skin were visualized and quantified. Both SRB and RBHE were observed to lie primarily in the lipid multilamellae region surrounding the corneocytes within the stratum corneum. From the TPM scans, the changes in the concentration gradient and the vehicle to skin partition coefficient of each probe induced by the oleic acid chemical enhancer action were calculated relative to the control sample (not exposed to oleic acid), and subsequently applied to theoretically derived mathematical expressions of transdermal transport to quantitatively characterize the oleic acid-induced relative changes in the skin diffusion coefficient and in the skin barrier diffusion length of the permeant. For the hydrophobic probe RBHE, the permeability enhancement was primarily driven by an increase in the vehicle to skin partition coefficient, leading to an increase in the steepness of the concentration gradient across the skin. The primary oleic acid-induced changes in the transport properties of the hydrophilic probe SRB included increases in the vehicle to skin partition coefficient and in the skin diffusion coefficient. These findings utilizing the TPM methodology and data analysis described here demonstrate that, in addition to providing three-dimensional
images that clearly delineate probe distributions in the direction of increasing skin depth, the subsequent quantification of these images provides additional important insight into the mechanistic changes in transdermal transport underlying the visualized changes in probe distributions across the skin.

1.6.2. Chapter 4: Skin Morphological Heterogeneity

To further validate the methodology introduced in Chapter 3 using TPM, larger skin areas were examined to evaluate the impact of the inherent skin morphological heterogeneity on the valid quantification of transdermal transport properties. A novel application of high-speed two-photon microscopy (HTPM) was utilized to determine the optimum number of skin sites required to accurately determine the changes in transdermal transport properties incurred globally, over a clinically relevant area of the skin. In contrast to the 4-6 skin sites (100 μm by 100 μm area per site) examined in Chapter 3 (see also Yu et al., 2001), the present study accounted for the fluorescent probe distributions at 400 consecutive skin sites, covering a total skin area of 2mm by 2mm. The oleic acid-induced changes in the transdermal transport properties of the model hydrophobic probe, rhodamine B hexyl ester, and of the model hydrophilic probe, sulforhodamine B, for the present 400-skin site study, exhibited different dependencies on sample size for each probe. Whereas the examination of 6 skin sites captures the relative changes in the global transdermal transport properties of the hydrophobic probe, the valid assessment of these changes for the hydrophilic probe requires a significantly larger sample size of at least 24 skin sites.
1.6.3. Chapter 5: Evaluation of Wide-Area Variations in Microscale Skin Transport Parameters

The application of high-speed two-photon fluorescence microscopy (HTPM) to examine transdermal transport processes has enabled the noninvasive visualization of permeant spatial distributions over a larger, more clinically relevant wide-area of skin (see Chapter 4 and Yu et al., 2002). In Chapter 5, the rate-limiting steps in transdermal transport are elucidated for a hydrophobic and a hydrophilic model probe through a novel analysis of the oleic acid-induced changes in the statistical distributions of two transdermal transport parameters over the 400 human cadaver skin sites imaged. Our results indicate that partitioning from the vehicle into the stratum corneum (SC), for the hydrophilic probe, and that diffusion through the SC intercellular lipid multilamellae, for the hydrophobic probe, constitute the rate-limiting steps in transdermal transport. Furthermore, at each skin site imaged, the fluctuations in the probe free energy for partitioning and in the probe concentration gradient from the respective 400 skin site average values were captured using surface plots. The number of skin sites displaying free-energy fluctuations increased for the hydrophobic probe and decreased for the hydrophilic probe as a result of the oleic acid enhancer action. The hydrophobic probe concentration gradient fluctuations remained insensitive to the oleic acid enhancer action, while the number of intensity gradient fluctuations for the hydrophilic probe increased.

Finally, good agreement was found between the microscopic transport parameter distributions over the 400 skin sites scanned and the histograms of the macroscale flux measurements for different hydrophilic and hydrophobic model drugs compiled from the literature. This finding suggests that the HTPM analysis discussed here can be utilized to
predict the macroscale permeability distributions in a population that result from the application of different chemical enhancer formulations.

1.6.4. Chapter 6: Evidence for Intracorneocyte Permeant Diffusion Using Dual-Channel Imaging of Skin Autofluorescence and Probe Spatial Distribution Intensities

The conclusions presented in Chapters 3, 4, and 5, that contribute to the identification of the transdermal transport rate-limiting steps for the hydrophobic and for the hydrophilic probes examined, capture the wide-area, average global fluorescent probe transdermal transport properties. To understand the oleic acid-induced changes in probe spatial distributions with respect to the SC structural features that underlie the globally obtained changes in transdermal transport properties, focus was placed in this chapter on characterizing the probe spatial distributions with respect to the corneocyte structures using dual-channel HTPM.

In this novel application of HTPM, introduced for the first time in this thesis, one channel detects fluorescence emission wavelengths characteristic of the endogenous (intrinsic) skin fluorophores (green channel), while the other channel detects the rhodamine-based probe intensity emission at a different wavelength range of the fluorescence emission spectrum (red channel). Hence, the question addressed in Chapter 6 examines the role of intracorneocyte diffusion in transdermal transport, and how oleic acid enhancer action influences the penetration of the model hydrophobic and hydrophilic probes, used in the previous chapters, into the corneocyte structures. Instead of treating the SC as a composite structure in which the average probe spatial distributions are evaluated, this
chapter considers the probe pixel intensity spatial distributions constituting the 9 consecutive skin sites imaged to account for the corneocyte structures within the SC. Relative to the skin autofluorescence intensity image, that delineates the corneocyte spatial positions, the probe fluorescence intensity spatial distribution at the precise same skin location were quantified using the average correlation length, a conventional standard used to characterize the decrease in the fluorescence intensity signal between two spatial positions.

In these studies, the relative difference between the average correlation lengths of the probe intensity and of the autofluorescence intensity distributions serves as a quantitative indicator of probe penetration into the corneocyte region. The relative differences calculated between the average correlations lengths were then verified using a linear image intensity deconstruction analysis, in which the probe intensities within individual corneocyte structures were evaluated. The quantitative results presented in Chapter 6 verify the existence of fluorescent probe diffusion into the corneocytes, such that probe partitioning from the lipid intercellular region into the corneocyte followed by intracorneocyte diffusion provides a secondary transport pathway. In this thesis, it is proposed that intracorneocyte diffusion is driven by the probe concentration gradient existing from the intercellular space (rich in probe) into the corneocytes (deficient in probe), and that the corneocytes act as permeant diffusion sinks in transdermal transport. Due to the hydrophilic nature of the corneocytes, the mechanism of oleic acid enhancer action for the hydrophobic fluorescent probe and for the hydrophilic fluorescent probe differ.
The visualization and quantification of these two different mechanisms of oleic acid chemical enhancer action revealed that oleic acid induces increases in the hydrophobic fluorescent probe localization to the intercellular region and in the hydrophilic fluorescent probe partitioning from the intercellular lipid multilamellae into the corneocyte region. The oleic acid-induced changes observed at the SC structural level further substantiates the two different rate-limiting steps (hydrophobic probe lateral diffusion and hydrophilic probe vehicle to skin partitioning) determined in the previous chapters. The localization of the hydrophobic probe to the intercellular region subjects its transport pathway to the constraints imposed by the lateral diffusion. For the hydrophilic probe, however, the increased lipid to corneocyte partitioning reflects the lack of constraints placed on its diffusional path; oleic acid has facilitated the increased vehicle to SC partitioning that was determined to be the rate-limiting step.

1.6.5. Chapter 7: Investigation of Skin Pretreatment with Chemical Enhancer Formulations, and Preliminary Examination of Protein Delivery Using Oppositely-Charged Surfactants

In addition to the development of the two-photon microscopy methodology, and the application of this methodology to elucidate the mechanisms of chemical enhancer action, transport experiments using side-by-side diffusion chambers and human cadaver skin were performed to evaluate the effects of different chemical enhancer formulations on enhancing transdermal drug delivery. The effect of the permeant physicochemical properties on permeant transdermal transport was evaluated using a variety of different radiolabeled model drugs that spanned the molecular weight range of 158 Da to 1000 Da, for the hydrophobic permeants, and 152 Da to 5000 Da for the hydrophilic permeants.
In addition to examining the passive permeabilities of these permeants, the skin was exposed to different chemical enhancer formulations. These formulations included skin exposure to standard solutions of PBS, PBS and Ethanol (EtOH), and a mixture of PBS, EtOH, and 5% oleic acid. In addition to the constant exposure of the skin to these three formulations, skin pretreatment with the mixture of PBS, EtOH, and 5% oleic acid, followed by skin exposure to either PBS or by PBS and EtOH, were also evaluated. Finally, the effect of pairing proteins with oppositely-charged surfactants (Aerosol OT and SDS) on facilitating protein transdermal transport was investigated.

The skin pretreatment experiments further verified that, for the hydrophilic model permeants examined (polyethylene glycol, PEG, of 400, 900, and 4000 Da molecular weights), drug partitioning is indeed the rate-limiting step in transdermal transport. For the hydrophobic permeants, the permeability enhancements measured utilizing the pretreatment procedure did not yield permeability enhancements comparable to those exhibited by the hydrophilic model drugs. Hence, lateral diffusion through the tortuous lipid intercellular matrix remains the rate-limiting step for the hydrophobic permeants examined, despite the increased permeant partitioning induced by the skin pretreatment weakening of the skin barrier properties.

The pairing of oppositely-charged surfactants with the model protein oxytocin did not demonstrate the feasibility of delivering protein-surfactant pairs transdermally. The impermeability of the skin barrier to proteins currently warrants the application of other
skin barrier modification techniques, such as, sonophoresis, electroporation, and iontophoresis that induce greater mechanical stresses on the SC barrier.

1.7. Conclusions

The descriptions of the skin barrier and morphology presented in Sections 1.2.1 and 1.2.2 have provided the physical basis for the existence of transdermal transport pathways through the lipid multilamellar region for hydrophobic and hydrophilic permeants. The more detailed description of the physicochemical properties of the SC lipids that comprise the intercellular region (see Section 1.2.3) included a discussion of the lipid packing conformations associated with the liquid crystalline, gel, and solid crystalline phases of the lipid multilamellae. The heterogeneity of the lipid multilamellae composition and the conditions under which different lipid packing conformations might be assumed, highlight the impact of the permeant physicochemical properties on transdermal transport, as well as the potential for enhancing transdermal transport by altering the lipid packing conformations to induce lipid fluidization using chemical enhancer formulations. The significance of the permeant physicochemical properties (as reflected in the $K_{o/w}$ and MW values), as well as of the SC structural heterogeneity, on transdermal transport was described further in Section 1.3 utilizing empirically-derived mathematical relationships. In light of the extensive number of mathematical expressions that relate permeant transdermal transport to its physicochemical properties (MW and $K_{o/w}$) and to the SC composition ($P_{psc}$, $P_{pol}$, and $P_{aq}$), an understanding of the mechanisms of chemically-induced transdermal transport responsible for the modifications of these relationships remains limited to the qualitative descriptions presented in Section 1.4. Hence, this thesis aims to further elucidate the current
understanding of the mechanisms of chemical enhancer action through the introduction of novel transdermal transport visualization and quantification techniques that reflect the current developments in instrumentation technology. The results of these investigations and their discussion are presented in the subsequent chapters with the hope of shedding new light on the mechanisms underlying oleic acid-induced transdermal transport, as well as of providing additional fundamental insight into the selection of effective compounds for the development of effective chemical enhancer formulations.
1.8. References


Chapter 2

Experimental Methods Utilized to Evaluate Chemically-Induced Transdermal Transport Mechanisms

2.1. Introduction

The experimental results presented in this thesis were obtained using the experimental methodologies described in this chapter. The details of these methods will be referenced frequently in the subsequent chapters, and they include: (i) the measurement of skin permeability using side-by-side diffusion cells (see Section 2.2), (ii) the skin chemical enhancer pretreatment protocol (see Section 2.2), (iii) the measurement of oleic acid transport (see Section 2.3), (iv) the evaluation of protein permeability (see Section 2.4), (v) the measurement of skin conductivity (see Section 2.5), (vi) the preparation of skin samples for the two-photon microscopy studies (see Section 2.6), and (vii) the determination of the fluorescent probe octanol-PBS partition coefficients (see Section 2.7). The details of the methodologies used to visualize and quantify the fluorescent probe spatial distributions will be presented in Chapter 3, Section 3.2, Chapter 4, Section 4.2, Chapter 5, Section 5.2, and Chapter 6, Section 6.2.
2.2. Permeability Measurement: Side-by-Side Diffusion Cells

Full-thickness human cadaver skin from the abdominal area (National Disease Research Interchange, Philadelphia, PA), acquired 10-20 hours post-mortem, was stored at -80°C and utilized within 3-6 weeks. Upon use, the skin was thawed at room temperature (25°C), and the fat from the dermal side was removed. Skin samples of 2.25 cm² were mounted in side-by-side diffusion cells (9mm diameter, PermeGear, Riegelsville, PA), and then immersed in phosphate buffered saline, PBS (0.01M Phosphate buffer, 0.0027M KCl and 0.137 NaCl, pH=7.4, Sigma, St. Louis, MO) for the measurement of skin conductivities (see Section 2.5 below). A schematic illustration of a side-by-side diffusion cells is shown in Figure 2-1, where C_d and C_r refer to the permeant concentrations in the donor and the receiver cells, respectively, and the diffusion cross-sectional area is denoted by A. The skin sample is placed between the two diffusion cell compartments.

![Skin Sample](image)

Figure 2-1: Schematic illustration of a side-by-side diffusion cell.
2.2.1. Preparation of Heat-Stripped Human Cadaver Skin

Heat-stripped human cadaver skin, comprising the SC and the viable epidermis of the skin, was prepared by heating the full-thickness skin sample to 60°C in a well-stirred PBS bath for 1 minute. The skin sample was then placed on a surgical diaper (VWR, Bridgeport, NJ) with the SC side facing up. Using a stainless steel spatula (VWR, Bridgeport, NJ), the epidermis along with the SC were gently removed using a controlled scraping motion. Upon complete removal of the heat-stripped skin, samples were mounted in the diffusion cells and skin conductivity measurements were made following the methodology described in Section 2.5.

2.2.2. Donor Solution Preparation

2.2.2.1. Preparation of Fluorescent Probe Donor Solution

For the fluorescent probes, donor vehicle solutions of rhodamine B hexyl ester (RBHE) and of sulforhodamine B (SRB) (Molecular Probes, Eugene, OR) consisting of ethanol (100%, Pharmco™ Products, Brookfield, CT) and PBS (Sigma, St. Lious, MO), at a 1:1 ratio, by volume, were prepared at concentrations of 0.5 mg/ml and 0.7 mg/ml, respectively. In preparing the solutions containing the model chemical enhancer (oleic acid), an additional 5% by volume of oleic acid (99%, Sigma, St. Louis, MO) was added to the PBS/ethanol probe solutions. Skin samples were left in contact with well-stirred donor solutions for 48 hours, until the steady-state diffusion profile was established.

2.2.2.2. Preparation of Radiolabeled Fluorescent Probe Solutions

Control (no chemical enhancer) and chemical enhancer solutions of RBHE and SRB, prepared as described above, were radiolabeled with the equivalent tritiated probe

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(American Radiolabeled Chemicals, St. Louis, MO) at 1 μCi/ml. Following the procedure described in the literature (see also Section 2.2), the permeability experiments were performed (Johnson et al., 1996) in triplicate using heat-stripped skin (HSS), (Peck et al. 1994; Li et al., 1998) with each of the four probe-vehicles, RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer, serving as the donor solution for each set of experiments. The skin conductivities were measured before and after the experiments, as described below (see Section 2.3) for each sample examined. The presence of radiolabeled permeants was detected with a liquid scintillation analyzer (2200CA Tri-Carb®, Packard Instrument Company, Downers Grove, IL), and the steady-state permeant permeabilities were calculated utilizing Eq.(2.1) below:

\[
P = \frac{dN_r}{dt} \frac{1}{A\Delta C}
\]  

(2.1)

where P is the permeability, \(\frac{dN_r}{dt}\) is the amount of permeant in the receiver cell at time t, A is the area of the skin exposed to the permeant, and \(\Delta C\) is the difference in concentration between the two cells.

2.2.2.3. Preparation of Radiolabeled Model Drug Solutions

The radiolabeled permeants (American Radiolabeled Chemicals, St. Lious, MO and NEN, Boston, MA) listed in Table 7-1 (see Chapter 7) were prepared at concentrations of 1 μCi/ml in the donor vehicle of interest. The standard donor vehicles evaluated consisted of PBS, PBS/EtOH (50/50 v/v), and a mixture of 50/50 PBS/EtOH (v/v) with 5% oleic acid (by volume). The permeabilities of these radiolabeled permeants were determined
using the full-thickness human cadaver skin model and the scintillation counter detection methodology described earlier in Section 2.2.

2.3. Skin Pretreatment Protocol

Human cadaver skin samples were pretreated with solutions of PBS/EtOH and of PBS/EtOH and 5% oleic acid for a specified number of hours, ranging from 3 hours up to 14 hours. Following skin pretreatment, the donor compartments were rinsed with PBS/EtOH, skin conductivities were measured in PBS (see Section 2.5), and the donor compartments were again filled with either PBS or PBS/EtOH, now containing the radiolabeled permeant (1 µCi/ml) under evaluation until the end of the diffusion cell experiment. The drug permeabilities resulting from the pretreatment procedure were determined using the scintillation counter method described earlier (see Section 2.2.2). The selection of the 3-hour pretreatment duration for a majority of the experiments described in Chapter 7 is discussed in Section 7.3.3.1.

2.4. Evaluation of Oleic Acid Transport

Heat-stripped human cadaver skin (Li et al., 1998) was inserted between the donor and the receiver compartments of the diffusion cells. Three sets of different experiments were performed. Each experimental set was repeated in triplicate. For each set, the stratum corneum side of the heat-stripped skin was pretreated with 1:1 PBS:ethanol and an additional 5% by volume of oleic acid containing a trace amount of radiolabeled 14C-oleic acid (1 µCi/ml) for 3 hours, while the receiver compartments of the diffusion cells were filled with PBS only. Upon removing the pretreatment solution, a PBS/EtOH solution was used to rinse off the oleic acid on the surface of the skin while keeping the
samples clamped in the diffusion cells. PBS was then placed in the donor cell, and the conductivities of each skin piece were measured (see Section 2.5). The contact of the PBS/EtOH solution with the skin lasted for less than 2 minutes, so that on the time scale of the experiments, the quantity of EtOH entering the skin could be considered negligible. PBS was not used alone to rinse the oleic acid off the skin because of the low oleic acid solubility in PBS.

After the conductivities of the skin were measured in PBS, the donor compartments were replaced with PBS/EtOH. For Set 1 (see Table 7-6 in Chapter 7), PBS/EtOH was placed in the donor compartment for the remainder of the experiment. For Set 2 (See Table 7-6 in Chapter 7) PBS/EtOH was placed in the donor compartment for 14 hours following the skin pretreatment, and was subsequently replaced with PBS for the remainder of the 72 hour experiment. For both Set 1 and Set 2, the entire volume of the donor and of the receiver compartments were sampled at specific intervals over 72 hours to monitor the transport of the $^{14}$C-oleic acid from the skin into the diffusion cell compartments of the donor as well as of the receiver. $^{14}$C-oleic acid transport from the skin into these two different diffusion cell compartments was again measured using the protocol described in Section 2.2 above.

In addition, skin samples subjected to the pretreatment were removed from the diffusion cells, rinsed with PBS/EtOH, and then dissolved with tissue solubilizing agent Soluene (Packard Instruments, Meriden, CT). The dissolved samples were examined with a scintillation counter to determine the amount of the $^{14}$C-oleic acid. These samples served
as a control in determining the fraction of oleic acid that entered the skin due to the oleic acid pretreatment, and are referred to as Set 3 in Table 7-6 in Chapter 7.

2.5. Evaluation of Protein Permeability

2.5.1. Chemical Enhancer Pretreatment

Heat-stripped human cadaver skin was pretreated with PBS/EtOH and 5% oleic acid for five hours following the methodology described in Section 2.3. Skin conductivity changes in the skin were then measured in PBS (see Section 2.5). The skin was then contacted with PBS/EtOH for 12 hours, and subsequently rinsed with PBS/EtOH. The conductivities were again measured in PBS. The second pretreatment step was utilized based on evidence that PBS/EtOH further increases the skin permeability following pretreatment with PBS/EtOH and 5% oleic acid.

Citrate buffer (pH 4.6, Sigma Chemicals, St. Louis, MO) was then placed in the receiver cells. At a pH of 4.6, the oxytocin protein has an overall positive charge. The donor solutions contained 0.3 mM oxytocin (Sigma Chemicals, St. Louis, MO) in citrate buffer with a 1 μCi/ml of tritium-labeled oxytocin (NEN, Boston, MA). ³H-oxytocin permeabilities were measured using the methodology described in Section 2.3.

2.5.2. Preparation of Protein-Surfactant Pairs

The donor solution was prepared by adding a trace quantity of ³H-oxytocin to a solution containing an equimolar quantity of the anionic surfactant Aerosol-OT (AOT) (Sigma Chemicals, St. Louis, MO) and oxytocin, or an equimolar quantity of the anionic surfactant sodium dodecyl sulfate (Sigma Chemicals, St. Louis, MO) and oxytocin, in
citrate buffer. The citrate buffer was used to maintain an overall positive charge on the oxytocin while the equimolar quantity of AOT (or of SDS) is added as the counter ion. Skin conductivity measurements were taken (see Section 2.5), and $^3$H oxytocin permeabilities were measured, as described in Section 2.2. The cost of oxytocin remains the limiting factor in the number of experiments performed, as well as in the concentration chosen for the protein and the surfactant. The effects of using higher AOT concentrations below the critical micelle concentration (CMC) of AOT were not addressed here.

2.6. Skin Conductivity Measurement

Skin conductivities for all the skin samples examined were measured as an indicator of the skin barrier integrity (Kalia and Guy, 1995, Kontturi et al., 1993, Peck et al., 1994). Ag-AgCl electrodes (E242, In Vivo Metric, Healdsburg, California) were connected to an AC signal generator (HP 33120A 15MHz Function Arbitrary Waveform Generator, Hewlett Packard, Palo Alto, California) in series with a digital multimeter (Radio Shack®, Fort Worth, TX). One electrode was placed in each compartment of the diffusion cell, filled with PBS, and a voltage drop of 100 mV was applied across the skin. To a 10 Hz current reading, the skin conductivity criterion of >10 (kΩ cm$^2$)$^{-1}$ was applied (Rossel et al., 1988), with skin samples not meeting this criterion replaced by intact skin.

The final skin conductivities of the samples were again measured as described above to determine the changes in the skin barrier function due to the chemical enhancer action, as skin conductivity measurements have frequently served as a useful indicator of the skin barrier function, and skin conductivity increases have similarly been found to correlate
well with permeant permeability increases (Sims et al., 1991). Compared to the skin samples for which no changes in skin conductivity were measured after 48 hours of contact with PBS only (the control case – no chemical enhancer), the skin samples treated with the oleic acid chemical enhancer indeed lowered the skin barrier function. The measured changes in skin conductivities for the samples kept in contact with the oleic acid solution are comparable to the 100 to 200 fold skin conductivity increases cited in the literature for skin treated with chemical enhancers (Inamori et al., 1994, Li, et al., 1998).

2.7. Skin Preparation for TPM Imaging

After the skin exposure to the donor solutions described in Section 2.2, each skin sample was removed from the diffusion cell and rinsed with PBS/ethanol (1:1, by volume), and then blotted with a Kimwipe (Kimberly-Clark®, Roswell, GA) to ensure removal of any excess fluorescent probes present on the surface of the skin. The circular area of the skin exposed to the probes was cut out with a surgical carbon steel razor blade (VWR Scientific, Media, PA), and then sealed in an imaging chamber (2.5mm Coverwell™, Grace Bio-Labs, Bend, OR) with a No. 1, 22mm square cover slip (VWR Scientific, Media, PA) contacting the SC side. A drop of PBS was added to the chamber to prevent drying of the sample.

2.8. Determination of the Fluorescent Probe Octanol-PBS Partition Coefficients

To obtain a calibration curve of the fluorescent probe intensity versus the fluorescent probe concentration, solutions of varying probe concentrations were prepared by diluting
the stock for each fluorescent probe. The probe fluorescence intensity at each known probe concentration was then measured with a fluorescence system (Photon Technology International, Monmouth Junction, NJ).

2 mL of each probe solution in PBS (~0.05 mg/ml for RBHE, and ~0.07 mg/ml for SRB) were vortexed for 30 seconds with 2ml of octanol (Fisher Scientific, Pittsburgh, PA) in a glass vial. Experiments were performed in triplicate. The vortexed mixtures were then allowed to equilibrate for 24 hours. The aqueous phases were measured with the fluorescence system, and the corresponding concentration was determined utilizing a calibration curve of the fluorescent probe concentration versus the probe fluorescence intensity. Following the measurement of the initial and the final fluorescent probe concentrations in the aqueous phase, the quantity of probe in the octanol phase was determined by the difference between the initial and the final fluorescent probe concentrations of the aqueous phase. The fluorescent probe octanol-PBS partition coefficient was calculated by taking the ratio of the final fluorescent probe concentration in the octanol phase and that in the PBS phase. The PBS-octanol partition coefficients obtained here (log $K_{O/PBS} = 2.49 \pm 0.18$ and $-0.45 \pm 0.0045$ for RBHE and SRB, respectively) illustrate that for the fluorescent probe concentrations used, RBHE and SRB act as the model hydrophobic (positive $K_{O/PBS}$) and hydrophilic molecules (negative $K_{O/PBS}$), respectively.
2.9. References


Chapter 3

In-Vitro Visualization and Quantification of Oleic Acid-Induced Changes in Transdermal Transport Using Two-Photon Microscopy

3.1. Introduction

3.1.1. Chemical Enhancement of Transdermal Drug Delivery

Chapter 1 described the experimental techniques, including Fourier transform infrared spectroscopy, differential scanning calorimetry, and nuclear magnetic resonance spectroscopy, utilized in mechanistic studies of chemically-induced transdermal drug permeability enhancement (Schaefer and Redelmeier, 1996). For oleic acid, a well-known chemical enhancer, these techniques have been helpful in identifying the mode of chemical enhancer action as a fluidization of the lipid multilamellae which form the continuous phase of the SC (Francoeur, et al., 1990, Schaefer and Redelmeier, 1996). The correlation found between increased transdermal drug flux and increased lipid fluidity strongly suggests that oleic acid alters the packing structure of the stratum corneum lipids to increase lipid fluidity and reduce skin resistance to drug transport (Golden, et al., 1987). Furthermore, the mechanism of oleic acid chemical enhancer action has been
associated with the formation of phase separated oleic acid domains in the SC multilamellar lipids (Ongpipattanakul et al., 1991). While these studies (Francoeur, et al., 1990, Golden, et al., 1987, Schaefer and Redelmeier, 1996) have clearly elucidated the role of oleic acid in altering SC lipid ordering, the changes in the micro-scale transport properties of the permeant, including the vehicle to skin partition coefficient, the skin diffusion coefficient, and the skin barrier diffusion length, that underlie the experimentally measured increases in the transdermal permeability of the permeant, remain to be clarified. The novel application of two-photon scanning fluorescence microscopy (TPM) to visualize and quantify the oleic acid-induced changes in these micro-scale transdermal transport properties will be presented in this chapter.

3.1.2. Two-Photon Scanning Fluorescence Microscopy

Laser scanning confocal fluorescence microscopy (LSCM) has been utilized to visualize hydrophobic and hydrophilic fluorescent probe spatial distributions across the skin (Kuijk-Meuwissen et al., 1998, Turner and Guy, 1997); however, three-dimensional imaging using this technique is limited by increased light scattering within the medium. In LSCM, the fluorescent probe penetration is, therefore, often imaged by sectioning skin samples perpendicular to the skin surface such that the field of view captures the fluorescent probe distribution from the SC to the dermis all in one plane. Because skin sample sections often undergo exposure to chemicals and mechanical forces in the curing, setting, and sectioning stages of the sample preparation procedure, the presence of artifacts in the final sample remains a potential risk.
As a noninvasive method for directly visualizing the skin structural features, two-photon scanning fluorescence microscopy (TPM) has been successfully used to image in vivo human skin (Masters et al., 1998). More recently, TPM has been utilized to visualize in vitro permeant spatial distributions across human cadaver skin.\textsuperscript{1,2,3} In TPM, tight focusing in a laser scanning microscope, combined with a femtosecond pulsed laser, enables a dye molecule to simultaneously absorb two long-wavelength photons to reach its excited state (Denk et al., 1990). The quadratic dependence of the excitation probability results in greater three-dimensional depth discrimination. The long wavelength photon source used in TPM allows deeper light penetration into media with a high degree of scattering (Denk et al., 1995, Diaspro, 1999, Soeller and Cannell, 1999), thus making it well suited for imaging the skin (Masters, et al., 1998). Other attributes of TPM include its use of a large area signal detector that maximizes the photon collection efficiency, and the localization of the excitation volume to the focal region which results in minimized photodamage and photobleaching (Masters et al., 1997, Periasamy et al., 1999).

Despite the application of TPM to visualize probe distributions in the skin (Grewal et al., 2000), to date, TPM scans of fluorescent probe distributions in the skin have not been rigorously analyzed to quantitatively characterize the changes in the transdermal transport properties resulting from chemical enhancer action. The structural complexity of the SC (see Chapter 1, Section 1.2) poses significant challenges to the quantitative analysis of


average probe transport properties in the skin. This analysis relies on an accurate assessment of the fluorescent probe concentration distributions throughout the skin depth. To demonstrate the quantitative analysis methodology described below in detail, the SC was treated as a membrane in which the corneocytes and lipid multilamellae are collectively considered in the quantification of the fluorescent probe distributions in the SC. This approach, in which the corneocytes were not excluded from the analysis of transdermal permeant transport, has been commonly employed by other researchers (Mak et al., 1990, Naik, et al., 1995, Pirot et al., 1997). Hence, the ensuing description of the TPM methodology utilized in the transdermal transport studies and in the quantification of the changes detected in the microscale transport properties relative to the control case, introduce, for the first time, the novel application of TPM to this in vitro model skin system to elucidate the mechanism of chemical enhancer action (see Section 3.2).

In the studies reported in this chapter, three-dimensional images of fluorescent probe spatial distributions across full-thickness human cadaver skin were constructed utilizing TPM data. Oleic acid-induced changes in the probe concentration gradient, as well as in the microtransport properties of the probe, including the skin diffusion coefficient, the skin barrier diffusion length, and the vehicle to skin partition coefficient, all of which influence the experimentally measured permeability of the probe across the skin, were quantified relative to the control case (without oleic acid) based on a theoretical analysis of these three-dimensional images to shed light on the mechanisms of chemically-enhanced transdermal transport.
3.1.3. Theoretical Description of Transdermal Transport

Chapter 1, Section 1.2 described the function of the stratum corneum (SC) as the primary barrier to transdermal drug transport (Bommannan et al., 1990). The two-photon images discussed in Section 3.3.1 will highlight permeant diffusion across the stratum corneum and a portion of the underlying VE, both of which are depicted in the schematic illustration of the skin presented in Chapter 1, Figure 1-1.

The steady-state flux, $J$, of a permeant across the SC membrane in the absence of convective solvent flow is commonly described using the one dimensional Fick's First Law of diffusion, namely,

$$ J = -D \frac{dC}{dz} \quad (3.1) $$

where $D$ is the effective permeant diffusion coefficient in the membrane, and $dC/dz$ is the concentration gradient in the $z$-direction, or the direction of increasing SC depth (Peck et al., 1996, Pugh et al., 1998). From Eq.(3.1), changes in the permeant flux are driven by changes in two microtransport properties, $D$ and $dC/dz$. Additional transport properties that contribute to the experimentally measured permeant skin permeability include the permeant vehicle to skin partition coefficient, $K$, and the skin barrier diffusion length, $l$.

The skin barrier diffusion length, $l$, represents the thickness of the skin region over which the greatest barrier to permeant transdermal transport is exhibited. In discussions of percutaneous absorption, in which a constant donor solution concentration and a zero concentration receiver solution were assumed, the mathematical models derived for
transdermal transport yield expressions for diffusion in one dimension (Pirot, et al., 1997, Scheuplein, 1967). With \( z/R \ll 1 \), where \( z \) and \( R \) are the skin depth and the skin diffusion area radius, respectively, and with the donor solution-imposed concentration gradient in the \( z \)-direction, the mathematical expression for multidimensional transport can be simplified to one dimension (Deen, 1998).

Currently, experimental methods are not available to directly determine the permeant diffusion coefficient in the skin in vitro, and SC models have relied on measurements of diffusion coefficients in liposomes of various lipid compositions (Johnson et al., 1996). In this chapter, we will focus on the oleic acid enhancer induced changes in the relevant transport properties discussed above, relative to the experimental control (without the enhancer), for each probe examined. The quantification of the relative change for each transport property under investigation is reported as an enhancement factor, \( E \), which is the ratio of the value of the transport property with and without the chemical enhancer. From TPM data, the relative change in the vehicle to skin partition coefficient for each probe can be directly determined. Consequently, the relative change in the probe diffusion coefficient due to oleic acid enhancer action can be calculated using the following expression relating the probe flux, \( J \), to its permeability, \( P \) (Schaefer and Redelmeier, 1996):

\[
P = \frac{J}{\Delta C}
\]  

(3.2)

where \( \Delta C \) is the difference in the probe concentration between the donor and the receiver compartments of the side-by-side diffusion cell used to measure \( P \). For a given value of
$\Delta C$, the permeability enhancement, $E_P$, which is the ratio of the permeant permeability in the presence of the chemical enhancer to that of the control (without the chemical enhancer), can be obtained by using Eq. (3.2) in Eq. (3.1), and then taking the ratio of the resulting expression for the chemical enhancer (E) and the control (C) samples. Specifically,

$$E_P = \frac{D_E}{D_C} \left\{ \frac{\left( \frac{dC}{dz} \right)_E}{\left( \frac{dC}{dz} \right)_C} \right\}$$

(3.3)

where the first derivative, or the slope, of the linear permeant concentration profile (discussed later) established in the SC at steady-state conditions is a constant quantity. Note that $E_P = P_E / P_C$ can be calculated from the ratio of the measured transdermal permeabilities of each permeant at the two conditions (E and C) examined. Recall that, in general, the permeant permeability can be obtained as follows:

$$P = \frac{1}{A \Delta C} \left( \frac{dN}{dt} \right)_r$$

(3.4)

where $(dN/dt)_r$ is the change in the amount of permeant over time in the receiver compartment, and $A$ is the area of the skin exposed to the donor solution. Moreover, for the steady-state transport of a permeant through biological membranes, the permeant partition coefficient between the donor solution and the skin, $K$, and the skin barrier diffusion length, $l$, also play significant roles in determining the magnitude of the permeability coefficient, $P$. Specifically (Schaefer and Redelmeier, 1996),
\[ p = \frac{K_D}{l} \quad (3.5) \]

Using Eq.(3.5), one can express the permeability enhancement, \( E_p \), in the following alternate useful form:

\[ E_p = \frac{P_E}{P_C} = \frac{E_k E_D}{E_l} \quad (3.6) \]

where the partition-coefficient enhancement, \( E_k \), the diffusion-coefficient enhancement, \( E_D \), and the skin barrier diffusion length enhancement, \( E_l \), are given, respectively, by

\[ E_D = \frac{D_E}{D_C} \quad (3.7) \]

\[ E_K = \frac{K_E}{K_C} \quad (3.8) \]

and

\[ E_L = \frac{l_E}{l_C} \quad (3.9) \]
Substituting Eq.(3.3) in Eq.(3.6), the skin barrier diffusion length enhancement, $E_l$, can be expressed in the following useful form:

$$E_l = \frac{E_K}{E_g} \quad (3.10)$$

where

$$E_g = \frac{\left(\frac{dC}{dz}\right)_E}{\left(\frac{dC}{dz}\right)_C} \quad (3.11)$$

is the concentration-gradient enhancement. Eq.(3.10) allows the calculation of the skin barrier diffusion length enhancement, $E_l$, utilizing quantities that are readily available through TPM experiments. Indeed, as shown in Sections 3.3.5 and 3.3.6, both the concentration-gradient enhancement, $E_g$, and the partition-coefficient enhancement, $E_K$, can be extracted solely from the data obtained from the TPM three-dimensional scans of the fluorescent probe spatial distributions to determine the skin barrier diffusion length enhancement, $E_l$, which describes the relative change in the thickness of the permeant diffusion barrier in the z-direction resulting from the chemical enhancer action.
3.2. Materials and Methods

Full-thickness human cadaver skin samples were prepared for TPM imaging following the methodology described in Chapter 2, Sections 2.2 and 2.7. Skin barrier integrity was confirmed using the methodology described in Section 2.6 of Chapter 2.

3.2.1. Fluorescent Probe Properties

The two fluorescent probes (Molecular Probes, Eugene, OR), rhodamine B hexyl ester perchlorate (RBHE) and sulforhodamine B (SRB), served as the hydrophobic and the hydrophilic model drugs, respectively, and were selected based on their similarity in molecular structure, disparity in octanol-water partition coefficient, and fluorescence emission range. Figures 3-1(a) and 3-1(b) show the chemical structures of the two fluorescent probes.

![Chemical structures of (a) rhodamine B hexyl ester (RBHE), the hydrophobic model drug, and (b) sulforhodamine B (SRB), the hydrophilic model drug (see Table 3-1 for a summary of some useful properties of the two fluorescent probes).](image)

Figure 3-1: Chemical structures of (a) rhodamine B hexyl ester (RBHE), the hydrophobic model drug, and (b) sulforhodamine B (SRB), the hydrophilic model drug (see Table 3-1 for a summary of some useful properties of the two fluorescent probes).
Their molecular weights (MW), fluorescence absorption (ab) and emission (em) spectral peaks (ab/em), and log $K_{O/PBS}$ (where $K_{O/PBS}$ is the experimentally determined octanol-PBS partition coefficient (see Chapter 2, Section 2.7)) are listed in Table 3-1. While similar in structure, slight differences in the functional groups of RBHE and SRB result in octanol-PBS partition coefficients that vary by three orders of magnitude. The absence of the sulfate groups ($SO_3^-$) and the addition of the hexyl ester moiety ($C_6H_{13}COO$) in RBHE, compared to SRB, impart a greater tendency for hydrophobic interactions. The log $K_{O/PBS}$ values (see Chapter 2, Section 2.7) measured for RBHE and SRB are 2.49 and -0.45, respectively, clearly reflecting their roles as the hydrophobic and the hydrophilic model drugs.

<table>
<thead>
<tr>
<th>Probe</th>
<th>RBHE</th>
<th>SRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW (Da)</td>
<td>627</td>
<td>559</td>
</tr>
<tr>
<td>ab/em (nm)</td>
<td>556/578</td>
<td>565/586</td>
</tr>
<tr>
<td>log $K_{O/PBS}$</td>
<td>2.49 ±0.18</td>
<td>-0.45 ±0.0045</td>
</tr>
</tbody>
</table>

| Table 3-1: Fluorescent probe properties of the hydrophobic (RBHE) and the hydrophilic (SRB) model probes examined.\(^a\) |

\(^a\)The chemical structures of RBHE and SRB are illustrated in Figures 3-1(a) and 3-1(b), respectively. MW, molecular weight of each probe; ab/em, the probe spectral absorption (ab) and emission peaks (em); log$K_{O/PBS}$, measured values of the log of the probe PBS-octanol partition coefficients (described in Chapter 2, Section 2.8).

\(^b\)Values are reported as mean ± SD. Experiments were performed in triplicate.
In addition to their physicochemical properties, these two probes were selected to minimize the effects of the skin autofluorescence, characterized by absorption and emission peaks of 488 nm and 515 nm, respectively (Masters, et al., 1997). Specifically, under the 780nm laser excitation, the fluorescence signals of the probes, their spectral absorption/emission peaks at 556 nm /578 nm (RBHE) and 565 nm /586 nm (SRB), have minimal overlap with that corresponding to the skin autofluorescence. The minimal overlap between the fluorescence emission spectra of the selected model probes and the intrinsic skin autofluorescence will be further exploited in Chapter 6 in a novel application of TPM to elucidate the probe spatial distributions with respect to the skin structural features, namely, the corneocytes and the intercellular region. Photon counts at each depth of the skin samples, kept in contact with the vehicles and in the absence of any fluorescent probes, maintained an average value of one and lower, with standard deviations of up to three photon counts. These values clearly indicate that the signal detected in the presence of the fluorescent probe does indeed originate from the fluorescent probe alone, since the samples exposed to the fluorescent probes yielded average photon counts that are greater by two orders of magnitude.

3.2.2. Two-Photon Microscope

The TPM instrument used in this study is shown in Figure 3-2, with a general description of the instrumentation given elsewhere (So et al., 1998). In the studies presented in this chapter, a femtosecond ti-sa laser (Tsunami®, Spectra-Physics, Mountain View, CA) pumped by a 5W diode-pumped, solid-state CW laser (Millennia®, Spectra-Physics, Mountain View, CA) provided the two-photon excitation at 780nm. The microscope system consisted of a modified inverted microscope (Axiovert 100TV, Zeiss,
Figure 3-2: Schematic illustration of the two-photon scanning microscope.

Thornwood, NY), a 40X objective lens (Zeiss F Fluar, NA .13), and an objective micropositioner (model P-721 PIFOC®, Physik Instrumente, Germany). X-Y scans are achieved using a scanner unit (Cambridge Technology, Watertown, MA), and photon pulses from the photomultiplier are discriminated against dark noise with an amplifier-discriminator unit (model F-100T, Advanced Research Instrument Corporation, Boulder, CO).

3.2.3. Data Collection

Per skin sample, four to six TPM scans of the fluorescence probe spatial distributions at different skin coordinates, or skin sites, were collected for each of the four treated skin
samples (RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer). The laser power (~2mW) was adjusted such that the fluorescence intensity emitted by the sample remained below the detection saturation threshold. The signal detection saturation threshold, below which a linear correlation exists between the fluorescence counts detected and the quantity of fluorescent probe present in the sample, was determined utilizing standard probe solutions of known concentrations.

3.2.4. Data Analysis

Three-dimensional images of the TPM data were examined with Spyglass® Slicer (Spyglass, Inc., Lexington, MA) data visualization software on a personal computer (Dimension XPS D300, Dell, Round Rock, TX). A corresponding average probe fluorescence spatial distribution profile was calculated with an in-lab subroutine (Eric Ho, Massachusetts Institute of Technology, Cambridge, MA) for each three-dimensional scan. At each depth (z-coordinate) scanned, the subroutine averages the fluorescence counts associated with the pixels in the 256 by 256 pixelated image representing the imaged field. For each site analyzed by the subroutine, a plot of the average fluorescence count versus the corresponding skin depth generates the site-specific average fluorescent probe spatial distribution profile (SSDP) that is the quantitative representation of the three-dimensional visualization generated with Spyglass® Slicer.

3.2.5. Generation of Fluorescent Probe Intensity Profiles

Per skin sample, the surface of each of the four to six sites examined, corresponding to \( z = 0 \, \mu m \), was defined by the scanning depth yielding the highest average fluorescence count. The definition of these surfaces normalized the different SSDP’s and enabled the
subsequent calculation of the sample average intensity profile (SAIP) for each probe-vehicle combination based on the four to six sites surveyed. In calculating each SAIP, the fluorescence counts associated with each individual SSDP corresponding to a given z-coordinate were averaged. This average was then plotted versus the corresponding z-coordinate to yield the SAIP, $I(z)$. The calculated standard deviations from the SAIP represent the site to site variations in probe penetration within a particular skin sample.

3.2.6. Fluorescent Probe Intensity Gradient Calculation

The intensity gradient (or the slope) corresponding to each SAIP was calculated by performing a linear regression for the data points comprising the first 11 axial scans, or 8 μm, of the skin, a region encompassing the drastic decrease in probe intensity for the control cases (in the absence of chemical enhancer) to which chemical enhancer-induced changes in probe transport are compared. The error in the intensity gradient corresponding to each SAIP is expressed as the range in which a 95% confidence level exists (see Table 3-2).

Because of the direct correlation between the probe fluorescence intensity and the probe concentration established with the standard probe solutions described above, the shape of the SAIP provides a good representation of the probe concentration profile in the skin sample. Hence, the calculation of the concentration-gradient enhancement, $E_g$, for each probe results from substituting the values of $dl/dz$, listed in Table 3-2 in Section 3.3.6, by those of the corresponding probe concentration gradients, $dC/dz$ (see Eq.(3.11)).
Moreover, the intensity gradient of each SSDP was calculated in the same manner, and then averaged to assess the relationship between the sample average intensity gradient and the corresponding site specific intensity gradient.

3.2.7. Quantification of Changes in the Probe Vehicle to Skin Partition Coefficient

For each probe, the concentration difference between the donor and the receiver diffusion cell compartments, $\Delta C$ (see Eq.(3.2)), was approximated by the probe donor concentration, $C_d$, because $C_d / C_r >> 1$, where $C_r$ is the probe concentration in the receiver compartment. Hence, under these infinite sink conditions, $C_d$ remains a fairly constant quantity in the control and the chemical enhancer permeability studies for both SRB and RBHE.

With a constant $C_d$ value for the control and the enhancer solutions for each probe, and given that $K = C_{sl0}/C_d$ (Schaefer and Redelmeier, 1996), where $K$ is the probe vehicle to skin partition coefficient and $C_{sl0}$ is defined as the probe concentration in the skin at the surface (at $z=0$ μm)(Deen, 1998, Pirot, et al., 1997), the probe vehicle to skin partition-coefficient enhancement, $E_K$, was approximated for each probe by taking the ratio of the average fluorescence intensity at the skin surface (corresponding to $z = 0$ μm) for the enhancer and the control samples. Specifically,

$$
E_K = \frac{I_E(z)_{z=0}}{I_C(z)_{z=0}} \quad (3.12)
$$
where $I_E(z)$ and $I_C(z)$ represent the average intensities of the probe for the enhancer and the control cases, respectively. For these studies, the skin volume associated with the concentration of the probe detected at the skin surface corresponds to the volume defined by the area of the field scanned and the two-photon laser beam point size in the axial or $z$-direction. Because the relative change in the probe vehicle to skin partition coefficient is the quantity of interest here, for the same skin surface volume, only the average probe surface intensity is considered.

3.3. Results

3.3.1. Three-Dimensional Visualization of Fluorescent Probe Spatial Distributions in the Skin

To illustrate the qualitative details that one can obtain using the TPM $z$-scans, representative three-dimensional images of the probe spatial concentration (or intensity) distributions for one site of the four sites examined per skin sample are shown in Figures 3-3(a), 3-3(b), 3-3(c), and 3-3(d). Each three-dimensional construct represents a field of 112 μm by 112 μm and a depth of 32 μm from the surface of the skin. The color scales used for these images are also shown in Figure 3-3, where regions in red (corresponding to 300) reflect regions of high probe concentration and regions in blue (corresponding to 0) reflect regions of low probe concentration. The $C$ in each figure points to structures resembling corneocytes.
Figure 3-3: Three-dimensional probe distributions in the upper 32 \( \mu \text{m} \) of the skin sample. (a) and (b) depict the hydrophobic probe (RBHE) spatial distributions resulting from the control and the chemical enhancer vehicles, respectively. (c) and (d) depict the hydrophilic probe (SRB) spatial distributions resulting from the control and the chemical enhancer vehicles, respectively.
3.3.1.1. Hydrophobic Probe RBHE

Figures 3-3(a) and 3-3(b) illustrate the spatial distributions of the hydrophobic probe RBHE across the thickness of the skin sample (32 μm) without (the control) and with the chemical enhancer oleic acid, respectively. Illustrating that the SC acts as the primary barrier to transdermal transport, the majority of the drop in intensity for RBHE occurs over the uppermost layers of the skin, approximately over 10 μm, limiting the fluorescence signal detection beyond that depth (see Figures 3-3(a) and 3-3(b)).

3.3.1.2. Hydrophilic Probe SRB

Figures 3-3(c) and 3-3(d) illustrate the spatial distributions of the hydrophilic probe SRB across the skin sample (32 μm). For the hydrophilic probe, SRB, in the control condition (see Figure 3-3(c)), the majority of the drop in probe intensity also occurs in the uppermost layers of the skin. In the presence of the oleic acid enhancer, however, the gradual decline of SRB concentration across the SC and into the VE (see Figure 3-3(d)) suggests that the chemical enhancer has reduced the SC barrier properties such that the contribution of the VE to the overall skin barrier becomes more noticeable.

3.3.2. Similarities between RBHE and SRB

For both RBHE and SRB, the presence of oleic acid is qualitatively observed to increase the quantity of each probe on the surface of the skin (compare Figures 3-3(b) and 3-3(a) for RBHE, and Figures 3-3(d) and 3-3(c) for SRB), which is indicative of increased partitioning of the probe into the skin. Moreover, the quantities of RBHE and SRB detected in the control samples are significantly lower as they lie in the blue region of the
color scale (see Figures 3-3(a) and 3-3(c)). Increases in both the partition coefficient and the concentration gradient of the probe due to the presence of oleic acid should contribute to an increase in the overall transdermal transport of the probe, as described by Eqs. (3.1) and (3.5).

Based on these three-dimensional images, regions of high probe concentration surround the corneocytes, suggesting that the primary transport pathway across the skin is through the lipid multilamellar regions between the corneocytes for both the hydrophobic and the hydrophilic model drugs examined. Because the resolution of these two-photon microscope images is limited by the laser scanning spot size of ~1 μm, molecular scale details are not visually accessible. Nevertheless, as discussed above and shown below, changes in the transport microenvironment can be inferred from a quantification of these images.

3.3.3. Differences between RBHE and SRB

Although both the hydrophobic and the hydrophilic model drugs examined appear to traverse the skin primarily via the intercellular route, the differences observed in the nature of the probe spatial distributions throughout the skin samples suggest that oleic acid enhancer action operates through different mechanisms for these two model drugs. These qualitative differences include the pronounced decrease observed in the average intensity for the hydrophobic probe over the first 8 μm of the skin sample, represented by the changes in the color scale from green to blue and from red to blue (see Figures 3-3(a) and 3-3(b)), for the control and the enhancer samples, respectively. A similar decrease in probe fluorescence signal, from green to blue (see Figure 3-3(c)), was detected for the hydrophilic probe over the first 16 μm in the control skin sample, but its spatial
distribution in the presence of oleic acid enhancer changed, showing the perfusion of hydrophilic probe throughout the 32 µm of the skin sample (see Figure 3-3(d)), highlighted by the regions in red. Insight into these differences in transport mechanisms can be gained from the quantitative analysis of the two-photon data which is presented next, and the examination of a larger number of skin sites that is introduced in Chapter 4.

3.3.4. Quantification of the Fluorescent Probe Spatial Distributions in the Skin

Figure 3-4: Sample average intensity profiles of RBHE. Key: sample average intensity counts calculated at each skin depth for the control (■) and the enhancer (□) vehicles. The average photon counts is plotted versus the corresponding skin depth. The error bars indicate one standard deviation from the sample average intensity of the four sites sampled.
Figures 3-4 and 3-5 show the measured average photon counts versus skin depth, representing the respective average concentration profiles

representing the respective average concentration profiles$^4$ of the hydrophobic (RBHE) and the hydrophilic (SRB) model drugs as described in Section 3.2.5. The average intensity profile at the skin surface ($z=0$ μm) is proportional to the magnitude of the probe skin-vehicle partition coefficient, and the slope of the curve corresponds to the probe concentration gradient.

![Graph showing average photon counts versus skin depth.](image)

**Figure 3-5:** Sample average intensity profiles of SRB. Key: sample average intensity counts calculated at each skin depth for the control (■) and the enhancer (□) vehicles. The average photon counts is plotted versus the corresponding skin depth. The error bars indicate one standard deviation from the average intensity of the four sites sampled.

---

$^4$ The estimated instrumentation error in the $z$ direction is 5%.
3.3.5. Quantification of the Relative Change in the Probe Vehicle to Skin Partition Coefficient, \( E_K \)

The higher probe concentrations detected at the surfaces of the three-dimensional representations of the oleic acid treated samples (see Figures 3-3(b) and 3-3(d)) agree with their corresponding probe intensity profiles in which four-fold increases in the average photon counts are measured at \( z=0\mu m \) (compare the □ and the ■ in Figures 3-4 and 3-5). A calculation of the probe partition-coefficient enhancement, \( E_K \), utilizing the \( I_E \) and \( I_C \) values obtained from the SAIPs at \( z=0\mu m \), yields values of 4.33 ± 3.45 and 4.56 ± 2.05 for the hydrophobic and the hydrophilic probes, respectively. The partitioning of both the hydrophobic and the hydrophilic probes from the control solution (1:1 PBS:ethanol) into the skin is enhanced to a similar degree in the presence of the oleic acid chemical enhancer. For both probes, the large error in \( E_K \) arises from the site to site variation of the probe average fluorescence profiles, as seen in Figures 3-4 and 3-5, in which the error bars reflect the variation in the SSDP detected at each skin depth (\( z \)-coordinate). The deduced \( E_K \) values further substantiate the increased partitioning of both RBHE and SRB from the vehicle to the skin in the presence of the oleic acid chemical enhancer shown in Figures 3-3(b) and 3-3(d), respectively.

3.3.6. Quantification of the Relative Change in the Probe Intensity Gradient, \( E_g \)

In addition to the partition-coefficient enhancement, the intensity gradients were calculated (see Section 3.2.6) for the SAIP’s in Figures 3-4 and 3-5, and the results are reported in Table 3-2 with respect to the slope and \( R^2 \) corresponding to the linear regression. The error corresponding to the slope calculations represents the statistical
range in which a 95% confidence level exists. As expected from the SAIP’s, the intensity gradients for RBHE and SRB in the control vehicles are similar, with values of $-7.66 \pm 0.21$ counts/$\mu$m and $-8.37 \pm 0.38$ counts/$\mu$m, respectively. However, the presence of the oleic acid chemical enhancer significantly increases the steepness of the intensity gradient of RBHE ($dI/dz = -41.16 \pm 1.67$ counts/$\mu$m), while having little effect on the SRB intensity gradient ($dI/dz = -10.42 \pm 0.58$ counts/$\mu$m). Based on the slope values determined, the concentration-gradient enhancements are $E_g = 5.37 \pm 0.26$ for RBHE and $E_g = 1.24 \pm 0.09$ for SRB, indicating that the RBHE flux enhancement results from an increase in the concentration gradient, one of the factors driving permeant flux across the skin (see Eq. (3.1)), as well as by increased probe partitioning into the skin.

<table>
<thead>
<tr>
<th>Probe</th>
<th>$dI/dz$ (counts/$\mu$m)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBHE (C)</td>
<td>$-7.66 \pm 0.21$</td>
<td>0.99</td>
</tr>
<tr>
<td>RBHE (E)</td>
<td>$-41.16 \pm 1.67$</td>
<td>0.99</td>
</tr>
<tr>
<td>SRB (C)</td>
<td>$-8.37 \pm 0.38$</td>
<td>0.98</td>
</tr>
<tr>
<td>SRB (E)</td>
<td>$-10.42 \pm 0.58$</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*The reported intensity gradients, $dI/dz$, are the slopes of the SAIP corresponding to the first 8 $\mu$m of each skin sample examined, and were obtained from linear regressions (see Section 3.2.6). The control and the enhancer vehicles are denoted by (C) and (E), respectively. The error reported for $dI/dz$ reflects the range of intensity gradient values that fall within a 95% confidence level based on the precision of the linear fit.*
3.3.7. Quantification of Site Specific Probe Intensity Gradients

The average intensity gradients calculated from the SAIP’s were compared to the intensity gradients obtained from each specific site examined by performing a linear regression of the SSDP’s. The four to six SSDP’s (not shown) that generate each SAIP revealed variations in probe intensity at each depth that were twice as large as those observed at the corresponding depth of the SAIP. These statistics suggest that the error bars associated with each SAIP may be reduced with increased sampling of different sites on the skin sample. Based on the statistics of a normal distribution, the error associated with each SAIP should decrease by the square root of the number of SSDP’s utilized in the analysis.

Table 3-3 reports the individual site specific probe intensity gradient values followed by the average of those values for each of the SSDP’s belonging to each of the four cases examined (RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer). The control and enhancer cases corresponding to RBHE for the SAIP intensity gradient (-7.66 ±0.21 and -41.16 ±1.67) and the average of the SSDP intensity gradients (-7.66 ±3.72 and -41.17 ±26.65) revealed nearly identical means. While the averages of the SSDP intensity gradients (-9.65 ±2.58, control and -15.43 ±4.47, enhancer) calculated for SRB were, in general, more negative in value than the corresponding SAIP intensity gradients (-8.37±0.38, control and -10.42±0.58, enhancer), the differences between the two intensity gradient calculations are ultimately captured within their error bars. The
analysis of the SSDP intensity gradients presented above suggests that the application of the SAIP in the methodology introduced here serves as an average representation of the different SSDP's sampled.

Table 3-3: SSDP linear regression results for each site examined.a

<table>
<thead>
<tr>
<th>Site No</th>
<th>RBHE</th>
<th></th>
<th>SRB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dl/dz</td>
<td>R²</td>
<td>dl/dz</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>(C)</td>
<td></td>
<td>(E)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-3.18</td>
<td>0.99</td>
<td>-20.30</td>
<td>0.97</td>
</tr>
<tr>
<td>2</td>
<td>-5.93</td>
<td>0.99</td>
<td>-20.30</td>
<td>0.98</td>
</tr>
<tr>
<td>3</td>
<td>-13.00</td>
<td>0.96</td>
<td>-52.80</td>
<td>0.96</td>
</tr>
<tr>
<td>4</td>
<td>-9.42</td>
<td>0.98</td>
<td>-88.70</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>-6.78</td>
<td>0.99</td>
<td>-40.60</td>
<td>0.95</td>
</tr>
<tr>
<td>6</td>
<td>--</td>
<td>--</td>
<td>-24.29</td>
<td>0.95</td>
</tr>
</tbody>
</table>

aThe linear regression for each SSDP was performed for the first 11 axial scans at each site to obtain the intensity gradient, dl/dz. The value of dl/dz for each site is reported for the control (C) and enhancer (E) cases. The R^2 value following each reported dl/dz value describes the accuracy of the linear fit.

bFor each group of SSDP's, the average value, AVG, of the intensity gradients calculated for the respective number of sites imaged per skin sample is reported as the mean±SD.

A discussion of the changes in the skin barrier diffusion length and in the skin diffusion coefficient of the permeant deduced from the permeability enhancement, $E_P$, the concentration-gradient enhancement, $E_g$, and the partition-coefficient enhancement, $E_K$, for RBHE and SRB is presented next.

### 3.3.8. Quantification of Oleic Acid-Induced Changes in Transdermal Transport Properties

The enhancements of the concentration gradient, $E_g$, the vehicle to skin partition coefficient, $E_K$, the permeability coefficient, $E_P$, the skin barrier diffusion length, $E_l$, and
the diffusion coefficient, $E_D$, are shown in Figure 3-6. The error bars represent one standard deviation from the average value, except for $E_g$, for which the error bars indicate a 95% confidence level in the results (described in Section 3.2.7). Factors contributing to the enhanced permeability values for the hydrophilic probe ($E_P = 9.59 \pm 2.92$) and the hydrophobic probe ($E_P = 7.35 \pm 1.00$) are immediately revealed by the $E_g$ and $E_K$ values. The $E_g$ values corresponding to the probe concentration gradients increased by $5.37 \pm 0.26$ (gray bar in Figure 3-6) and $1.24 \pm 0.09$ (striped bar in Figure 3-6), for the hydrophobic (RBHE) and the hydrophilic (SRB) probes, respectively. Despite the negligible change in the concentration gradient obtained for SRB in the presence of oleic acid, the value for $E_K$ ($4.56 \pm 2.05$, striped bar in Figure 3-6) suggests that the main driving force for flux enhancement in this case is due to the SRB increased affinity for the stratum corneum.

As shown in Figure 3-5, the average intensity profile for SRB maintains a similar shape in both the presence and the absence of oleic acid, but is translated in the direction of increasing intensity by a factor of four in the presence of oleic acid. For RBHE, both $E_g$ and $E_K$ clearly indicate increases in the average concentration gradient and in skin partitioning in the presence of oleic acid with values of $5.37 \pm 0.26$ and $4.33 \pm 3.45$ (gray bars in Figure 3-6), respectively.
Figure 3-6: Relative changes in transport parameters. Key: RBHE (gray bars), SRB (striped bars). With the exception of $E_g$, the error bars indicate one standard deviation from the mean. The error bars corresponding to $E_g$ for both probes represent the upper and the lower limit of a 95% confidence level.

These results presented for both the hydophilic and the hydrophobic probes are consistent with the two-layer transdermal diffusion model proposed by Yamashita et al. (Yamashita et al., 1995), who used mannitol (polar) and 6-mercaptopurine (nonpolar) as the model drugs in a system that accounts for the existence of both a polar and a nonpolar permeant transport route through the SC. For both routes, their results showed that the oleic acid action increased their model parameter associated with permeant partitioning from the vehicle to the skin (Yamashita, et al., 1995).
3.3.9. Quantification of Oleic Acid-Induced Changes in the SC Microtransport Environment as Reflected in $E_D$ and $E_I$

The oleic acid-induced changes in the hydrophilic probe microtransport environment that promote increased transdermal diffusion are discussed next. From the relative changes in the intensity gradient, $E_g$, and in the skin partition coefficient, $E_K$, one can subsequently estimate the changes that arise in the permeant skin barrier diffusion length, reflected in $E_I$, and in the permeant skin diffusion coefficient, reflected in $E_D$. $E_I$ is calculated using Eq.(3.10), in which the permeant skin barrier diffusion length enhancement is simply the ratio of $E_K$ and $E_g$, the two enhancement factors readily obtained using the SAIP's shown in Figures 3-4 and 3-5. By applying Eq.(3.6), in which $E_D$ can now be obtained as a function of $E_P$, $E_K$, and $E_I$ upon rearranging the equation, the quantitative changes in the permeant skin diffusion coefficient can be obtained.

For the hydrophilic probe, SRB, in the presence of oleic acid, an increase in the skin barrier diffusion length was calculated, as well as clearly visualized in Figure 3-5 (□). Based on Eq.(3.5), an increase in the in skin barrier diffusion length would contribute to a decrease in the permeability, but the concomitant increase in probe partition coefficient, $E_K = 4.56 \pm 2.05$, and diffusion coefficient, $E_D = 7.70 \pm 2.41$, offsets the increase in skin barrier diffusion length, $E_I = 3.66 \pm 1.67$

In contrast to the changes estimated for the microtransport properties of the hydrophilic probe, the skin barrier diffusion length and diffusion coefficient of the hydrophobic probe, RBHE, show minor deviations from the control condition in the presence of oleic acid, as reflected in the values of $E_I = 0.81 \pm 0.64$ and $E_D = 1.37 \pm 0.20$, respectively.
The deduced values for both the skin barrier diffusion length enhancement and the diffusion-coefficient enhancement are discussed below, first for the hydrophilic probe, SRB, and subsequently, for the hydrophobic probe, RBHE.

3.4. Discussion

3.4.1. Implications of Changes Detected in the Hydrophilic Probe Transport Properties

The observed increase in the skin barrier diffusion length \((E_I = 3.66 \pm 1.67)\) for the hydrophilic probe in the presence of oleic acid suggests the creation of a microtransport environment in the SC which is more amenable to hydrophilic drug transport such that the predominant skin barrier now includes both the SC and the viable epidermis. As revealed by the increase in the diffusion coefficient \((E_D = 7.70 \pm 2.41)\), the permeant microenvironment has noticeably changed to allow for the increased mobility of SRB. Therefore, the diffusion of the hydrophilic permeant exhibits a greater sensitivity to changes in the lipid environment because of its inherent incompatibility with the SC lipid environment under passive conditions. The precise significance of the four-fold increase in the skin barrier diffusion length, as well as the eight-fold increase in the skin barrier diffusion coefficient will be investigated in Chapter 4, where the validity of these studies with respect to a clinically more relevant skin area of 2mm by 2mm is examined.
3.4.2. Implications of Changes Detected in the Hydrophobic Probe Transport Properties

Compared to the significant increase detected in the skin barrier diffusion length for the hydrophilic probe, the minor change in this value for the hydrophobic probe \( (E_t = 0.81 \pm 0.64) \) indicates that, even in the presence of the oleic acid chemical enhancer action, the stratum corneum comprises the primary barrier to transdermal transport, as the deeper layers of the skin do not contribute to the transport barrier delineated by the drop in the probe concentration across the first 8 μm of the skin. The minor effect of oleic acid on the microtransport environment of the hydrophobic probe, as reflected in the \( E_D \) value of \( 1.37 \pm 0.20 \), can be attributed to the natural intrinsic compatibility of the hydrophobic probe with the SC lipid environment.

The increase in SC lipid fluidity associated with the oleic acid chemical enhancer action, in this case, is primarily manifested by the increased partitioning of the hydrophobic probe into the skin, reflected in \( E_K = 4.33 \pm 3.45 \), followed by the increased concentration gradient driving transdermal diffusion, reflected in \( E_K = 5.37 \pm 0.26 \). The error bars shown on the SAIP curves reflect the heterogeneity of the SC and result from the spread in the individual pixel intensities for each SSDP. We believe that these large variations (twice as large as those observed for the SAIP’s) in the SSDP’s are mainly dominated by the skin location morphological variation on the micron scale. In the presence of oleic acid chemical enhancer action, the variance for both RBHE (Figure 3-4, □) and SRB (Figure 3-5, □), increases, as reflected in the increased size of the error bars relative to the
control cases (Figures 3-4 and 3-5, ■). In light of this observation, additional variance may also result from the nonuniform action of the oleic acid. A heterogeneous distribution of oleic acid throughout the SC may result from the formation of separate domains of oleic acid pools within the SC lipids (Ongpipattanakul, et al., 1991).

Based on the data presented, the variance due to SC morphology may be reduced by sampling over larger areas at each site in the skin sample. However, as the sampling area increases, the intrinsic heterogeneity of the skin properties and the nonuniform action of the oleic acid chemical enhancer may become more evident. The site to site variations observed in the skin model are further addressed in Chapter 4.

3.5. Conclusions

The advantages of TPM in providing three-dimensional images detailing the chemical enhancer-induced changes in fluorescent probe distributions in the upper 32 μm of skin samples, with respect to important structural features such as the corneocytes and the lipid multilamellar region, have been demonstrated in this chapter. More importantly, we have presented, for the first time, a methodology that quantitatively characterizes the significance of the visualized differences in these three-dimensional images with respect to the commonly investigated permeant transport parameters. The agreement between the apparent changes in probe concentration gradient, probe partitioning, probe diffusion coefficient, and probe skin barrier diffusion length, estimated using this method, and the phenomenological explanations of permeability enhancement detailed in the literature illustrates the potential of two-photon microscopy in elucidating the mechanisms of transdermal transport processes at the mechanistic level. The results presented above for
this in vitro skin model system, in addition to the noninvasive nature of TPM, reinforces the potential applicability of utilizing this method in future studies to understand the changes underlying the experimentally measured permeabilities for in vivo skin systems.

In Chapter 4, the methodologies introduced in this chapter will be applied to a skin area 100 times larger than the combined areas of the 4-6 skin sites examined here. Based on the variability of the global changes in the oleic acid-induced transdermal transport properties observed in the wide-area skin imaging performed in the next chapter, the valid skin sample size will be determined for the hydrophobic and the hydrophilic model fluorescent probes examined in this chapter.
3.6. References


Chapter 4

Topographic Heterogeneity in Transdermal Transport Revealed by High-Speed Two-Photon Microscopy: Determination of Representative Skin Sample Sizes

4.1. Introduction

The recent application of two-photon microscopy (TPM) to visualize model drug spatial distributions across human cadaver skin, introduced in Chapter 3, has enabled the noninvasive elucidation of oleic acid-induced changes in transdermal transport properties (see also Yu et al., 2001). Compared with other visualization techniques, the advantages of TPM include increased three-dimensional depth discrimination, increased signal collection efficiency, and reduced skin sample photobleaching and photodamage (Masters et al., 1997). In Chapter 3, the quantification of the relative changes (enhancement values) in the vehicle to skin partition coefficient, $E_K$, the skin intensity gradient, $E_g$, the skin diffusion coefficient, $E_D$, and the skin barrier thickness, $E_l$, of the probes used was based on a limited sampling of 4 to 6 different skin sites per skin sample, where each skin site covers 100 μm by 100 μm. Moreover, the site-to-site
variations in probe spatial distributions were attributed to the intrinsic heterogeneity of the skin morphology (Yu, et al., 2001). While the morphological variations in skin topology have been pertinent to dermatological research (Huzaira et al., 2001), the consequences of the skin morphology on the variability of transdermal permeant distributions over a clinically relevant skin area have not yet been addressed, and is the focus of this chapter.

In this chapter, the permeant spatial distributions over 400 consecutive sites in excised human cadaver skin were captured to reveal the variability of transdermal transport over a more clinically relevant area of 2mm by 2mm, using High-Speed Two-Photon Microscopy (HTPM) (Kim et al., 1999). In Chapter 3, the restriction to small sample sizes (4-6 skin sites) (Yu, et al., 2001) was largely due, in part, to the slow image acquisition times (0.5 hours per skin site), which are typical of instruments with microscopic resolution such as conventional TPM imaging systems. The development of HTPM has subsequently increased data acquisition rates by two orders of magnitude (Kim, et al., 1999), thus enabling the scanning of multiple skin sites per skin sample over a relatively short period of time. Specifically, for the 400 skin sites examined in this chapter, the total scanning time remains below 1.5 hours, compared with the 200 hours required by the conventional TPM for the same number of skin sites.

This chapter evaluates the sample-size dependence of the oleic acid-induced enhancement values introduced in Chapter 3 (see also Yu, et al., 2001). Based on the methodology described below, the optimum number of skin sites that capture the global
variations in transdermal transport was determined for the hydrophobic and the hydrophilic model permeants examined.

4.2. Materials and Methods

4.2.1. Sample Preparation

Human cadaver skin (National Disease Research Interchange, Philadelphia, PA) was prepared following the methods described in Chapter 2, Sections 2.2 and 2.7. Skin barrier integrity was evaluated using the procedure described in Chapter 2, Section 2.6. Sulforhodamine B (SRB), the model hydrophilic probe, and rhodamine B hexyl ester (RBHE), the model hydrophobic probe, (Molecular Probes, Eugene, OR) were prepared at 0.33 mg/ml for both the control and the model chemical enhancer vehicles (Yu, et al., 2001).

4.2.2. High-Speed, Two-Photon Microscope and Data Analysis

400 consecutive skin sites in a 2mm by 2mm area of each skin sample were scanned from the skin surface to a depth of 21 μm (at 0.7 μm intervals) using the high-speed, two-photon microscope described in a separate study (Kim, et al., 1999). The 400 different consecutive skin sites (each with an area of 100 μm by 100 μm) were computationally stitched together at a specified skin depth. The islands of dark regions, most apparent in Figure 4-1(b), reflect locations of incomplete SC contact with the microscope slide coverslip due to the skin surface roughness, and have been omitted from the data quantification results presented below.
The quantification methodology, introduced in Chapter 3, was applied in the present study to determine the enhancement values. In Chapter 3, the relative change in the probe vehicle to skin partition coefficient, $E_K$, was defined as the ratio of the average probe intensities at the surface of the skin ($z=0$) for the enhancer and the control samples (see Chapter 3, Section 3.2.7). In the 400-skin site study presented in this chapter, $E_K$ is redefined as the ratio of the zero-order linear regression coefficients (the intercepts) of the linear region of the wide-area average intensity profile for the enhancer and the control cases. This linearly regressed value reflects the probe intensity at the surface of the skin where $z=0$ μm, and yields a 95% confidence interval that reduces the error contributions to $E_K$.

### 4.2.3. Determination of Representative Skin Sample Sizes

For each sample size (6, 12, 24, and 48 skin sites), ten different sets of transport enhancement values for each probe were calculated based on ten randomly selected sets of skin sites from the control and the enhancer skin samples. For each sample size, the relative variation, $V$, from the wide-area values calculated for each transport enhancement property were quantified utilizing the following equation (Johnson, 2000):

\[
V = \frac{1}{\langle E \rangle} \sqrt{\frac{\sum_{i}^{N} (E_i - \langle E \rangle)^2}{(N-1)}}
\]  

(4.1)

where $N$ denotes the 10 different sets of skin sites associated with each sample size evaluated, $E_i$ represents the transport property enhancement value obtained for data set $i$, and $\langle E \rangle$ is the wide-area value of the transport property enhancement.
4.3. Results and Discussion

4.3.1. Visualization of Wide-Area Axial Scans

For each skin sample, a selected fraction of the total 4mm² skin area evaluated is shown in Figure 4-1. Figures 4-1(a), 4-1(b), 4-1(c), and 4-1(d), depict the spatial heterogeneity of probe distributions captured over a small portion of the total skin area imaged. The green box in Figure 4-1(a) represents the area encompassed by one microscope field of view. For each image in Figure 4-1, a comparison of the probe spatial distributions covered by one field of view with the corresponding skin fraction shown illustrates the need to examine multiple skin sites to fully capture the site to site variations in permeant transport.

For both RBHE and SRB, oleic acid-induced increases in the probe partitioning into the stratum corneum (SC) are revealed by the increased intercellular probe intensities that are represented by the light blue regions in the color scale shown in Figure 4-1. In addition to the increased probe distribution throughout the intercorneocyte regions, Figure 4-1(b) illustrates an increase in the heterogeneity of the probe distribution, with regionalized areas of high probe concentration (regions in white).
Figure 4-1: Selected regions from the 4mm² total skin area scanned are shown for the (a) RBHE control, (b) RBHE oleic acid, (c) SRB control, and (d) SRB oleic acid cases of the probe distributions close to the skin surface. The colorbar shows the pseudo-color scale, ranging from a low intensity of $I_0$ to a high intensity of $I_n$, utilized to represent the relative probe intensities. For (a), (b), (c), and (d), $I_n = 100$. To account for the increased range of intensities, $I_n = 3000$ for (a) and $I_n = 10000$ for (b). $I_n = 5000$ for both (c) and (d). Regions of highest probe intensities are depicted in white.
In contrast to the similar probe intensity distributions observed in an overwhelming majority of the 400 skin sites scanned for the RBHE control (Figure 4-1(a)), Figure 4-1(c) illustrates the nonuniform spatial distribution of the hydrophilic probe SRB typical of the 2mm by 2mm area scanned. The black to dark blue colors marking the intercellular regions observed in Figure 4-1(c) reflect the low skin permeabilities to hydrophilic permeants such as SRB. Furthermore, the lighter blue regions in Figure 4-1(c) illustrate a localization of SRB transport that is consistent with the proposed existence of aqueous ‘shunt’ transport pathways (Scheuplein, 1967, Sznitowska et al., 1998). In the presence of oleic acid, the overall increase in SRB skin penetration is accompanied by the appearance of distinct intercellular regions of higher probe concentrations that are highlighted in light blue (see Figure 4-1(d)).

The wide-area axial scans presented in Figure 4-1 unquestionably demonstrate the advantages of HTPM in efficiently increasing the sample area (100-fold) to provide a dramatically improved visualization of the range of variations in probe distributions over a clinically more relevant area of skin (2mm by 2mm). From these scans, the skin area represented by one field of view (100 μm by 100 μm) clearly does not validly capture the variability of the probe distributions over the 400 consecutive skin sites scanned.

4.3.2. Quantification of Relative Changes in Transdermal Transport Properties

As shown in Figure 4-2, the predominant effects of oleic acid action based on the 400-skin site analysis for the hydrophilic (SRB) and for the hydrophobic (RBHE) probes are
the enhancements in the vehicle to skin partitioning ($E_K = 4.77\pm0.83$ and $10.22\pm0.55$, respectively) and in the concentration gradient ($E_g = 4.81\pm1.86$ and $9.93\pm1.50$, respectively). The physically pertinent $E_i$ values of approximately unity obtained for both probes indicates that the SC remains the primary barrier to transdermal transport, and brings greater validity to relative changes in transport properties calculated for both probes based on the 100-fold increase in sample size.

![Graph showing transport property enhancement values for different conditions.](image)

**Figure 4-2:** Relative changes in oleic acid-induced transport. Key: 400-skin site RBHE (gray bars), 400-skin site SRB (white bars), RBHE-limited sample size (black bars), SRB-limited sample size (checkered bars). The wide-area values correspond to the transport enhancement values obtained in this study utilizing the data from the 400 skin sites scanned, whereas the limited sample size values refer to those reported in Chapter 3 (see also Yu, et al., 2001) based on 4-6 skin sites.
Good agreement exists for the hydrophobic probe $E_K$, $E_g$, $E_D$, and $E_I$ values between the 400-site study (Figure 4-2, gray bars) and the 4 to 6-site study presented in Chapter 3 (Figure 4-2, black bars). The changes in the transport properties for the hydrophilic probe, however, exhibit a high degree of variability between these two very different skin sample sizes. This can be seen by comparing the white bars in Figure 4-2, that represent the 400-skin site hydrophilic probe enhancement values, and the checkered bars in Figure 4-2, that represent the 4-6 skin site enhancement values. Moreover, the hydrophilic 4-6 skin site $E_I$ value of $3.66 \pm 1.67$ predicts an increase in the skin barrier thickness, suggesting that, in the presence of oleic acid, the primary transport barrier extends beyond the SC layer. This $E_I$ value deviates from unity and is inconsistent with the role of the SC as the primary barrier to transdermal transport.

4.3.3. Validity of Limited Site Sampling in Elucidating Relative Changes in Transdermal Transport Properties

To address the inconsistency found between the hydrophilic probe enhancement values based on 4-6 skin sites (see Chapter 3, Figure 3-6) and the values obtained based on the 400-skin site analysis, sample sizes reflecting a smaller number of skin sites (6, 12, 24, and 48 sites) were evaluated for both the hydrophilic and the hydrophobic probes to determine the feasibility of inferring chemical enhancer induced changes in global transport properties based on a limited sample size.

The relative insensitivity of the enhancement values to sample size for the hydrophobic probe is further substantiated by the mild decreases in the relative variation, $V$, calculated using Eq.(4.1), with increases in sample size. The fractional deviations of approximately
36%, 27%, 40%, and 33% were obtained for the hydrophobic $E_K$, $E_g$, $E_D$, and $E_l$ values using the 6-skin site sample size. Increasing the sample size to 48 skin sites decreases the relative variance values to approximately 17%, 21%, 26%, and 15%, respectively. In optimizing the sample size, increasing the sample size beyond 6 skin sites does not provide substantial increases in the accuracy. Considering the variations in the human skin permeabilities described in the literature, with intersample variations in drug permeability as large as 40% (Southwell et al., 1984), and intrasample drug permeability variations of 20-30% (Noonan and Gonzalez, 1990), the relative variances of enhancement values resulting from a 6-skin site sample size fall within the range of variation expected due to the inherent heterogeneity of the skin morphology.

![Graph showing relative variation of transport properties](image)

**Figure 4-3: Hydrophilic probe transport property enhancement relative variation.** Key: 6 skin sites (black bars), 12 skin sites (gray bars), 24 skin sites (white bars), and 48 skin sites (striped bars). The strong dependence of the relative variation (see Eq.(4.1)), reported in percentage form along the y-axis, is shown as a function of the sample size on the x-axis for each transport enhancement value. The data table below the chart lists the values of the relative variations calculated.
For the hydrophilic probe, however, Figure 4-3 shows the strong dependence of V on the 4 different sample sizes evaluated. Compared to the 6-skin site V values for $E_K$, $E_g$, $E_D$, and $E_l$ of 75%, 98%, 62%, and 21%, respectively, significantly decreased values of approximately 13%, 17%, 18%, and 9%, respectively, were obtained using the 48-skin site sample size. The optimum sample size for the hydrophilic probe is 24 skin sites, at which the maximum decrease in the relative variance is observed with the least number of increases in the skin sites. At the 24-skin site sample size, the relative variance values of approximately 15% to 26% all fall within the criteria cited above that describe the expected variability of human skin.

4.4. Conclusions

The variations in the probe spatial distributions observed over the 400 consecutive skin sites imaged, suggest that, for future applications of other microscopy visualization techniques, the validity of the global changes in skin barrier function deduced from the evaluation of a limited skin sample size warrants additional scrutiny. A more detailed analysis of the hydrophobic and the hydrophilic probe distributions examined in this chapter over the 400 different skin sites will be presented in Chapter 5, where the rate-limiting transdermal transport step in the presence of the oleic acid chemical enhancer action for each probe is deduced.
4.5. References


Chapter 5

Elucidation of Oleic Acid-Induced Changes in Transdermal Permeant Pathways Using Wide-Area Analyses of Variations in Microscale Transport Parameters

5.1. Introduction

The transdermal route of drug delivery provides a noninvasive method of administering pharmaceutically relevant compounds over a sustained period (Gay, 1996, Naik et al., 2000). The inherent advantages exhibited by transdermal drug delivery have been described in Chapter 1, Section 1.1. Due to our limited understanding of the mechanisms of chemically-induced transdermal drug delivery, the successful formulation of these chemical enhancer vehicles, however, has resulted primarily from the systematic trial-and-error analysis of different chemical enhancer systems.
In this chapter, the mechanisms of chemical enhancer action responsible for the transdermal transport of rhodamine B hexyl ester (RBHE) and sulforhodamine B (SRB), the model hydrophobic and hydrophilic fluorescent probes, respectively, used in Chapters 3 and 4, were elucidated using the novel High-Speed Two-Photon Microscopy (HTPM) wide-area skin imaging methodology introduced in Chapter 4 (see also Kim et al., 1999, Yu et al., 2002). Using the methodology described in Chapter 4, Section 4.2, (see also Yu et al., 2001) the effects of the model chemical enhancer oleic acid on two transport parameters – the vehicle to skin partition coefficient and the skin concentration gradient of each probe – were evaluated over 400 consecutive skin sites, where each skin site captures a 100 μm by 100 μm microscope field of view.

The spatial variability of the two transport parameters at each of the 400 individual skin sites imaged were captured using surface plots of the fluctuations in the probe free energy for partitioning and in the probe intensity gradient relative to the corresponding 400 skin site values calculated for each skin sample examined. These surface plots illustrate the influence of oleic acid on the inherent heterogeneity of the skin barrier, and give rise to the specific statistical distributions for the two transport parameters that are exhibited over the 400 skin sites. Histograms depict the frequencies of the values obtained for each of the two transport parameters over the 400 skin sites, and the statistical distributions of the two transport parameters that these histograms represent are characterized by their corresponding skewness and kurtosis values.
The skewness and the kurtosis describe the symmetry and the peakedness of the shapes of statistical distributions, respectively, and will be discussed in more detail in Section 5.3.4. Hence, these histograms reflect the probability distributions for each transport parameter over the skin area scanned. The quantitative descriptors of skewness and kurtosis (see Section 5.1.1) enable the systematic comparison of the changes in the shapes of the transport parameter distributions arising from the oleic acid enhancer action. From the changes in the distributions of these two transport parameters, the rate-limiting steps in transport for the hydrophobic and the hydrophilic permeants examined were determined with respect to the permeant partitioning and the permeant diffusion steps of transport. Furthermore, these oleic acid-induced changes in transport parameter distributions over the 400 different skin sites examined provided further evidence supporting the existence of permeant specific transport pathways resulting from the intrinsic skin morphology.

5.1.1. Skin Heterogeneity

The inherently complex morphology of the stratum corneum imparts a high degree of heterogeneity to this skin layer. These topological variations in skin morphology have been described in the literature (Huzaira et al., 2001), and the microscale drug spatial distributions arising from the skin heterogeneity were visualized using HTPM and discussed in Chapter 4 (see also Yu, et al., 2002). The variations in the hydrophilic and the hydrophobic drug transdermal permeabilities using diffusion cells have also been characterized (Kasting et al., 1987, Southwell et al., 1984, Williams et al., 1992), with reported intersample variations in drug permeability as large as 40% (Southwell, et al., 1984), and intrasample drug permeability variations of 20-30% (Noonan and Gonzalez, 1990). Furthermore, the statistical distributions of the number of skin samples
corresponding to a measured skin permeability indicated that the hydrophobic permeants (estradiol, ethanol, and isopropanol) exhibited more symmetric distributions of skin permeabilities about the mean compared to the hydrophilic permeants (terbutaline sulfate and sodium diclofenac) (Liu et al., 1993). For Gaussian, or normal, distributions, the mean equals the median (the value representing the 50th percentile of the data). Deviations of the median from the mean reflect asymmetric data distributions.

For larger data sets, such as the ones acquired utilizing HTPM, the geometric shapes of the data distributions can be quantified utilizing skewness and kurtosis values, which describe the asymmetry about the mean and the peakedness of the distribution profiles, respectively. Eq.(5.1) below describes the skewness ($\mu_3$) and the kurtosis ($\mu_4$):

$$
\mu_k = \frac{\sum_{all\ x} (x - \lambda)^k}{\sigma^k}
$$

(5.1)

where $x$ is the sample value, $\lambda$ is the mean, and $\sigma$ is the standard deviation (Johnson, 2000). Normal, or symmetric, distributions have skewness values of 0, whereas positive and negative skewness values correspond to distributions with tails extending to the right and to the left of the mean, respectively. Moreover, the kurtosis value describes how prone a distribution is to outliers, with a kurtosis value of 3 representing a normal distribution. Kurtosis values greater than 3 are outlier prone, and thus exhibit flatter distribution profiles, whereas kurtosis values less than 3 correspond to peaked distribution profiles. By comparing the changes in the skewness and the kurtosis values associated with the two transport parameter distributions examined, the effects of oleic
acid enhancer action on the SC morphology and on the associated reduction in skin barrier function can be examined in a quantitative systematic manner. Because the hydrophobic and the hydrophilic permeant transdermal pathways arise from the inherent SC morphology, the observed changes in the two transport parameters distributions induced by oleic acid also serve as an indicator of corresponding changes in the lipoidal and the aqueous pore pathways described below.

5.1.2. Proposed Permeant Pathways

The stratum corneum (SC), the uppermost 10-20 μm of the skin, serves as the primary barrier to transdermal transport of permeants (see Chapter 1, Section 1.1). The SC has been described utilizing the brick and mortar model in which the corneocytes (bricks) lie in a continuous phase of lipid multilamellae (mortar). Permeant partitioning into the SC, followed by permeant diffusion through the SC, are the two main steps in a simplified description of the permeation mechanisms across the SC (Sznitowska et al., 1995). Because the lipid multilamellae of the intercorneocyte region form the only continuous phase through the SC, this intercellular region has been proposed to be the primary transdermal diffusion pathway, for both hydrophilic and hydrophobic permeants (Johnson et al., 1997, Potts and Guy, 1992, Suhonen et al., 1999). Within the intercellular transport region, however, the terms ‘lipoidal’ and ‘aqueous pore’ pathways have been used to describe the two different transport pathways that distinguish the mechanisms underlying the hydrophobic and the hydrophilic permeant transdermal transport, respectively (Hirvonen et al., 1998, Manabe et al., 1996, Sznitowska et al., 1998).
Lateral diffusion through the intercellular region was shown to constitute the rate-limiting step in transdermal transport for a series of hydrophobic probes in a model skin system of lipid multilamellar liposomes utilized to mimic the lipoidal pathway of these permeants. (Johnson, et al., 1997) However, the current understanding of the ‘aqueous pore’ pathway remains less developed (Sznitowska, et al., 1998), although the lacunar regions of the intercellular space have provided a morphological basis for the hydrophilic permeant transdermal transport pathway (Menon and Elias, 1997).

In the presence of oleic acid, increased permeant transdermal transport has been attributed to SC lipid multilamellae fluidization (Garrison et al., 1994, Golden et al., 1987, Green et al., 1988, Mak et al., 1990, Naik et al., 1995, Wartewig et al., 1998). These oleic acid-induced changes in the SC barrier function were quantified in Chapter 3 using Two-Photon Fluorescence Microscopy (TPM), in which the relative changes in transdermal transport properties (enhancement values), including the probe vehicle to skin partition coefficient, $E_K$, and the probe concentration gradient, $E_g$, were determined (Yu, et al., 2001). In the studies presented in Chapter 3, the probe intensity at the skin surface (a depth of $z = 0 \mu m$) and the probe skin intensity gradient reflected the probe vehicle to skin partitioning and the probe concentration gradient, respectively. Hence, in Chapter 3, Section 3.1.3, $E_K$ and $E_g$ correspond to the ratio of the probe surface intensities and the ratio of the probe intensity gradients, respectively, in the presence and in the absence of oleic acid.
The weakening of the SC barrier to enable increased permeant penetration can also be described by a decrease in $G^*$, the free energy associated with the work required for entry of a permeant into the SC lipid multilamellae (Cevc et al., 1996, Mitragotri et al., 1999). $G^*$ can be related to the probe vehicle to skin partition coefficient, $K$, utilizing the following relation:

$$K = \exp\left(-\frac{G^*}{RT}\right) \quad (5.2)$$

where $R$ and $T$ are the gas constant (8.314 J/mol K) and the absolute temperature of the system, respectively (Cevc, et al., 1996). According to Eq.(5.2), probe partitioning into the skin increases exponentially with decreases in the free energy, $G^*$. In the case of the HTPM studies reported in this chapter, the relative magnitudes of the probe vehicle to skin partitioning are reflected in the magnitudes of the probe surface intensities (see also Yu, et al., 2001). Through this direct relationship between the probe vehicle to skin partition coefficient and the probe surface intensity, the histograms representing the frequency of each measured surface intensity over the 400 skin sites examined depict the corresponding probability distributions of the probe partition coefficient and, hence, of the free energy, $G^*$, associated with probe partitioning.

The spatial variability of the skin barrier resulting from the inherent skin morphological heterogeneity, which gives rise to the 400 skin site surface intensity distributions, can be observed through the fluctuations in the free energy of probe partitioning at each skin site, $i$, relative to the 400 skin site global average, $G_{\text{fluct},i}$. Note that $G_{\text{fluct},i}$ is the difference
between the free energy specific to one of the 400 skin sites examined and the 400 skin site average free energy. These fluctuations in the free energy can be determined using Eq. (5.3) below:

\[
\frac{G_{\text{fluct},i}}{RT} = -\ln \left( \frac{K_i}{K_A} \right)
\]  

(5.3)

where \( K_i \) is the partition coefficient at the individual skin site, \( i \), and \( K_A \) is the global average vehicle to probe partition coefficient. In the studies reported in this chapter, \( K_i \) and \( K_A \) are captured by the surface intensity at skin site, \( i \), and by the average surface intensity over the 4mm\(^2\) skin area surveyed, respectively. The subscript, \( A \), can correspond to the control skin sample (C) or to the enhancer skin sample (E) for the cases of \( K_C \) and \( K_E \), respectively.

The relative change in the probe vehicle to skin partition coefficient, \( E_K \), is quantified as the ratio of the 400-skin site enhancer surface intensity and the 400-skin site control surface intensity (Yu, et al., 2001). In other words, \( E_K = K_E / K_C \) can also be described by the relationship, \( E_K = \exp((-\Delta G^*)/RT) \) where \( \Delta G^* = G_E^* - G_C^* \), and the subscripts E and C denote the enhancer and the control samples, respectively. Hence, the change in the free energy, \( \Delta G^* \), can be determined by the following equation:

\[
\Delta G^* = -RT \ln(E_K)
\]  

(5.4)

The relevance of \( G_{\text{fluct},i} \) and of \( \Delta G^* \) in further elucidating the mechanism of chemical enhancer action will be discussed in Section 5.3.2.
While the oleic acid-induced changes in the first step of transdermal transport, probe partitioning, is revealed through changes in the free energy of probe partitioning, the oleic acid-induced changes in probe diffusion through the SC, the subsequent step of probe transport across the SC, can be inferred through the changes in the probe concentration gradient across the SC. In Chapter 3, the oleic acid-induced changes in the probe intensity gradient captured the effect of chemical enhancer action on the probe concentration gradient (see also Yu, et al., 2001). The probe concentration gradient, in turn, controls the probe flux across the SC based on the one-dimensional Fickian diffusion relation, \( J = -D(dC/dz) \), where \( J \) is the probe flux in the direction of increasing skin depth, \( z \), \( D \) is the probe skin diffusion coefficient, and \( (dC/dz) \) is the probe concentration gradient across the SC (see Chapter 3, Section 3.1.3). From this relation, the probe flux increases with an increase in the steepness of the concentration gradient which, in these studies, is captured by the intensity gradient, \( dI/dz \). Hence, the individual skin site fluctuations in the probe intensity gradient, \( (dI/dz)_{\text{fluct,i}} \), reflect the fluctuations in the probe flux relative to the 400 skin site average, and further illustrates the effect of the oleic acid enhancer on the probe diffusion throughout the SC. Accordingly, \( (dI/dz)_{\text{fluct,i}} = (dI/dz)_i - (dI/dz)_A \), where \( (dI/dz)_i \) is the intensity gradient at site, \( i \), and \( (dI/dz)_A \) is the 400 skin site intensity gradient for which the subscript, \( A \), refers to the control case (denoted by C) or the enhancer case (denoted by E). These fluctuations in the probe flux will be examined further in Section 5.3.3.
5.2. Materials and Methods: High-Speed, Two-Photon Microscope and Data Analysis

Human cadaver skin samples were prepared following the methodology described in Chapter 4, Section 4.2. From the wide-area transdermal transport studies presented in Chapter 4 (see also Yu, et al., 2002), the 400-skin site probe surface intensity, \( I_{z=0} \), and the 400-skin site probe intensity gradient, \( dI/dz \), were quantified for each of the four cases examined (RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer). Table 5-1 lists the average as well as the median of the slope and of the intercept values that result from the 400 individual linear regressions performed corresponding to each skin site imaged. As described in Chapter 3, Sections 3.2.6 and 3.2.7 (see also Yu, et al., 2001), these slope and intercept values reflect the probe concentration gradient and the probe vehicle to skin partition coefficient, respectively. These two transport parameters, which enable the calculation of the enhancer-induced changes in the probe vehicle to skin partitioning, \( E_K \), and in the probe skin concentration gradient, \( E_g \), were determined from a linear regression of the 400-skin site probe intensity profile, which quantifies the probe intensity as a function of skin depth based on the HTPM scans performed at skin depths of 0.7 \( \mu m \) intervals over a skin depth of 0 \( \mu m \) to 21 \( \mu m \). The 400-skin site median of the 400-skin site intensity profile measured per sample was also determined as an indicator of the asymmetry in the probe intensity distributions.

The number of maxima and minima corresponding to the fluctuations in the free energy, \( G_{\text{fluct},i} \), that were determined using Eq.(5.3), and to the fluctuations in the probe intensity gradient, \( (dI/dz)_{\text{fluct},i} \), were further quantified. For each skin sample considered, the skin
sites for which $G_{\text{fluct},i}$ exceeded 1RT were counted as maxima, whereas the number of minima corresponded to skin sites revealing $G_{\text{fluct},i}$ values below -1RT.

**Table 5-1: Central tendencies of transport parameters.**

<table>
<thead>
<tr>
<th></th>
<th>INTENSITY GRADIENT(^a)</th>
<th>SURFACE INTENSITY(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>median</td>
</tr>
<tr>
<td>RBHE (C)</td>
<td>57 ± 36</td>
<td>55</td>
</tr>
<tr>
<td>RBHE (E)</td>
<td>266 ± 198</td>
<td>240</td>
</tr>
<tr>
<td>SRB (C)</td>
<td>6.9 ± 8.9</td>
<td>3.4</td>
</tr>
<tr>
<td>SRB (E)</td>
<td>67 ± 56</td>
<td>54</td>
</tr>
</tbody>
</table>

The intensity gradient and the surface intensity are represented, respectively, by the first-order (slope) and zero-order (intercept) linear regression parameters of the first ten points in the linear region of the intensity profile generated at each of the 400 skin sites examined. The mean and the median values were calculated based on these individual linear regression results of 400 different skin sites in each sample. The control and the enhancer samples are denoted by (C) and (E), respectively.

The mean is reported as the mean ±SD.

\(^a\) The absolute value of the intensity gradient is reported in units of counts/μm.

\(^b\) The surface intensity is reported in units of counts/pixel.

The arbitrary selection of ±1RT as the criterion for determining the number of extrema, coincidentally, reflects the thermal energy. As a basis of comparison, the thermal energy, or 1RT per mole, roughly indicates the strength of an interaction, where interactions with
energies exceeding 1RT dominate over the disorganizing effects of thermal motion (Israelachvili, 1992).

For the intensity gradient fluctuations, maxima and minima were defined by the skin sites that exhibited intensity gradient values exceeding the range of values encompassed by the corresponding 400 skin site intensity gradient value plus and minus the reported standard deviation (SD), respectively (see Table 5-1). Recall that 1 standard deviation, by definition, includes 68.27% of the 400 intensity gradients measured. Table 5-2 lists the number of maxima and minima determined for each skin sample examined based on the application of the ±1SD criteria to each of the 400 skin sites imaged.

Table 5-2: Quantification of the 400 skin site free energy and intensity gradient extrema.

<table>
<thead>
<tr>
<th></th>
<th>Free-Energy Fluctuation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Intensity-Gradient Fluctuation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no. of maxima</td>
<td>no. of minima</td>
</tr>
<tr>
<td>RBHE (C)</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>RBHE (E)</td>
<td>92</td>
<td>28</td>
</tr>
<tr>
<td>SRB (C)</td>
<td>113</td>
<td>34</td>
</tr>
<tr>
<td>SRB (E)</td>
<td>29</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Maxima correspond to skin sites where $G_{\text{free,1}} > 1$RT, and minima correspond to skin sites where $G_{\text{free,1}} < -1$RT. In order to quantify the number of maxima and minima, the activation energy fluctuation criterion of 1RT, the thermal energy, above and below the mean was employed.

<sup>b</sup>Maxima and minima are defined by skin sites exhibiting $\frac{dI}{dz}$ values exceeding the corresponding 400 skin site average slope values shown in Table 5-1 by +/- 1 SD, respectively.

For the intensity gradient fluctuations, the sensitivity of the number of extrema determined to the criterion applied was evaluated by applying 10 different criteria to the same set of 400 intensity gradient fluctuation values for each sample. The 10 additional
criteria applied for evaluating the number of intensity gradient extrema begin with the mean ± 0.2 SD and increase at intervals of ± 0.2 SD, where the mean ± 2SD is the last criterion examined. For each probe, the ratio of the total number of extrema (minima + maxima) in the enhancer case and in the control case (referred to as the extrema ratio) was used to verify the robustness of the number of extrema determined depending on the specific criterion applied. Variability in the value obtained for the extrema ratio upon the application of the different criteria indicates that the number of extrema determined is sensitive to the criterion utilized.

In these studies, the 10 additional extrema ratios that correspond to each of the 10 different criteria evaluated were averaged, where the standard deviation of these 10 values from the mean extrema ratio value served as an indicator of the variability in the number of extrema determined. Hence, large standard deviations reflect a strong dependence of the extrema number determined on the criterion applied.

For the hydrophobic probe, the extrema ratios determined under each criterion were insensitive to the specific intensity gradient criterion used, as reflected in the mean extrema ratio of 0.99±0.11. However, the hydrophilic probe extrema ratios exhibited greater sensitivity to the criterion used, with a peak extrema ratio of 2.42 at the criterion of ±0.8 SD. At the criterion of ±1SD used to determine the number of extrema in Table 5-2, the mean ratio exhibits a slightly lower value of 2.00. In light of these two values, the average extrema ratio of 1.36±0.47, based on the 10 extrema values, reflects the large variability introduced at the criteria of ±0.8 SD and ±1SD. By excluding these two
values, the mean extrema ratio for SRB becomes 1.17± 0.14, where the SD has decreased from 0.47 to 0.14. Thus, the ±1SD criterion implemented in Table 5-2 identifies the range of intensity gradient fluctuations over which oleic acid enhancer action is most prominent for the hydrophilic probe. The implications of these sensitivity studies with respect to oleic acid enhancer action will be discussed further in Section 5.3.3.

5.3. Results and Discussion

5.3.1. Probe Concentration Distribution in the Stratum Corneum

![Graph showing intensity profiles across skin depth](image)

Figure 5-1: Hydrophobic probe (RBHE) intensity profiles. Key: wide-area average intensity (solid line, dotted line) and 400 skin site median intensity (■, ▲) for the control and the enhancer samples, respectively. The error bars indicate 1 SD from the wide-area average intensity of the 400 skin sites surveyed per sample.
The 400 skin site probe average intensity profiles, which reflect the probe concentration as a function of skin depth across the SC, are shown in Figures 5-1 and 5-2 for RBHE and SRB, respectively. In both Figures 5-1 and 5-2, the solid and dashed lines indicate the average intensity, for the control and the enhancer cases, respectively. The triangles

![Intensity vs Skin Depth](image)

**Figure 5-2: Hydrophilic probe (SRB) intensity profiles.** Key: wide-area average intensity (solid line, dotted line) and wide-area median intensity (■, ▲) for the control and the enhancer samples, respectively. The average photon counts per pixel is plotted vs. the corresponding skin depth. The error bars indicate 1 SD from the wide-area average intensity of the 400 skin sites surveyed per sample.

and the squares depict the median intensities all as a function of skin depth, for the control and the enhancer cases, respectively. For both the hydrophobic probe RBHE (Figure 5-1) and the hydrophilic probe SRB (Figure 5-2), the primary drop in intensity occurs over the first 10-20 μm for both the control and the enhancer cases. This drop in probe intensity corresponds well with the 10-20 μm thickness of the SC, the primary
barrier to transdermal transport (Yu, et al., 2001). The error bars in Figures 5-1 and 5-2 reveal the skin site to skin site variability of the intensity profiles measured for each skin sample, and reflect the inherent skin heterogeneity.

The effects of the SC morphology on the spatial variations in probe transport are visualized in Figures 5-3(a), 5-3(b), 5-4(a), and 5-4(b), where contour plots illustrate the relative fluctuations in the free energy of probe partitioning at each skin site, $G_{\text{fluct,}i}/RT$, defined in Eq.(5.3). The z-axis of Figures 5-3(a), 5-3(b), 5-4(a), and 5-4(b) represents $G_{\text{fluct,}i}/RT$ as a function of the x-y spatial position of the skin site imaged. Each unit on the xy plane corresponds to 0.1 mm, and the value of $G_{\text{fluct,}i}/RT = 0$ corresponds to skin sites with free energies equal to the global values. Hence, $G_{\text{fluct,}i}/RT = 0$ reflects the globally detected skin barrier resistance to probe transport. These microscale distributions of probe free energy depict the spatial fluctuations in SC barrier functionality in response to probe hydrophilicity as well as to chemical enhancer action. From Eq.(5.2), the probe vehicle to skin partition coefficient, K, decays exponentially with increases in the probe free energy. Accordingly, in Figures 5-3 and 5-4, the peaks correspond to skin sites with higher than average barrier resistance (smaller K values) and the valleys reflect high permeant transport regions (larger K values). The 3-dimensional surfaces in Figures 5-3 and 5-4 map the fluctuations in the free energy over the SC topology that are responsible for the skin barrier function.
Figure 5-3: Spatial fluctuations in free energy. Surface plots illustrate $G_{\text{fluct}}/RT$ (represented on the z-axis), defined in Eq.(5.3), as a function of the spatial coordinates displayed in the x-y plane for (a) the hydrophobic probe RBHE-control and (b) the RBHE-enhancer. Each unit along the x- and the y- axes corresponds to a length of 0.1 mm, such that the total 4mm$^2$ skin area is depicted.
In the absence of oleic acid, the SC barrier to RBHE, shown in Figure 5-3(a), exhibits a more uniform distribution of free energies compared to the RBHE-enhancer case, shown in Figure 5-3(b). To further quantify the distributions in Figures 5-3(a) and 5-3(b), the number of maxima, or peaks, was found to increase from 31 to 92, and the number of minima, or valleys, was found to increase from 2 to 28 as a result of the oleic acid enhancer action (see Table 5-2). This provides striking quantitative evidence of the perturbation of the SC barrier resulting from the oleic acid enhancer action.

For the hydrophilic probe SRB, however, the control case exhibits a large total number of extrema, with 113 maxima and 34 minima (see Figure 5-4(a)). In contrast to the hydrophobic control mean surface intensity of 551 ± 281 counts/pixel, the 6-fold lower mean surface intensity of 84 ± 90 counts/pixel listed in Table 5-1 for the hydrophilic control corresponds to little or no probe partitioning. The inherently low permeability of the SC to the hydrophilic probe that is revealed in the low probe partitioning can be further visualized over the area defined by x= 0 to 20 units and y= 0 to 10 units in Figure 5-4(a). Here, the existence of higher SRB free energies (positive deviations from $G^\text{f}_{\text{fluct.}}/RT = 0$) over a significant region of the total 4mm$^2$ for the control case suggests that transport of the hydrophilic probe is limited to a small number of higher transport regions encompassed by the energy minima shown in Figure 5-4(a). Out of the 400 skin sites examined, 28% of the total skin area manifests free energy barriers exceeding the 400-skin site average by 1RT, the criterion established earlier. Compared with the 113 maxima, the 34 free energy minima that lie 1RT below the sample average comprise
8.5% of the total 4mm² skin area examined. These domains of higher transport remain consistent with the proposed existence of ‘aqueous pore’ pathways through which hydrophilic probes can traverse the lipoidal SC matrix as a result of inherent imperfections in the SC lipid multilamellae (Menon and Elias, 1997, Sznitowska, et al., 1998).

In the presence of oleic acid (see Figure 5-4(b)), the number of peaks decreases from 113 to 29 and the number of valleys decreases from 34 to 4. These decreases in the number of extrema, that are associated with the previously reported oleic acid-induced increase in probe vehicle to skin partitioning of $E_K = 10.22\pm0.55$ (see Chapter 4), signal the increased uniformity in probe transport possibly resulting from the chemical enhancer-induced formation of additional hydrophilic probe pathways. With respect to the aqueous pore pathway for the hydrophilic probe, the mechanism of chemical enhancer action has been described by increased SC hydration resulting from enlarged pore pathways (Yamashita et al., 1995), as well as by the increased continuity of the SC lacunar domains (Menon and Elias, 1997). Hence, the decrease in the number of minima from 34 to 4 indicates that, in the presence of oleic acid, the localized transport regions comprise solely 1% of the skin area examined as a result of uniform increases in hydrophilic probe transport over the 4mm² skin area examined. The SC barrier response to the oleic acid enhancer action is displayed in Figure 5-4(b), where the reduction in the skin sample free energy, evident in the 10-fold increase in the 400 skin site surface intensity, enables an increased probability of probe partitioning resulting from an increase in the number of skin sites possessing free energies that lie within $\pm1RT$ of the sample average.
Figure 5-4: Spatial fluctuations in free energy. Surface plots illustrate $G_{\text{fluct}}/RT$ (represented on the z-axis), defined in Eq.(5.3), as a function of the spatial coordinates displayed in the x-y plane for (a) the hydrophilic probe SRB-control and (b) the SRB-enhancer. Each unit along the x- and the y- axes corresponds to a length of 0.1 mm, such that the total 4mm$^2$ skin area is depicted.
Accordingly, the 400-skin site transport property enhancement values measured arise from these microscale changes in the SC barrier revealed by the spatial distributions of the fluctuations in the free energy. For the hydrophobic probe RBHE, these changes resulted in transport property enhancement values of $E_K = 4.77 \pm 0.83$ and $E_g = 4.81 \pm 1.86$, for the vehicle to skin partition coefficient and the concentration gradient, respectively, whereas for the hydrophilic probe SRB, enhancement values of $E_K = 10.22 \pm 0.55$ and $E_g = 9.93 \pm 1.50$ were calculated, respectively (see Chapter 4).

### 5.3.2. Oleic Acid-Induced Changes in the Permeant Free Energy

From Eq.(5.3), the 400 skin site $E_K$ values shown above for the hydrophobic and the hydrophilic probes correspond to oleic acid-induced decreases in the free energy requirement for probe transport from the vehicle into the stratum corneum of $1.56RT$ and $2.32RT$, respectively. The reduction in the free energy barrier results in a higher probability for the permeants to enter into the SC, resulting from the reduction of the transport free energy barrier required for the permeant transfer from the vehicle into the skin. The alteration of the skin barrier properties described by the reduction in the transport free energies from the control to the enhancer cases can be observed in Table 5-1, in which the oleic acid-induced increases in probe vehicle to skin partitioning are summarized. The median surface intensity increases from 511 counts/pixel to 1991 counts/pixel for the hydrophobic probe as a result of the chemical enhancer action. For the hydrophilic probe, the median surface intensity increases from 60 counts/pixel to 766 counts/pixel as a result of the chemical enhancer action. Since the median captures the $50^{th}$ percentile of the data distributions, the oleic acid-induced increase in the median
surface intensity reflects the increased probability of a skin site possessing the altered barrier properties conducive to increased drug transport.

These oleic acid-induced increases in the surface intensity median values signal corresponding increases in probe penetration at each skin site. In addition to increasing the probe penetration at each skin site, the oleic acid-induced positive shifts in the surface intensity distributions of the 400 skin sites for both the hydrophobic and the hydrophilic probes also indicate increases in the fractional skin area facilitating probe transport. Hence, the oleic acid-induced decreases in $\Delta G^*$ increase the fractional area of the skin which is amenable to probe partitioning. Accordingly, the global transport enhancement resulting from the oleic acid enhancer action arises from an increased capacity for transport at each skin site resulting from the reduced skin barrier function, as well as from an increased fraction of skin area contributing to permeant diffusion.

5.3.3. Oleic Acid-Induced Fluctuations in the Permeant Intensity Gradient

Following probe partitioning into the SC, probe diffusion through the SC is revealed by the probe intensity gradient across the SC. Figures 5-5(a), 5-5(b), 5-6(a), and 5-6(b) show the fractional fluctuations in the intensity gradient at each of the 400 skin sites examined, for the hydrophobic control, the hydrophilic enhancer, the hydrophilic control, and the hydrophilic enhancer cases, respectively. The spatial coordinates for each skin site are depicted by the x-y coordinates, while the z-axis shows $(dI/dz)_{\text{fluct,i}}$ normalized by $(dI/dz)_A$. 

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5.3.3.1. Hydrophobic probe

The oleic acid-induced changes in the intensity gradient fluctuations reported in Table 5-2 indicate minor increases in the total number of extrema for the hydrophobic probe, with 121=52+69 extrema for the control case and 126=67+59 extrema for the enhancer case. The corresponding surface plots in Figures 5-5(a) and 5-5(b) further illustrate the similarity in the intensity gradient fluctuations between the control and the enhancer cases of the hydrophobic probe, respectively.

Compared to the oleic acid-induced increases in the free energy fluctuations shown in Figures 5-3(a) and 5-3(b), the effect of the chemical enhancer action on the lateral diffusion step of transdermal transport for the hydrophobic probe is relatively minor with respect to the increase in the number of extrema from 121 to 126 for the intensity gradient fluctuations observed over the 4mm² skin area examined (see Table 5-2). The increase in the 400 skin site mean surface intensity from 551±281 counts/pixel to 2546±2176 counts/pixel is followed by an increase in the 400 skin site mean intensity gradient from 57±36 counts/μm to 266±198 counts/μm (see Table 5-1). Despite this overall increase in the driving force of probe flux, the insensitivity of the intensity gradient fluctuations in response to the oleic acid-induced increases in the free energy fluctuations that reflect the probe vehicle to skin partitioning suggests that changes in the lateral diffusion pathways do not correlate with sites of increased probe partitioning. This relationship between the two transdermal transport steps provides additional insight into the mechanism of chemical enhancer action and will be addressed further in Section 5.3.4.
Figure 5-5: Spatial fluctuations in the hydrophobic probe (RBHE) concentration gradient. Surface plots illustrate $\frac{dI/dz}{dz_{fluct,i}}$ normalized by $\frac{dI/dz_C}{dI/dz_C}$ and $\frac{dI/dz_E}{dI/dz_E}$ (represented on the z-axis), as a function of the spatial coordinates displayed in the x-y plane for (a) the RBHE-control and (b) the RBHE-enhancer, respectively. Each unit along the x- and the y- axes corresponds to a length of 0.1 mm, such that the total 4mm$^2$ skin area is depicted.
Figure 5-6: Spatial fluctuations in the hydrophilic probe (SRB) concentration gradient. Surface plots illustrate $\frac{dI}{dz_{\text{fluct},i}}$ normalized by $\frac{dI}{dz_C}$ and $\frac{dI}{dz_E}$ (represented on the z-axis), as a function of the spatial coordinates displayed in the x-y plane for (a) the SRB-control and (b) the SRB-enhancer, respectively. Each unit along the x- and the y- axes corresponds to a length of 0.1 mm, such that the total 4mm² skin area is depicted.
5.3.3.2. Hydrophilic Probe

For the hydrophilic probe, oleic acid increases the total number of extrema, while decreasing the magnitude of the intensity gradient fluctuations from the control case (Figure 5-6(a)) to the enhancer case (Figure 5-6(b)). These significant increases in the total number of fluctuation extrema resulting from chemical enhancer action are shown in Table 5-2, where a total of 50 extrema for the hydrophilic control case and a total of 99=61+38 extrema for the hydrophilic enhancer case are reported. Compared to the significant decrease in free energy fluctuations from 147 extrema to 33 extrema (see Table 5-2), oleic acid induces significant increases in the intensity gradient fluctuations that represent changes in the lateral diffusion step of transdermal transport. The strong correlation between the decrease in free energy fluctuations and the increase in the intensity gradient fluctuations suggests that oleic acid-induced changes in the probe vehicle to skin partitioning step of transport lead to corresponding changes in the probe lateral diffusion step of transport. From the sensitivity studies performed in Section 5.2, the ±1SD criterion, applied to determine the number of intensity gradient fluctuation extrema, reflects the magnitude of the fluctuations associated with the oleic acid chemical enhancer action. Recall from Section 5.2 that the oleic acid-induced fluctuations are most prominent at the ±1SD criterion, where a two-fold increase in the number of extrema is observed.

The significance of these findings in elucidating the mechanisms of chemical enhancer action will be addressed in Section 5.3.5, where the statistical distributions of the surface
intensities and of the intensity gradients resulting from these fluctuations in the probe free
energy, $G_{\text{fluct},i}$, and in the probe intensity gradient, $(dI/dz)_{\text{fluct},i}$, are discussed.

5.3.4. Statistical Distribution of Transport Parameters for the
Hydrophobic Probe RBHE

The variations detected in the probe spatial distributions over the wide-area scanned
naturally reflect the intrinsic variations in SC morphology that control RBHE transport

![Bar charts showing the distribution of skin site intensities and intensity gradients for control and enhancer cases.](image)

Figure 5-7: Hydrophobic probe (RBHE) transport parameter distributions. Histograms of
the surface intensity distributions for the control and the enhancer cases are shown in (a) and
(b), respectively. (c) and (d) depict the intensity gradient distributions for the 400 skin sites
examined in the control and the enhancer samples, respectively.
(see Chapter 4). Hence, the oleic acid-induced increase in transport and localization of high transport regions also reflects the changes in the SC morphology resulting from the chemical enhancer action.

The histograms presented in Figures 5-7(a) to 5-7(d) capture the mechanistic responses underlying the reduced SC barrier function resulting from exposure to oleic acid for the hydrophobic probe RBHE. Figures 5-7(a) and 5-7(b) depict the surface intensity distributions for the control and the oleic acid samples, respectively, whereas the corresponding intensity gradient distributions are shown in Figures 5-7(c) and 5-7(d), respectively. In the absence of oleic acid, the RBHE surface intensities at each site display a symmetric distribution (Figure 5-7(a)), with a few outliers at the higher end of the surface intensity spectrum. This distribution reflects the uniform probe spatial distribution in the wide-area visualization reported in Chapter 4 (see also Yu, et al., 2002), as well as in the free energy fluctuation (see Figure 5-3(a)), and illustrates the inherent preference of the hydrophobic probe for the ‘oily’ microenvironment of the lipoidal transport pathway. The distribution of transport parameters for the control case is also consistent with the symmetric drug flux distribution over multiple skin samples observed for neutral permeants in diffusion cell experiments (Liu, et al., 1993).

In the presence of oleic acid, the skin barrier becomes more permeabilized to allow for the positive shift in surface intensities from a mean of 551 ±281 counts/pixel to 2546 ±2176 counts per pixel over the 400 different skin sites (see Table 5-1). The oleic acid-induced perturbation of the skin barrier revealed by increases in the free energy
fluctuations can be visualized in Figures 5-3(a) and 5-3(b) for the control and the enhancer cases, respectively. This positive shift captures the increase in the probability of probe partitioning into each of the 400 skin sites examined, due to increased transport through a greater fractional area of the exposed SC. A comparison of the surface intensity distributions in Figures 5-7(a) and 5-7(b) indicates that the 400 skin sites in the control case all display surface intensities below 1991 counts/pixel, whereas in the presence of oleic acid, the surface intensity of 1991 counts/pixel defines the RBHE median. Surface intensities between 1991 counts/pixel and the upper bound of 12000 counts/pixel shown in the x-axis in Figure 5-7(b) are displayed by 50% of the skin sites examined. The increased number of skin sites displaying the oleic acid-induced increase in probe partitioning that these histograms reveal remains consistent with the increased solute partitioning resulting from the increased lipid fluidity associated with the oleic acid enhancer action (Golden, et al., 1987).

Comparing Figures 5-7(c) and 5-7(d), however, the oleic acid-induced changes in the hydrophobic probe surface intensity distribution profile was not as prominent for the intensity gradient distributions upon examining the corresponding skewness and kurtosis values. Despite the overall increase in the magnitude of the average intensity gradient per skin site, from 57 ± 36 counts/μm to 266 ± 198 counts/μm (see Table 5-1), the similar skewness values of 0.89 for the control and 1.04 for the enhancer shown in Figure 5-8 reflect minor changes in the shape of the distribution profiles resulting from the oleic acid enhancer action. The comparable, relatively flat distribution profiles for both cases are further captured by the similarity in their kurtosis values of 4.05 and 4.07 shown in
Figure 5-8 (see Intensity Gradient RBHE (KR)). The similarity in the distribution profiles for the control and the enhancer skin samples reflects the close resemblance of their normalized intensity gradient fluctuations depicted in Figures 5-5(a) and 5-5(b), respectively. Hence, the oleic acid-induced changes in SC morphology that give rise to the changes detected in the surface intensity distributions do not lead to the corresponding changes in the intensity gradient distributions.

The observed independence between the responses of the surface intensity and the intensity gradient distributions remains consistent with the proposal that diffusion through the tortuous pathway of the intercellular region is the rate-limiting step (Johnson, et al., 1997) for the transdermal transport of the hydrophobic probe. Although similar enhancement values were measured in Chapter 4, Section 4.3.2 for the probe vehicle to

![Figure 5-8: RBHE and SRB data distribution characterization. Key: control vehicle (gray bar), enhancer vehicle (black bar). The skewness (SK) and kurtosis (KR) values calculated for the histograms shown in Figures 3 and 5 using Eq.(5.3) are plotted for the hydrophobic (RBHE) and the hydrophilic (SRB) probes. The data table shows the numerical values of SK and KR associated with the surface intensity (left panel) and the intensity gradient (right panel) distributions of the two probes.](image)
skin partition coefficient (\(E_K=4.77\pm0.83\)) and the concentration gradient (\(E_g=4.81\pm1.86\)),
the changes in the microscale distributions underlying these two enhancement values
enable the identification of lateral diffusion as the rate-limiting step for the transdermal
transport of the hydrophobic probe.

### 5.3.5. Statistical Distribution of Transport Parameters for the Hydrophilic Probe SRB

The localization of high transport regions to the limited number of minima observed in
Figure 5-4(a) for the SRB-control case (see Table 5-2) is also evident in the SRB-control
surface intensity distribution shown in Figure 5-9(a). The hydrophilic probe surface
intensities of the control sample reveal a high degree of positive skewness (2.14 in Figure
5-8), with the outliers corresponding to the skin regions possessing low free energies. A
majority of the sites remain impermeable to SRB, illustrated by the large fraction of
positive \(G_{\text{fluct},i}\) values shown in Figure 5-4(a). Similarly, the intensity gradient distribution
for the control case (see Figure 5-9(b)) reflects a high degree of positive skewness (2.47
in Figure 5-8). The maxima shown in Figure 5-6(a) illustrate well the intensity gradient
outliers that contribute to the reported positive skewness. These distributions reflect the
inherently low permeability of the skin to hydrophilic permeants, where transdermal
transport is described by ‘aqueous pore’ pathways. The presence of these ‘aqueous pore’
pathways responsible for the restricted transport of the hydrophilic permeant are revealed
by the outliers in the positive region of the surface intensity and intensity gradient
distributions. These data are in agreement with the positively skewed flux distributions of
hydrophilic permeants observed for multiple skin samples in diffusion cell studies
Figure 5-9: Hydrophilic probe (SRB) transport parameter distributions. Histograms of the surface intensity distributions for the control and the enhancer cases are shown in (a) and (c), respectively. (b) and (d) depict the intensity gradient distributions for the 400 skin sites examined in the control and the enhancer samples, respectively.

The increase in the skin permeability to the hydrophilic probe becomes evident in the redistribution of the surface intensities shown in Figure 5-9(c). The overall shift in the 400 skin site mean surface intensity from $84 \pm 90$ counts/pixel to $838 \pm 467$ counts/pixel (see Table 5-1) indicates an increased probability of probe partitioning into the SC. This increased probability of partitioning can be observed in the increased number of skin sites extending into the positive region of the surface intensity distribution profile shown in
Figure 5-9(c). The change in the shape of the distribution profile can be quantified by a
decline in the skewness from 2.14 to 0.90 (see Figure 5-8), such that a more symmetric
data distribution exists about the mean (see Figure 5-9(c)). A similar decrease in
skewness from 2.47 to 1.25 was calculated for the 400 skin site intensity gradients (see
Figure 5-8) and observed in the more symmetric distribution profile shown in Figure 5-
9(d). The narrowing of the relative difference between the mean and the median surface
intensities, described by the decrease in the skewness and the kurtosis of the distribution
profile, also indicates that the probability of skin barrier function reduction at a given
skin site approaches the more familiar Gaussian distribution. Figure 5-4(b) illustrates the
oleic acid-induced changes in the free energy fluctuations underlying the surface intensity
distributions for the enhancer case.

The skewness and kurtosis values that describe the shapes of these SRB-enhancer
distributions agree well with the distributional shapes achieved for the hydrophobic
control case. For the surface intensity distributions, the skewness of 0.9 and the kurtosis
of 3.47 for the hydrophilic enhancer case are similar to the skewness of 0.77 and the
kurtosis of 3.50 for the hydrophobic control case (see Figure 5-8). Similarly, the intensity
gradient distribution for the hydrophilic enhancer case yields a skewness of 1.25 and a
kurtosis of 4.35 which are comparable to the values of 0.89 and 4.07, respectively, for the
hydrophobic control case (see Figure 5-8). The oleic acid-induced changes in the
hydrophilic probe microtransport environment are evident in the increased similarity in
the transport parameter distributions between the hydrophilic enhancer case (Figures 5-
9(a) and 5-9(d)) and the hydrophobic control case (Figures 5-7(a) and 5-7(c)). The
microscale changes underlying the measured SRB permeability enhancement gives rise to transport parameter distributions that reflect the diffusion through the intercellular regions, characteristic of the hydrophobic permeant control case. These changes indicate an increase in the hydrophilicity of the hydrophilic probe transport microenvironment that is consistent with the enhancer-induced swelling and confluence of SC lacunar regions to create continuous aqueous pathways across the skin (Menon and Elias, 1997). Moreover, oleic acid has also been suggested to increase the pore sizes of the hydrophilic permeant pathway (Yamashita, et al., 1995).

The oleic acid-induced changes in SC morphology captured by the changes in the transport parameter distributions reflect changes in the aqueous pore pathway that increase the probability of hydrophilic probe partitioning into the SC. Furthermore, the increased probe partitioning is followed by evidence of similar changes in the spatial variation of hydrophilic probe diffusion through the aqueous pores that are captured by the intensity gradient distribution. Figure 5-6(b) illustrates the oleic acid-induced spatial fluctuations in the intensity gradients over the 400 skin sites examined that are responsible for the intensity gradient distribution shown in Figure 5-9(d). The decrease in the skewness from 2.14 to 0.9 and of the kurtosis from 8.06 to 3.47 for the hydrophilic probe surface intensity distributions is followed by similar, marked decreases in these values for the intensity gradient distributions (see Figure 5-8). Indeed, for the intensity gradient distributions, the skewness decreases from 2.47 to 1.25 and the kurtosis decreases from 10.51 to 4.35. The good correlation between the surface intensity and the
intensity gradient distributional responses to the oleic acid enhancer action indicates that *probe partitioning is the rate-limiting transport step for the hydrophilic probe*.

**5.3.6. Inherent Link between Macroscale and Microscale Transport Parameter Distributions**

The positively-skewed site surface intensity and intensity gradient distributions exhibited by the hydrophilic probe SRB (see Figures 5-9(a) and 5-9(b)), as well as the relatively more symmetric hydrophobic probe RBHE data distributions (see Figures 5-7(a) and 5-7(c)) for the control cases, reflect the flux distributions of terbutaline and estradiol in 42 and 49 skin samples, respectively, based on the more labor and material intensive diffusion cell experiments (Liu, et al., 1993). While inter- and intra- skin sample permeability variations have been widely reported (Kasting, et al., 1987, Liu, et al., 1993, Southwell, et al., 1984, Williams, et al., 1992), statistical analyses of intrasample permeability variabilities of permeants have been limited by the small area of skin samples available, coupled with the need to satisfy a prerequisite sample area dictated by the diffusion cell apparatus. The correlation between the 400 skin site intrasample transport data distributions and the collection of permeability data cited in the literature (Liu, et al., 1993) further reinforces the inherent link between the microscale transport variations detected utilizing the HTPM methodology described in this chapter and the macroscale transport parameters quantified by the commonly measured drug permeability using diffusion cell measurements.
5.3.7. Implications of the Transport Parameter Statistical Distributions with Respect to Oleic Acid-Induced Alterations of Permeant Pathways

With respect to the inherent heterogeneity of the skin morphology at the micro-scale level, the skewness and the kurtosis values that were presented in this chapter provide insight into the relative accessibility of the hydrophobic and the hydrophilic probe transport pathways, in the absence and in the presence of the chemical enhancer oleic acid. The number of statistical outliers observed for the distributions of the surface intensities and the intensity gradients of the hydrophilic probe SRB in the control case, coupled with the relatively low, median values centered at 50 counts/pixel and 3.4 counts/μm, respectively, illustrates the inaccessibility of the aqueous pore pathways over a majority of the skin wide area examined. The inaccessibility, or limited presence, of these pathways is consistent with the inherent impermeability of the skin to hydrophilic permeants. These transport parameter statistical outliers in the hydrophilic control case also provide additional evidence for the heterogeneity of the skin barrier, over which these hydrophilic permeant pathways are distributed nonuniformly. In the presence of oleic acid, however, the increase in symmetry and the decrease in the number of outliers (observed for the statistical distributions of the hydrophilic probe (SRB) surface intensities and intensity gradients reflect the increased accessibility of the hydrophilic permeant pathways, signaling an increase in the ‘porosity’ of the SC membrane resulting from changes in the SC morphology.
For the hydrophobic probe (RBHE), the symmetric distributions for both the probe surface intensities and the probe intensity gradients over the 400 skin sites examined reflects the hydrophobic nature of the SC barrier, as well as the inherent accessibility of the lipoidal pathway to the hydrophobic permeants. The positive shift in the median values of 511 to 1991 counts/pixel and 50 to 766 counts/μm for the hydrophobic probe surface intensity and intensity gradient, respectively, indicate that oleic acid induces alterations to the SC barrier morphology that enable overall increased probe accessibility. Despite the global increase in hydrophobic probe transdermal transport that is visualized, and quantified using these two transport parameters, over the wide area imaged, the oleic acid-induced increase in the number of maxima in the free-energy from 31 to 92 and in the intensity gradient fluctuations from 52 to 67 (see Table 5-2), illustrate that the increase in hydrophobic probe transport also becomes localized to more specific domains.

The relationship between the oleic acid-induced changes in the statistical distributions of the transport parameters with respect to the specific permeant transport pathways will be examined in Chapter 6, where the probe spatial distributions with respect to the SC structural features will be elucidated.

5.4. Conclusions

The oleic acid-induced changes in the surface intensity and in the intensity gradient distribution profiles of the 400 consecutive skin sites surveyed, evident in the corresponding skewness and kurtosis values, suggest different rate-limiting steps in the transdermal transport of the hydrophobic and the hydrophilic probes examined. Probe partitioning and intercellular diffusion primarily accounted for the transdermal transport
resistance to the hydrophilic SRB probe and the hydrophobic RBHE probe, respectively, and highlights the existence of 2 different permeant pathways corresponding to the hydrophobic and the hydrophilic model probes. Furthermore, transport enhancement was attributed to the decreased free energy required for probe entry into the skin, promoting the increased probability of probe partitioning into the SC, as well as the increased fractional area of skin amenable to drug transport.

The intrasample microscale distributions of surface intensity and intensity gradient measured utilizing HTPM establishes the close relationship between the microscale variations in probe transport parameters and the macroscale intersample permeability variations described in the literature. The good correlation determined between the variations in characteristic transport parameters observed at two entirely different length scales highlights the potential of inferring drug permeability distributions for a population of skin samples from the more efficient HTPM wide-area sampling of model-probe intensity distributions utilizing one single skin sample.
5.5. References


Chapter 6

Visualization of Intracorneocyte Permeant Penetration Using Dual-Channel High-Speed Two-Photon Microscopy: Evidence of a Secondary Transdermal Transport Pathway

6.1. Introduction

In Chapters 3, 4, and 5, the advantages of using two-photon microscopy to examine a highly scattering and morphologically complex system such as the skin were highlighted. The evaluation of oleic acid-induced transdermal transport focused on the primary diffusion pathway of the intercellular lipid region that was well delineated by both the hydrophobic and the hydrophilic probes (see Figure 3-3 in Chapter 3 and Figure 4-1 in Chapter 4). In this chapter, the focus will shift to the role of the corneocytes, that are embedded within the continuous stratum corneum (SC) lipid multilamellar matrix (see Chapter 1, Section 1.2), with respect to the oleic acid-induced transdermal transport.

Until now, the study of chemical enhancer action has focused primarily on the alteration of the SC barrier properties resulting from a fluidization of the lipid multilamellar region
through which the permeants diffuse (Golden et al., 1986, Golčen et al., 1987). The increased lipid disorder resulting from lipid fluidization has been cited as being responsible for the increase in drug partitioning into the SC, as well as for the increase in the porosity of the SC membrane (Yamashita et al., 1995, Yoneto et al., 1998). Although chemical enhancer-induced deformation of the corneocytes has been described in studies using octyl glucoside (Lopez et al., 2000, Lopez et al., 2000), the role of the corneocyte structures in transdermal transport processes has been neglected, primarily due to their relative impermeability when compared to that of the lipid multilamellar region (Matoltsy, 1976). The corneocyte envelope has been implicated in providing SC structural integrity, as well as in contributing to the SC barrier function (Behne et al., 2000, Elias et al., 2000).

As a novel approach to identify the oleic acid-induced changes in intracorneocyte probe penetration, a dual-channel high-speed two-photon microscope (HTPM), used to filter the fluorescence emission wavelengths in the green color spectrum range from the red color spectrum range, enables the simultaneous visualization of the fluorescence signals resulting from the intrinsic skin autofluorescence in one channel and those signals resulting from the exogenous (or externally introduced) model fluorescent probe in the other channel. In Chapters 3, 4, and 5, the exogenous probe fluorescence was examined, independently of the corresponding skin autofluorescence image. In this chapter, for the first time, the exogenous probe spatial distributions in the skin are shown with respect to the intrinsic skin autofluorescence at precisely the same spatial coordinates (see Section 6.3.2).
Skin autofluorescence has been well studied in the literature, both in vivo and in vitro (Gonzalez et al., 2000, Kollias et al., 1998, Na et al., 2001). Using excitation wavelengths ranging from 340 nm to 380 nm, the skin autofluorescence emission spectrum was detected using a fluorescence spectrophotometer that revealed two major component bands centered at 450 nm and 520 nm, that corresponded to 75% and 25% of the total spectrum intensity, respectively (Na et al., 2000). These two peaks in the skin autofluorescence spectrum correspond to the fluorescence emission peaks of the endogenous (or intrinsic) skin fluorophores, that include collagen, elastin, aromatic amino acids such as tryptophan and tyrosine, and nicotinamide adenine dinucleotide, porphyrins, and flavin adenine dinucleotide (Kollias, et al., 1998). The structural details of the corneocyte-lipid interface, delineated by imaging the inherent skin autofluorescence, enables the quantitation of probe spatial distributions relative to the corneocyte structures in the same skin sample, at precisely the same spatial locations. Skin autofluorescence is detected using the green channel, whereas the rhodamine-based fluorescent probes were examined using the red channel (see Section 6.2.2). For the model hydrophobic and hydrophilic probes, rhodamine B hexyl ester (RBHE) and sulforhodamine B (SRB), respectively, the influence of oleic acid on the possible secondary transport pathway of permeant diffusion into the corneocytes will be investigated.

In this chapter, the visualization of the skin autofluorescence, along with the corresponding visualization of the probe spatial distributions, will be presented in
Sections 6.3.1 and 6.3.2. The quantitative analysis of the probe spatial distributions, relative to the fingerprint provided by the skin structural features delineated with the skin autofluorescence visualizations, will be presented in Sections 6.3.3 and 6.3.4, in which the average correlation lengths calculated for each image and the linear image intensity deconstruction analysis, described in the Materials and Methods section (Section 6.2), are discussed.

6.2. Materials and Methods

6.2.1. Skin Sample Preparation

Using the methodology described in Chapter 2, Section 2.2, excised human cadaver skin (National Disease Research Interchange, Philadelphia, PA) was mounted in side-by-side diffusion cells (Permegear, Riegelsville, PA). The probe control and enhancer vehicle solutions consisted of 50/50 v/v phosphate buffered saline (PBS)/ethanol and of the control solution containing an additional 5% oleic acid (Sigma Chemicals, St. Louis, MO), respectively. The probe concentrations for RBHE and SRB (Molecular Probes, Eugene, OR) were adjusted such that the exogenous fluorescent probe intensity signal detected was comparable to the endogenous SC autofluorescence intensity. The relatively low skin autofluorescence signal requires the application of increased HTPM laser power for the endogenous fluorophore excitation (described in Section 6.2.2 below). The dilution of the RBHE and SRB vehicles to 0.005 mg/ml (compared to the 0.33 mg/ml utilized in the previous studies reported in Chapter 3) enables the simultaneous imaging of the skin autofluorescence as well as of the exogenous fluorescent probe intensities, such that probe signal saturation is avoided. After 24 hours of skin exposure to the probe
solution, the samples were rinsed with PBS, and mounted on microscope slides as described previously (see Chapter 2, Section 2.7).

6.2.2. High-Speed Two-Photon Microscopy

A detailed description of the HTPM methodology utilized in these studies can be found in Chapter 4, Section 4.2.2 and in the literature (Kim et al., 1999). Modifications to the HTPM instrumentation include the use of a filter set (Chroma Technologoies, Brattleborough, VT) that separates the fluorescence emission signal into two wavelength ranges. The filter set consists of a dichroic mirror (part no. 530DCXR), a short pass filter (part no. E535SP), and a bandpass filter (part no. D630/40). The dichroic mirror reflects emission wavelengths below 530nm to one channel, where the short pass filter is applied. Fluorescence emission signals in the range of 610 nm to 650 nm, that are reflected from the dichroic mirror, are detected in the second channel using the bandpass filter. The first channel collects the emission wavelengths characteristic of the intrinsic skin fluorophores, and will be referred to as the green channel. The second channel, or the red channel, detects the probe fluorescence signal (see Table 3-1 in Chapter3 for a summary of the probe properties). 100 consecutive skin sites were imaged to cover a total skin area of 1 mm by 1mm and a depth of 21 μm, the thickness of the SC. Figure 6-1 shows the probe autofluorescence for the control and the enhancer vehicle treated skin samples in the absence of any fluorescent probe. Figures 6-2 and 6-3 show the skin images obtained using dual-channel HTPM (see Section 6.3 for a detailed discussion).
6.2.3. Image Analysis of Probe Spatial Distributions

6.2.3.1. Determination of the Skin Autofluorescence and the Probe Average Correlation Lengths

From the 100 consecutive skin sites imaged, a region consisting of 9 consecutive skin sites was selected for the image analysis methodology presented below. Because this chapter focuses specifically on the role of intracorneocyte diffusion in transdermal transport, these 3 x 3 skin sites were selected based on their representation of a continuous skin region well defined by the presence of regularly spaced corneocytes. To characterize and quantify the fluorescence emission images of probe spatial distributions for each probe-vehicle combination examined, the normalized two-point intensity autocorrelation function, shown in Eq.(6.1) below, was calculated using the MATLAB software (Mathworks, Natick, MA):

\[
C(\Delta x, \Delta y) = \frac{\sum (I(x, y) - < I >)(I(x + \Delta x, y + \Delta y) - < I >)}{\sum (I(x, y) - < I >)^2}
\]

(6.1)

where \(C(\Delta x, \Delta y)\) is the normalized two-point autocorrelation function, \(\Delta x\) and \(\Delta y\) are the x and y spatial coordinate distances between two pixels, \(I(x,y)\) is the pixel intensity at position (x,y), and \(<I>\) is the average pixel intensity over the total skin area imaged. For a distance of \((\Delta x, \Delta y) = (0,0)\), the two points evaluated have zero separation, and therefore, the two pixels evaluated are the same pixel. Hence, the normalized autocorrelation
function has a maximum value of 1 at \( C(0, 0) \), which corresponds to the greatest degree of correlation.

As \((\Delta x, \Delta y)\) take on larger values, the separation distance between the spatial coordinates of two pixels increases, and the value of the autocorrelation function decreases. In the studies reported in this chapter, the characteristic decay length, \( \tau \), will be used as a quantitative standard for comparing the distances over which pixel intensities no longer exhibit strong correlations. By convention, \( \tau \) is the distance \((\Delta x \text{ and } \Delta y)\) over which the normalized autocorrelation function, \( C(\Delta x, \Delta y) \), approaches \( 1/e \) of its maximum value, \( C(0,0)=1 \). The average correlation length, \( \tau \), over the \( x\)-\( y \) coordinate space is presented for each skin sample examined in Figure 6-4. These average correlation lengths for each skin sample arise from the curve fit of the distinct planes of the correlation function along the \( x \) and \( y \) axes, such that \( C(\Delta x=0,\Delta y) \) and \( C(\Delta x,\Delta y=0) \) are fit to Eq.(6.2) below using a MATLAB curvefitting subroutine:

\[
C(r) = e^{-a_1 r} \quad \text{(6.2)}
\]

where \( C(r) \) is the autocorrelation function as a function of \( r \), the two-point separation distance, that, for the pixilated images evaluated in this chapter, is captured by either \( \Delta x \) or \( \Delta y \) while the other coordinate is kept at a value of 0. The regression coefficient, \( a_1 \), of Eq.(6.2) yields the correlation distance, where \( \tau = 1/a_1 \). The four different \( \tau=1/a_1 \) values that result from the curve fit of each of the four autocorrelation function cross sections demarked by \( C(\Delta x \geq 0, \Delta y=0) \), \( C(\Delta x \leq 0, \Delta y=0) \), \( C(\Delta x = 0, \Delta y \geq 0) \), and \( C(\Delta x=0, \Delta y \leq 0) \),
are then equally weighted and averaged to produce the average correlation lengths reported in Figure 6-4.

Before introducing the correlation lengths, a short discussion describing the relevance of the arbitrarily determined correlation distance based on the convention adopted here is presented next. As described in Eq.(6.1), the two-point autocorrelation function, \( C(\Delta x, \Delta y) \), accounts for the average spatial variation over the image area examined. Note that products of the fluctuations in the signal intensity at each specific \( \Delta x \) and \( \Delta y \) values, \( (I(x+\Delta x,y+\Delta y) - \langle I \rangle) \), and the fluctuations in signal intensity over all the \( x \) and \( y \) values, \( (I(x,y) - \langle I \rangle) \) are summed over the entire coordinate space of the skin area to yield the autocorrelation function. \( I(x+\Delta x, y+\Delta y) \) captures the entire range of intensity signals possessing the specified spatial separation distance, \( \Delta x \) or \( \Delta y \), along each coordinate axis from \( I(x,y) \). Furthermore, \( I(x,y) \) includes each of the \( 36 \times 10^4 \) pixels present in the \( 3 \times 3 \) skin area examined. The correlation length, \( \tau \), hence, reflects a statistically significant average quantity, as it arises from the curve fit of the autocorrelation function to Eq.(6.2). The correlation length reflects the average spatial distance between two points, over which differences between the two signal intensities contribute to the decay of the autocorrelation function (see Eq.(6.1)) to \( 1/e \) of the normalized peak value of 1. At this point of greatest correlation (where \( C(\Delta x, \Delta y) = 1 \)), \( \Delta x \) and \( \Delta y \) are zero, indicating that the two signal intensities evaluated possess the same \( (x,y) \) coordinates, and hence, correspond to exactly the same pixels of the skin image.
The skin sample size dependence of the changes measured in the transdermal transport properties, described in Chapter 4, highlights the impact of the inherent skin heterogeneity on the variability of these measurements. In evaluating the specific question of oleic acid-induced intracorneocyte probe diffusion, the 3x3 skin sites were selected based on the visibility of the regularly spaced corneocyte structures. Compared to the wide-area studies presented in Chapters 4 and 5, where the average intensity profile of each skin site (100 μm by 100 μm) served as the fundamental unit of the 2mm by 2mm total skin area evaluated, the studies presented in this chapter focus on the probe spatial information pertaining to the length scale of the SC structural features (where the average corneocyte diameter is 30μm). These details, are therefore, lost in the calculation of the average intensity profile for each of the 400 skin sites examined. Hence, in this chapter, the basic unit evaluated is one pixel within each 3 x 3 skin site area, such that SC structural information is captured. Whereas the statistics presented previously (see Chapter 5) arose from the sampling of the average skin site intensity of 400 different skin sites, it is important to note that the autocorrelation function calculated in this chapter from the 9 consecutive skin site image reflects the sampling of each pixel intensity within the 600 pixel by 600 pixel corneocyte-rich skin area (36 x10^4 pixels). Note that the image stitching program utilized to assemble the individual skin sites imaged (see Chapter 4, Section 4.2.2) crops each 256 pixel by 256 pixel image of one skin site to approximately 200 pixels by 200 pixels as a result of the inherent pixel overlap implemented. The overlapping pixels allow for the construction of the wide skin area visualized by the stitching together of the individual skin sites. In short, the studies presented in this chapter address a specific question regarding SC structural components that requires the
examination of the HPTM images at a significantly smaller length scale. This length scale is limited by the HTPM pixel resolution of 0.4 µm/pixel, and hence, the sampling criteria presented in Chapter 4, based on skin sites, do not apply to the pixel-size sampling undertaken here.

\( \tau \) provides a quantitative basis from which oleic acid-induced changes in the width of the intercellular domain can be quantified. Recall that in earlier HTPM studies (see Figure 4-1 in Chapter 4), the visualization of both hydrophobic and hydrophilic model probe transport through the lipid multilamellar region of the SC confirmed this continuous intercellular matrix as the primary transdermal transport pathway. For the same, given SC spatial position, the intercellular region width for the image produced by each channel (the skin autofluorescence signal, detected in the HTPM green channel, and the exogenous model probe fluorescence intensity signal, detected in the red HTPM channel) can be quantified. A comparison of the average correlation lengths arising from these images, hence, provides a quantitative measure of the probe penetration into the corneocyte region.

The autocorrelation function was calculated for the image of skin autofluorescence and the corresponding image of probe spatial distributions in the SC to yield two \( \tau \) values. For the 0.3 mm x 0.3 mm skin area selected, differences between the average correlation length for the red channel, \( \tau_R \), and the autocorrelation length for the green channel, \( \tau_G \), highlight the spatial distribution of the probe in the SC relative to the corneocyte-lipid intercellular interface, established by the images of the intrinsic skin autofluorescence.
Probe diffusion beyond the intercellular region defined by the SC autofluorescence will yield $\tau_R$ values larger than $\tau_G$. Hence, skin samples displaying increased probe penetration into the corneocyte region will correspond to increased $\tau_R$ values from the control case to the enhancer case, whereas decreases in $\tau_R$ correspond to an increased localization of the probe in the intercellular region.

The average correlation length for the green channel, $\tau_G$, for which the skin autofluorescence is evaluated, should have similar values for each of the four samples evaluated (RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer), and thus serves as a consistent fingerprint image of the SC structural features. A comparison of the fluorescent probe spatial distribution images and the corresponding images of the inherent SC autofluorescence provides insight into the model probe transport pathways relative to the SC structural features. The difference between $\tau_G$ and $\tau_R$ provides a quantitative indicator of the penetration distance of the probe into the corneocytes. Using a paired t test for sample means, the statistical significance of the different values obtained for $\tau_G$ and $\tau_R$ was verified with $p<0.05$ (Microsoft Excel, Seattle, WA). Hence, the average correlation length obtained for each of the exogenous probe spatial distributions evaluated are statistically different than the corresponding average correlation length obtained from the probe autofluorescence.

6.2.3.2. Image Decomposition Analysis

The differences revealed between $\tau_G$ and $\tau_R$ provide a statistically robust, quantitative basis to evaluate the probe transport relative to the inherent skin structural features. Based
on the conventional definition, the correlation length is the distance over which the autocorrelation function decays from 1 to 1/e, and hence, the correlation length serves as a consistent measure of the change in the exogenous probe signal intercellular region width relative to the intercellular width defined by the skin autofluorescence. The trends detected for $\tau_C$ and $\tau_R$ reflect the spatial average autofluorescence and the probe intensity distributions over a 0.3 mm by 0.3 mm skin area, respectively. To confirm these spatially averaged trends with respect to the pixel intensities of the individual corneocytes and the corresponding intercellular regions that were visualized, a detailed analysis of the probe and of the autofluorescence intensities specific to selected corneocytes is described next.

To focus on the SC structural dimensions defined by the skin autofluorescence and the probe intensity signals, a representative subregion, spanning 150 pixels by 150 pixels, was selected from each 3x3 skin site area examined previously (see Section 6.2.3.1). Within each 150 pixel by 150 pixel image (shown in Figures 6-5 and 6-6), a linear path of 5 pixels in width (y coordinate) and 150 pixels in length (x coordinate), was decomposed into its pixel intensity values (see regions marked by the white rectangles in panels (a), (c), (d), and (f) in Figures 6-5 and 6-6) to determine the intercellular region width defined by the fluorescence intensity peak widths. At each position along the x coordinate, the intensities of the corresponding 5 pixels were averaged. The arbitrarily selected line width of 5 pixels accounts for the intensity variations along the y coordinate while limiting the inclusion of intensities arising from different structural features in the average value. The resulting plot of the fluorescence intensity spectrum for the green and the red channels, as a function of the x coordinate position, reveals the peak-to-peak
distance as well as the peak width, which indicate the corneocyte width and the intercellular spacing, respectively, along the 150 pixel linear region analyzed. These skin autofluorescence and probe intensity spectra are then normalized with respect to the corresponding maximum fluorescence intensity detected within the selected linear path. These normalized plots are shown in panels (b) and (d) of Figure 6-5 and 6-6. A detailed discussion of Figures 6-5 and 6-6 will be presented in Section 6.3.4.

6.3. Results and Discussion

6.3.1. Visualization of the Skin Autofluorescence

The intrinsic autofluorescence of 9 consecutive skin sites comprising an area of approximately 0.3 μm by 0.3 μm at a SC depth close to the skin surface (where the axial position, or z coordinate, is zero) for skin samples exposed to the control and the enhancer vehicles, both in the absence of probe, are shown in Figures 6-1(a) and 6-1(b), respectively. The corneocytes, depicted by the dark regions, are interspersed within the lipid multilamellae shown in green. The skin autofluorescence intensity scale is shown by the colorbar, in which autofluorescence intensities are shown by the corresponding shade of green, where $\text{AF}_0 = 0$ counts/pixel and $\text{AF}_n = 50$ counts/pixel. From Figures 6-1(a) and 6-1(b), the corneocytes are delineated by the intercellular lipid multilamellae based solely on the intrinsic skin autofluorescence. Hence, the skin autofluorescence images presented below (see Figures 6-2 and 6-3), corresponding to the dual-channel HTPM imaging of the four different cases examined (RBHE-control, RBHE-enhancer, SRB control, and SRB-enhancer), place the probe spatial distributions in the proper context of the skin structural
Figure 6-1: Autofluorescence of skin exposed to: (a) the control vehicle, and (b) the enhancer vehicle, both in the absence of probe.
features. The graphical overlapping of these images provides evidence for oleic acid-induced intracorneocyte probe diffusion, and is discussed next.

6.3.2. Visualization of Fluorescent Probe Spatial Distributions

The HTPM dual-channel imaging visualizations of the skin autofluorescence and of the exogenous model probe intensity are presented in Figures 6-2 and 6-3 for the hydrophobic probe RBHE and for the hydrophilic probe SRB, respectively. The panels of Figures 6-2 and 6-3 illustrate 3x3 skin site areas imaged using dual-channel HTPM for the control and the enhancer cases of the RBHE and of the SRB probe spatial distributions examined, respectively. For Figures 6-2 and 6-3, the first three vertical panels on the left-hand side depict the control case of skin exposure to the fluorescent probe, where: (a) the skin autofluorescence (green HTPM channel), (b) the probe spatial distribution at the same skin location (red HTPM channel), and (c) the overlapping of the two images are shown. The resulting images of skin exposure to the chemical enhancer and to the fluorescent probe are shown in the 3 vertical panels on the right-hand side, where panels (d), (e), and (f) depict the skin autofluorescence, the probe spatial distribution, and the overlay of these two images obtained at the same skin location, respectively. In both Figures 6-2 and 6-3, the skin autofluorescence intensity is again depicted by the green colorbar, where increasing intensities correspond to brighter green colors. \( AF_0 = 0 \) counts/pixel in both Figures 6-2 and 6-3, whereas \( AF_n = 100 \) counts/pixel in Figure 6-2 and \( AF_n = 50 \) counts/pixel in Figure 6-3. The probe intensities are reflected in the red scale color bar, where regions in black indicate low probe concentration and regions of high probe concentration are represented by white. The maximum intensities
displayed for the RBHE are $I_n = 1000$ counts/pixel in Figures 6-2 (b) and (e). For SRB, $I_n = 200$ counts/pixel in Figure 6-3(b) and $I_n = 1000$ counts/pixel in Figure 6-3(e). $I_0 = 0$ counts/pixel for both panels (b) and (e) in Figures 6-2 and 6-3.

6.3.2.1. Hydrophobic Probe (RBHE) Control Case

The black hexagonal regions that are surrounded by the green intercellular autofluorescence indicate a spacing regularity of corneocyte structures for the control case (see Figure 6-2 (a)). Based on the length of the 100 mm scalebar provided at the lower left of Figure 6-2(a), the corneocyte dimensions are approximately 30 μm, which is consistent with the 30-40 μm diameter cited in the literature (see Chapter 1, Section 1.2, Figure 1-1). The precise dimensions of these corneocytes will be discussed further when the quantification of model probe distributions in the corneocytes is presented in Section 6.3.4. The presence of the few green domains that do not surround corneocyte structures may be attributed to autofluorescence signals generated by additional intrinsic SC fluorophores. These domains are located at the center of Figure 6-2(a). A comparison of the skin autofluorescence image for the control RBHE case (Figure 6-2(a)) with the RBHE-control probe spatial distribution (Figure 6-2(b)), indicates that RBHE primarily traverses the intercellular region, as the corneocyte structures exhibit a lower fluorescence intensity compared with the probe intensity shown at the perimeter of these structures, that reflect the location of the intercellular lipid multilamellar region. This finding is consistent with the previous studies using HTPM aimed at elucidating the mechanisms of chemical enhancer action (see Chapter 5, Section 5.3.4 and Yu et al., 2002). The subtle differences between the images of the skin autofluorescence (Figure 6-2(a)) and of the RBHE probe spatial distribution (Figure 6-2(b)) become more apparent
in Figure 6-2(c), in which the probe fluorescence is displayed against a background of the intrinsic skin autofluorescence by overlaying Figure 6-2(b) above Figure 6-2(a). A comparison of Figure 6-2(c) with Figure 6-2(a) shows that the presence of the probe in the intercellular space has highlighted the previously not so visible corneocytes present in the green domain located close to the center of Figure 6-2(a). Furthermore, the differences between the autofluorescence and the probe signals illustrates the ability of HTPM to isolate the intrinsic skin autofluorescence from the exogenous probe intensity. In addition to the apparent differences between the SC features revealed through the skin autofluorescence and the probe intensity, the similarities between these two images are abundant. The green region not occupied by corneocyte structures in Figure 6-2(a) (located at the upper left-hand corner) is also present in Figure 6-2(b). The image overlay in Figure 6-2(c) illustrates that this domain exhibits signal from both channels, indicating the presence of the probe in the precise same region of skin autofluorescence. Additional differences between the intercellular region highlighted by the probe signal and the intercellular region defined by the intrinsic SC autoflourescence are not easily distinguished qualitatively. Hence, the application of the autocorrelation function analysis presented in Section 6.2.3.1 to quantitatively characterize these differences through the calculation of the average correlation distances, \( \tau_G \) and \( \tau_R \), is required, and will be presented in Section 6.3.3.

**6.3.2.2. Hydrophobic Probe (RBHE) Chemical Enhancer Case**

The structural features shown in Figure 6-2(d) for the RBHE (oleic acid) enhancer case do not display the same, regularly-spaced corneocyte structures highlighted by the intrinsic skin autofluorescence that is observed for the control case (see Figure 6-2(a)).
The corneocyte structures in Figure 6-2(d) appear rounder in shape, in contrast to the more hexagonally-shaped structures observed for the control RBHE skin autofluorescence image in Figure 6-2(a). Furthermore, the intercellular space appears to also include continuous green domains that are not occupied by corneocyte structures (see the upper and lower areas of Figure 6-2(d)). While causes of these apparent differences are not addressed in this thesis, the influence of oleic acid on inducing SC structural alterations in the lipid intercellular region, as well in the corneocytes, warrants a more extensive analysis. These differences in the corneocyte structure could be attributed to the inherent heterogeneity of the skin morphology, where variability in the corneocyte structure, as well as the corneocyte packing, has been well described (Plewig, 1970, Schatzlein and Cevc, 1998). Moreover, the oleic acid-induced fluidization of the lipid multilamellae may contribute to the observed changes in the SC structure that includes swelling of the intercellular region, disruption of the corneocyte lipid envelope, and loss of structural cohesion. These potential causes proposed for the observed differences between the control and the oleic acid-treated skin sample requires further investigation.

For Figure 6-2(e), in which the oleic acid-induced RBHE probe spatial distribution is shown, the spatial positions corresponding to the green domains observed in the lower portion of Figure 6-2(d) also reflect regions that do not reveal corneocyte structures. The probe delineation of rounder corneocyte structures becomes more apparent in the upper portion of the image, where corneocyte structures were not highlighted by the autofluorescence in Figure 6-2(d). The composite image presented in Figure 6-2(f)
illustrates the appearance of the corneocyte structures in the upper right area of the image when the probe signal is placed on the background of the autofluorescence channel.

Compared with the control case (Figure 6-2(b)), the increase in the nonuniformity of the probe spatial distributions, apparent in the microdomains of probe pools shown at the upper and the lower bright, white, regions of Figure 6-2(e), remains consistent with findings reported in the previous HTPM studies (see Chapter 4, Section 4.3.1), where oleic acid also induced the formation of hydrophobic probe microdomains. Although similar microdomains are visualized in the skin autofluorescence images at similar locations within Figure 6-2(d), the bright white region indicating high probe intensity within these pools does not map directly with the features highlighted in the autofluorescence region in Figure 6-2(d).

**6.3.2.3. Hydrophilic Probe (SRB) Control Case**

Figure 6-3(a) shows the SC autofluorescence signal for the hydrophilic probe (SRB) control case. The corneocytes are the dark polyhedral regions surrounded by the green autofluorescence of the SC intercellular region. These results agree well with the autofluorescence images obtained for the hydrophobic probe (RBHE) control case of skin autofluorescence (compare Figures 6-3(a) and 6-2(a)). The similar autofluorescence images for the hydrophobic probe and the hydrophilic probe control cases indicates that the intrinsic SC fluorescence is probe independent, and hence, can serve as a fingerprint of the SC structural features, relative to which the fluorescent probe spatial distributions can be mapped. A green domain present in the upper left-hand corner of Figure 6-3(a) can again be attributed to the endogenous SC fluorophores when compared with the exact
Figure 6-2: Visualization of the fluorescence intensity of: (a) the skin autofluorescence, (b) the RBHE probe intensity, and (c) the probe signal-autofluorescence overlay for the control case. The corresponding images for the chemical enhancer (oleic acid) case are shown in (d), (e), and (f), respectively.
same spatial position in Figure 6-3(b). The SRB probe spatial distribution shown in
Figure 6-3(b) further highlights the intercellular region. The overlapping of Figure 6-3(b)
with the corresponding SC autofluorescence shown in Figure 6-3(a) (see Figure 6-3(c))
reveals the corneocyte structures obscured by the endogenous SC fluorophore intensity in
that region of Figure 6-3(a). As observed in the wide-area visualization of hydrophilic
probe SRB distributions presented in Chapter 4, Section 4.3.1, SRB lies primarily within
the intercellular region. The appearance of SRB probe intensity within the corneocyte
region provides evidence for hydrophilic probe penetration into the corneocyte region.
The validity of this observation will be addressed in detail in Section 6.3.4.1, where the
intracorneocyte probe intensity distributions are quantified.

6.3.2.4. Hydrophilic Probe (SRB) Chemical Enhancer Case

For the hydrophilic probe (SRB) oleic acid case, high intensity domains are present at the
right-hand side of the SC autofluorescence image shown in Figure 6-3(d). The dark
regions representing corneocytes are again well defined by the green intercellular region
autofluorescence. These high intensity domains can be attributed to intrinsic skin
fluorophores upon comparing the differences between Figures 6-3(d) and 6-3(e), in which
the SRB probe spatial distribution is shown. The overlap of the SC autofluorescence and
of the SRB probe distribution images, shown in Figure 6-3(f), reveals that the regions of
high autofluorescence intensity (at the right-hand side) do not completely correlate to the
regions of high probe intensity. The oleic acid-induced changes in the intercellular probe
spatial distributions will be quantified next to address the mechanism of oleic acid
enhancer action with respect to intracorneocyte permeant penetration.
Figure 6-3: Visualization of the fluorescence intensity of: (a) the skin autofluorescence, (b) the SRB probe intensity, and (c) the probe signal-autofluorescence overlay for the control case. The corresponding images for the chemical enhancer (oleic acid) case are shown in (d), (e), and (f), respectively.
6.3.3. Fluorescence Intensity Average Correlation Length

The visualization of the intrinsic skin autofluorescence with the corresponding exogenous probe spatial distributions shown in Figures 6-2 and 6-3 reveals the qualitative differences between the signals detected with the two HTPM channels. The skin autofluorescence images shown in Figures 6-2(a), 6-2(d), 6-3(a), and 6-3(d) show that the corneocyte structures which appear in black are well delineated by the autofluorescence of the endogenous fluorophores in the intercellular region. Relative to the corneocyte structures and the intercellular region defined by the skin autofluorescence, the fluorescent probe diffusion pathways can hence be examined.

In this section, the extent of probe penetration into the corneocytes will be quantified using the average correlation length, \( \tau_R \), determined from the autocorrelation function described in Section 6.2.3.1. Recall that the average correlation length, by definition, corresponds to the average distance between two pixels over which the autocorrelation function, \( C(\Delta x, \Delta y) \), drops from a maximum value of 1 to a value of \( 1/e \). Based on this definition, then, the average correlation length serves as a consistent quantitative length scale to characterize the probe spatial distributions, and to make relative comparisons. With respect to the skin structural features, the decay in the fluorescence intensity signal over the average correlation length refers to the distance over which a pixel of maximum intensity decays to an intensity corresponding to an autocorrelation value of \( 1/e \). Since pixels of high intensity lie along the centerpoint of the width of the intercellular space, the average correlation length defines the distance extending from this centered position.
within the intercellular region to a position where the drop in pixel intensity leads to $C(\Delta x, \Delta y)=1/e$. In essence, the length over which this drop in intensity occurs reflects the apparent intercellular region half-width, such that increased probe penetration into the corneocyte region will result in an increased average correlation length. The increase in the average correlation length corresponds to the increase in probe intensity that is visualized at the perimeter of the corneocyte region. The magnitude of the increase in the average correlation length reflects the extent of the probe diffusion from the intercellular region toward the corneocyte center.

In the absence of probe signal, the apparent intercellular region half-width should remain similar from sample to sample, as the $\tau_G$ value reflects the spatial length over which the endogenous fluorophore emissions decay. This spatial length effectively, although not precisely, corresponds to the transition from the intercellular space to the intracorneocyte region. While the average correlation length does not represent the exact length of the intercellular space, the relative comparisons made between $\tau_R$ and the corresponding $\tau_G$, with $\tau_G$ serving as the reference average correlation length, provide an initial quantitative analysis of intracorneocyte probe diffusion. A more detailed analysis of the intracorneocyte probe spatial distributions will follow in Section 6.3.4, where the changes in the probe intensity from the perimeter of the corneocyte to the corneocyte center will be evaluated directly with the autofluorescence intensity over the same length. In this section, the spatial average correlation lengths will be considered.
In the following analysis, the probe average correlation length, $\tau_R$, will be compared to the corresponding autofluorescence average correlation length, $\tau_G$. The curve fit of each autocorrelation function yielded the average correlation lengths for each skin sample examined, following the method described in Section 6.2.3.1. These values are summarized in Figure 6-4. The gray bars and the black bars represent the average correlation lengths obtained for the probe spatial distribution, $\tau_R$, and for the SC autofluorescence, $\tau_G$, respectively, where the error bars reflect one standard deviation from the average of the four correlation lengths calculated per sample.

![Figure 6-4: Average correlation lengths associated with the probe, $\tau_R$ (gray bars), and with the autofluorescence, $\tau_G$ (black bars), signals examined for the skin areas evaluated. The error bars indicate one standard deviation from the average correlation length of the four different autocorrelation cross sections evaluated along the x and the y axes (see Section 6.2.3.1).](image-url)
The skin autofluorescence average correlation lengths (black bars, in Figure 6-4) obtained for each of the four skin samples examined were found to be comparable, with the RBHE-control, the RBHE-enhancer, the SRB-control, and the SRB-enhancer yielding \( \tau_G \) values of \( 13.49 \pm 0.57 \) pixels, \( 13.03 \pm 1.46 \) pixels, \( 8.32 \pm 0.35 \) pixels, and \( 13.51 \pm 6.30 \) pixels, respectively, where each pixel corresponds to \( 0.4 \mu m \). With the exception of the SRB-control skin sample evaluated, the skin autofluorescence average correlation lengths are comparable, having a value of about \( 5 \mu m \) (or 13 pixels). The average of the \( \tau_G \) values for the four samples examined yielded an average correlation length value of \( 4.83 \pm 1.01 \mu m \). As described previously, the precise value of the skin autofluorescence average correlation length serves as a reference length, from which probe penetration into the corneocyte can be quantified. The length of \( 4.83 \pm 1.01 \mu m \) corresponds well with the apparent width of the intercellular space, that is observed in the images presented in Figures 6-2 (a), 6-2(d), 6-3(a) and 6-3(d). This apparent intercellular space is visualized as a result of the endogenous fluorophores present at the perimeter of the corneocytes. Note that the apparent intercellular region width that is defined by the endogenous skin fluorophores has a value which is different from the \( 0.075 \mu m \) intercellular lipid multilamellae width cited in the literature (see Chapter 1, Section 1.2). Because the actual dimension of the lipid intercellular space is 4 times smaller than the HTPM pixel resolution of \( 0.4 \mu m/pixel \), the analyses presented in this chapter focuses on the probe spatial distributions relative to the corneocyte structures that are defined by the skin autofluorescence. Nevertheless, the good agreement found between the correlation lengths for the skin autofluorescence of each of the four skin samples examined serves as quantitative evidence supporting the similarity of the skin autofluorescence intensity
distributions visualized (Figures 6-2(a), 6-2(d), 6-3(a), and 6-3(d)). The similarity in the \( \tau_{G} \) values for the skin autofluorescence signals in each of the four cases examined, despite the skin exposure to the four different probe-vehicle combinations, quantitatively reinforces the use of the skin sample autofluorescence image, captured with the HTPM green channel, as a fingerprint of the SC structural features, relative to which probe transport (detected in the HTPM red channel) can be quantified.

Comparing the four black bars (\( \tau_{G} \)) with the corresponding gray bars (\( \tau_{R} \)) in Figure 6-4, an increase in the average correlation length is apparent when the probe spatial distribution images are evaluated. The \( \tau_{R} \) values for the RBHE-control, the RBHE-enhancer, the SRB-control, and the SRB-enhancer are 18.64 ± 4.13 pixels, 44.29 ± 8.32 pixels, 20.34 ± 4.11, and 22.86 ± 8.59 pixels, respectively. Unlike the skin autofluorescence average correlation lengths, the probe intensity average correlation lengths vary from sample to sample, with the greatest average correlation length displayed by the RBHE-enhancer sample. The average correlation lengths for the RBHE-control, the SRB-control, and the SRB-enhancer are comparable, and, since each pixel corresponds to 0.4 \( \mu \)m, translate to values of 7.46 ± 1.65 \( \mu \)m, 8.13 ± 1.64 \( \mu \)m, and 9.14 ± 3.44 \( \mu \)m, respectively.

Compared with the corresponding reference lengths determined for the skin autofluorescence signal, the statistical significance of the differences found between \( \tau_{R} \) and the corresponding \( \tau_{G} \) was confirmed. The \( p<0.05 \) for the paired 2 sample t-test of \( \tau_{G} \) and of the corresponding \( \tau_{R} \) value indicated that the 4 average correlation lengths for the
probe spatial distributions are indeed greater than those calculated for the skin autofluorescence. Hence, as discussed in Section 6.2.3.1, the probe transport pathway extends beyond the intercellular region delineated by the skin autofluorescence signal, and possibly into the corneocyte region for these four cases.

Between samples, however, the differences between the probe spatial distributions, reflected in the \( \tau_R \) values for each case considered, did not yield statistically discernable differences. Using the student t test, assuming two sample unequal variances, a value of \( p > 0.05 \) was found when the RBHE-control, the SRB-control, and the SRB-enhancer samples were compared with each other. The \( \tau_R \) values for these three cases, however, were statistically different from the significantly larger \( \tau_R = 44.29 \pm 8.32 \) pixels obtained for the RBHE-enhancer case. While the statistically significant differences found between the average correlation lengths for the skin autofluorescence and the corresponding probe intensity enable the quantitative characterization of probe penetration into the corneocytes within a skin sample, a comparison of the calculated probe average correlation lengths between skin samples exposed to different probe-vehicle combinations do not yield the same statistics. The sample to sample differences in the average probe spatial distributions, therefore, cannot be quantified utilizing solely their respective average correlation lengths.

The qualitative differences in the probe distributions immediately surrounding the corneocytes between Figures 6-2(b) (RBHE-control) and 6-2(e) (RBHE-enhancer) are quite evident, where the probe distribution appears to be more localized to two regions in
Figure 6-2(e): (i) the white colored ‘pools’ located at the top and at the bottom of Figure 6-2(e), and (ii) the intercellular region not included in these ‘pools’. The continuous ‘pools’ of high probe intensity evident in Figure 6-2(e) may contribute to the significantly larger average correlation length, as the drop in intensity from the center of these high probe intensity ‘pools’ to a spatial position displaying decreased probe intensities occurs over a longer length compared with the width of the apparent intercellular region. Despite the presence of these ‘pools’, the distribution of the RBHE probe in the neighboring intercellular regions (at the center of Figure 6-2(e)) appears more localized to the intercellular space when compared with Figure 6-2(b). This increase in probe localization to the centerpoint (see Section 6.3.2) of the intercellular region is made increasingly evident by the significantly darker corneocyte structures that the region surrounds compared with the control case (Figure 6-2(b)), where the corneocytes indicate a higher probe intensity corresponding to the orange color in the colorbar.

Based on the visualization of the hydrophobic probe (RBHE) control and enhancer cases, the action of oleic acid appears to increase probe pooling, as well as probe localization to the intercellular region. In the presence of the oleic acid chemical enhancer, the hydrophobic probe spatial distribution increases in heterogeneity. This was also observed in the wide-area scans of the RBHE-oleic acid samples presented in Chapter 4 (see Figure 4-1). In this section, the increased RBHE probe distributional heterogeneity is revealed by the isolated domains of high probe intensity in Figure 6-2(e). The presence of these high intensity probe pools, that span significantly larger lengths than the apparent intercellular region width, contributes to the large $\tau_R$ value (44.29 ± 8.32 pixels)
calculated for the case of the RBHE-enhancer case. Therefore, the $\tau_R = 44.29 \pm 8.32$ pixels also captures the spatial distribution of the probe pools within the skin sample. Considering the contribution of these concentrated probe domains (the white regions in Figure 6-2(e)) to the calculated autocorrelation function, the relatively larger $\tau_G$ value obtained for the RBHE-enhancer case remains consistent with this introduction of a larger length scale, over which the probe intensity decays. The evidence of probe penetration into the corneocytes provided by the average correlation lengths of 9 consecutive skin site area for the cases of the RBHE-control, the SRB-control, and the SRB-enhancer, as well a probe localization in the intercellular region for the case of the RBHE-enhancer, will be followed next by a detailed examination of the probe intensity distributions surrounding, as well as in specific, corneocyte structures.

6.3.4. Linear Image Deconstruction of Intracorneocyte Probe Intensity Distributions

The probe intensity distributions within specific corneocyte structures were evaluated based on the method described in Section 6.2.3.2, where the pixel intensities over a 5 pixel-wide line were averaged at each of the 150 pixel x-coordinate positions along the length of the line. The average intensity of the 5 pixels at each x-coordinate was then plotted to yield an intensity spectrum reflecting the probe intensity distribution along the 150 pixel linear path that traverses the widths of consecutive intercellular regions and corneocytes. The spatial positions of these linear paths are presented within the context of a 150 pixel by 150 pixel subsection, selected from each of the RBHE-control, the RBHE-enhancer, the SRB-control, and the SRB-enhancer cases (see Figures 6-2 and 6-3). The image deconstruction results are presented in Figures 6-5 and 6-6 for the hydrophobic
and the hydrophilic probes, respectively. Each Figure includes the probe spatial distribution in panel (a), the fluorescence intensity over the image width in panel (b), and the skin autofluorescence image in panel (c), for the control case. The corresponding images for the chemical enhancer (oleic acid) cases are shown in panels (d), (e), and (f), respectively. The white rectangles in panels (a), (c), (d), and (f) in Figures 6-5 and 6-6 demark the area spanned by the linear image deconstruction path.

The fluorescence intensity spectra shown for the RBHE-control, the RBHE-enhancer, the SRB-control, and the SRB-enhancer cases in Figures 6-5(b), 6-5(e), 6-6(b), and 6-6(e), respectively, represents the deconstruction of the fluorescence images along one dimension. In deconstructing the image, the specific width of the intercellular space can be determined for the probe fluorescence images (red lines) with respect to the skin autofluorescence images (green lines). Each of the intensity spectra depicts the fluorescence intensity (shown on the y-axis) as a function of the x coordinate position (shown on the x-axis) for the skin region highlighted by the white rectangles. Comparing the intensity spectra with the corresponding regions highlighted in the images shown in the panels above and below each intensity plot, the high intensity regions in the images correspond to the peaks, and the dark regions correspond to the valleys in these plots. Moreover, these peaks correspond to the centerpoint of the intercellular region width, and the decrease in probe intensity about this centerpoint reflects the probe concentration gradient from the intercellular region to the corneocyte region.
Differences between the intensity spectrum peak widths for the probe and the skin autofluorescence serve as an indicator of the probe diffusion pathway. In these image deconstruction studies, the width of the peaks corresponding to the skin autofluorescence intensity spectrum serves as a reference, relative to which the probe intensity spectrum peak widths are compared. Probe intensity spectrum peak widths that are greater than the corresponding skin autofluorescence peak widths indicate that probe diffusion extends beyond the intercellular region defined by the skin autofluorescence spectrum peak widths, and into the corneocyte region. On the other hand, probe intensity spectrum peak widths that are shorter than the peak widths of the autofluorescence intensity spectrum indicate a localization of the probe to the intercellular region, or that the probe remains within the bounds of the intercellular space defined by the skin autofluorescence intensity spectrum. While the autofluorescence intensity spectrum peaks correspond to the spatial positions of the intercellular regions along the linear decomposition path, the valleys, or the autofluorescence signal intensity minima, correspond to the corneocyte regions. The periodicity of the peak and valleys observed in the skin autofluorescence spectra presented below reflect the brick and mortar structure of the stratum corneum, where corneocyte structures alternate with the intercellular lipid regions. The probe and the corresponding autofluorescence intensity spectra are shown in Figures 6-5(b) and 6-5(e) for the hydrophobic probe, and in Figures 6-6(b) and 6-6(e), for the hydrophilic probe. An in-depth discussion of the spectra obtained in each of the four cases is presented below.
Figure 6-5: Peak-width image analysis of the hydrophobic probe intensity spectrum. For the control case, the linear fluorescence intensity deconstruction regions are demarked by the white rectangles in the images of the RBHE probe spatial distribution, shown in (a), and of the corresponding skin autofluorescence signal, shown in (c). The green and the red lines in (b) represent the skin autofluorescence and the probe intensity spectra, respectively. For the chemical enhancer (oleic acid) case, the white rectangles in (d) and (f) show the linear fluorescence intensity deconstruction regions for the images of the probe spatial distribution and of the skin autofluorescence, respectively. The green and the red lines shown in (e) depict the intensity spectra for the skin autofluorescence and for the probe, respectively. See the text for details.
6.3.4.1. Hydrophobic Probe

Figures 6-5(b) and 6-5(e) illustrate the intensity spectra for the RBHE hydrophobic probe in the control case and the enhancer case, respectively. For the RBHE-control sample (Figure 6-5(b)), note the good agreement between the peak locations for the probe and the autofluorescence signals at x positions of 11, 72, and 134 pixels. These peaks in the probe and in the autofluorescence intensity spectra, represented by the green line and the red line in Figure 6-5(b), respectively, correspond to the pixels of high intensity along the linear path demarked by the rectangular boxes for the autofluorescence image (Figure 6-5(c)) and for the probe image (Figure 6-5(a)), respectively. These intensity spectrum peak widths correspond to the apparent widths of the intercellular regions visualized in panels (a), (c), (d), and (e) of Figure 6-5. For the RBHE-control in Figure 6-5(b), the probe intensity spectrum (red line) envelopes the autofluorescence spectrum (green line). The wider probe intensity spectrum peaks, relative to the referenced peak widths of the corresponding autofluorescence spectrum, indicate that the probe transport extends beyond the intercellular region defined by the skin autofluorescence intensity spectrum and into the corneocyte structures. As established in Section 6.3.3, the pixel resolution of the HTPM (0.4 μm/pixel) does not capture the precise dimensions of the lipid multilamellar regions (0.075 μm wide), while the pixel distance from peak-to-peak reflects the diameter of the corneocytes lying along the linear path evaluated. Note that the autofluorescence peaks alternate with regions reflecting intensity minima. These regions extend the diameter of the corneocyte that is visualized in the corresponding skin autofluorescence image shown in Figure 6-5(c). The peak-to-peak distances for the
RBHE-control autofluorescence spectra represent the 60 pixel (or 24 μm) diameter of the corneocytes in these images, which is in good agreement with the 30 μm corneocyte diameter reported in the literature (Heisig et al., 1996). Compared with the zero intensity minima exhibited by the skin autofluorescence spectrum, the probe intensity spectrum minima never approach zero. The probe intensities detected within the corneocyte regions further corroborate the existence of intracorneocyte probe penetration driven by the probe concentration gradient from the intercellular region into the corneocyte region.

In the presence of oleic acid, however, RBHE becomes localized to within the defined intercellular space. The intensity spectra shown in Figure 6-5(e) indicate that the widths of the autofluorescence intensity peaks (located at x = 60 pixels and at x = 140 pixels) extend beyond the corresponding peak widths for the probe intensity spectrum. For both the autofluorescence and the probe intensity spectra shown in Figure 6-5(e), the minima represents a 0 intensity value indicating the absence of probe penetration into the corneocytes. Furthermore, the 80 pixel (36 μm) peak-to-peak distance enclosing the zero intensity region is again in good agreement with the reported corneocyte diameter (30 μm). This quantification of the oleic acid-induced localization of the RBHE probe to the intercellular region is consistent with the probe spatial distribution shown in Figure 6-2(a). The oleic acid-induced localization of the RBHE probe to discrete domains of approximately 20 μm in width, and wider (see white regions in Figure 6-2(e)), contributes to the calculated τR value of 44.29 ± 8.32 pixels that exceeds the τG value of 13.03 ± 1.46 pixels (see Section 6.3.3). In enhancing transdermal transport of the hydrophobic probe, the action of the oleic acid chemical enhancer is observed in the
localization of the RBHE distribution, first to the intercellular region, and second, to the larger domains of high probe intensity (white regions at the top and at the bottom of Figure 6-2(e)), that we have referred to as concentrated 'pools' of probe distribution in Section 6.3.2.2.
Figure 6-6: Peak-width image analysis of the hydrophilic probe intensity spectrum. For the control case, the linear fluorescence intensity deconstruction regions are demarked by the white rectangles in the images of the SRB probe spatial distribution, shown in (a), and of the corresponding skin autofluorescence signal, shown in (c). The green and the red lines in (b) represent the skin autofluorescence and the probe intensity spectra, respectively. For the chemical enhancer (oleic acid) case, the white rectangles in (d) and (f) show the linear fluorescence intensity deconstruction regions for the images of the probe spatial distribution and of the skin autofluorescence, respectively. The green and the red lines shown in (e) depict the intensity spectra for the skin autofluorescence and for the probe, respectively. See the text for details.
6.3.4.2. Hydrophilic Probe

Figure 6-6(b) shows the SRB-control sample intensity spectrum (red line), corresponding to the white rectangle highlighted in Figure 6-6(a), and the autofluorescence spectrum (green line), corresponding to the white rectangle highlighted in Figure 6-6(c). In Figures 6-6(b), the SRB-control autofluorescence spectrum (green line) exhibits two major peaks at x = 21 pixels and at x = 103 pixels, with minor peaks at x = 56 pixels and at x = 150 pixels. These four peaks correspond to the positions of the intercellular regions shown in the white rectangle demarking the linear path evaluated in the corresponding skin autofluorescence image (Figure 6-6(c)). The two minor peaks reflect weaker autofluorescence signals, or the presence of fewer intrinsic fluorophores within the corresponding intercellular regions. The average peak-to-peak distance of 43 pixels (17 μm) observed here reflects the corneocyte diameter within this skin area represented by Figure 6-5(c). Again, the dimensional variability of the SC corneocytes arises, where the average 17 μm corneocyte diameter observed here is half the length of the average corneocyte diameter described in the literature (Heisig, et al., 1996). Efforts to characterize the variability of the skin structural features in the literature remains a relevant aspect of dermatological research (Huzaira et al., 2001, Plewig, 1970).

The SRB-control probe intensity spectrum exhibits peaks at those four x-coordinate positions, as well as an additional peak at x= 120 pixels (see red line in Figure 6-6(b)). The probe intensity spectrum peak widths (see red line in Figure 6-6(b)) are greater than those corresponding to the skin autofluorescence peaks (see green line in Figure 6-6(b))
at the four x-coordinates noted. This difference between the probe intensity and the skin autofluorescence peak widths indicates that the probe does penetrate into the corneocyte region, although not to the same extent as for the SRB-enhancer case (described below). The minima corresponding to the SRB-control intensity spectrum in Figure 6-6(b) (see red line at x= 40 and 75 pixels) do reach the zero intensity value as the spatial position approaches the corneocyte center and away from the intercellular region. The additional peak detected only for the probe intensity at x= 120 pixels provides evidence of probe penetration into the corneocyte region. The peak-to-peak region of the autofluorescence spectrum (see green line in Figure 6-6(b)) from x= 103 to 150 pixels demarks the outer bounds of a corneocyte. The probe intensity peak at x=120 pixels, as well as the nonzero probe intensities detected within the corresponding corneocyte region (see red line in Figure 6-6(b) from x=103 to 150 pixels), indicate probe penetration within this corneocyte region. Compared with the differences between the hydrophobic probe peak widths and their corresponding autofluorescence peak widths, the hydrophilic probe control case remains localized to the intercellular region. This may be attributed to the inherently low skin permeability to hydrophilic molecules. The low SRB concentration in the SC intercellular region is observed in Figure 6-2(b), which in turn, generates a weaker concentration gradient, compared with the hydrophobic control, from the intercellular region into the corneocytes. The probe intensity spectrum (red line in Figure 6-6(b)) further reinforces the decreased probe diffusion into the corneocyte region relative to the RBHE-control case (red line in Figure 6-5(b)), as the probe intensity peaks decay to a segment exhibiting zero intensity. The length of the SRB-control probe zero intensity regions are shorter than the corresponding segments in the autofluorescence
spectrum, while being centered about the same x position (see Figure 6-6 (b)). The shorter zero probe intensity segments result from probe intensity increases at the endpoints of these segments that reflect the observed probe penetration at the periphery of the corneocyte structures (see Figure 6-6(a)).

The oleic acid-induced increase in the penetration of the hydrophilic probe (SRB) into the corneocyte region is observed in the spectra shown in Figure 6-6(e), in which the probe peak widths far exceed the autofluorescence peak widths that demark the boundary of the intercellular region. The spectral peaks at x= 20, 40, 70, and 120 pixels for both the probe (red line) and the autofluorescence (green line) intensities represent the spatial positions of the intercellular regions along the linear regions demarked by the white rectangle in Figure 6-6(d) and in Figure 6-6(f), respectively. The corneocyte regions are represented by the segments of zero intensity in the autofluorescence spectrum (green line in Figure 6-6(e)). The peak-to-peak width corresponding to the diameter of a corneocyte visualized (between x= 70 pixels and x= 120 pixels in Figure 6-6(f)) is 20 μm. Relative to the zero intensity segments that reflect the spatial positions of the corneocyte structures in the skin autofluorescence spectrum, the minima shown in the probe intensity spectrum (red line in Figure 6-6(e)) do not reach zero intensity, and thus provides evidence for the oleic acid-induced SRB diffusion into the corneocyte. The oleic acid-induced increased partitioning of SRB into the SC intercellular region, discussed in Chapters 4 and 5 (see Sections 4.3.2 and 5.3, respectively), increases the probe concentration in the intercellular region. The increased probe intercellular concentration, in turn, generates a greater concentration gradient from the intercellular region into the corneocyte region. The increased probe
penetration into the corneocytes that results from the increased intracorneocyte flux is seen in the increased distance into the corneocyte structures over which the drop in probe intensity occurs. The drop in probe intensity (see red line in Figure 6-6(e)) about the peak maximum reflects the concentration gradient driving SRB diffusion into the corneocytes. With respect to the corneocyte region confined by the autofluorescence spectrum peaks between x=70 and 120 pixels, the decrease in the probe intensity from the intercellular region centerpoint (spectrum peak at x=70 pixels), to the center of the corneocyte (spectrum minimum at x=90 pixels), captures the probe intensity gradient responsible for the increased probe penetration into the corneocyte region. For the hydrophilic probe, oleic acid acts to increase probe partitioning into the SC intercellular region. Recall that the fluorescence intensity spectra are normalized with respect to the maximum probe intensity within the linear path evaluated. Hence, the increased intracorneocyte probe concentration gradient is not apparent by comparing the intracorneocyte intensity gradients in Figure 6-6(e) with Figure 6-6(b), both of which display normalized intensities. This increase in value of the actual drop in probe concentration with respect to the x coordinate (the intensity gradient) from the control to the enhancer SRB cases becomes apparent when considering the oleic acid-induced 10-fold increase in the probe intensity normalization factor (see Chapter 4, Section 4.3.2). In contrast to its effect on the hydrophobic probe localization to the intercellular region, the increased concentration of SRB in the intercellular region contributes to the oleic acid-induced intracorneocyte penetration observed in Figure 6-6(f), where the probe intensity does not drop to zero in the corneocyte regions. The additional effects of oleic acid on altering the integrity of the
corneocyte lipid envelope to induce the increased SRB lipid-corneocyte partitioning warrants additional investigation.

The results presented in this chapter remain consistent with the results presented in Chapters 3, 4, and 5. In Chapter 3, the oleic acid-induced permeability enhancement was attributed to increased probe partitioning for both the hydrophilic and the hydrophobic probes. In Chapters 4 and 5, additional mechanistic insight was provided through the wide-area analysis of transdermal transport parameter distributions that identified the rate-limiting transport steps for the hydrophilic and the hydrophobic probes. Whereas probe vehicle to SC partitioning is the rate-limiting step for the hydrophilic probe, lateral diffusion was found to be the rate-limiting step for the hydrophobic probe. Based on the visual elucidation of transport pathways through the decomposition of the skin autofluorescence signals from the probe fluorescence signal, oleic acid was observed to increase intracorneocyte penetration for the hydrophilic probe and to localize hydrophobic probe diffusion to either the intercellular region or to the larger domains ('pools') of high probe intensity. The oleic acid-induced increase in SRB lipid to corneocyte partitioning, as a result of the increased partitioning of the SRB probe from the vehicle to the SC, promotes probe diffusion into the corneocytes. The availability of corneocyte diffusion as a secondary pathway suggests that the constraints to transdermal transport via lateral diffusion through the tortuous intercellular multilamellae no longer hold. Therefore, the oleic acid-induced increase in SRB probe diffusion into the corneocytes remains consistent with the earlier finding that the hydrophilic probe vehicle to SC partitioning is the rate-limiting step. Furthermore, the hydrophobic probe rate-
limiting step of lateral diffusion coincides with the oleic acid-induced localization of the hydrophobic probe to domains within the intercellular region. This conclusion remains consistent with the transport hindrance presented by the tortuous lipid multilamellae region for the hydrophobic probes established earlier by Johnson et al. (Johnson et al., 1997).

6.4. Conclusions

The novel application of dual-channel HTPM to delineate the oleic acid-induced changes in the permeant transdermal pathways has been introduced for the first time to elucidate the mechanisms of chemical enhancer action. The rate-limiting steps for the model hydrophobic and hydrophilic probes established in Chapter 5 was further verified and visualized through the application of dual-channel imaging followed by the image analysis techniques introduced in this chapter. Oleic acid was found to induce increases in the SRB intracorneocyte diffusion and in the localization of the RBHE probe diffusion to the intercellular region. For the hydrophilic SRB probe, probe partitioning from the vehicle into the SC is the rate-limiting step, beyond which, barriers to hydrophilic probe diffusion through the intercellular region are relatively less significant. The lack of diffusional constraints in the intercellular region is visualized in the oleic acid-induced increase in probe diffusion into the corneocyte region.

For the hydrophobic RBHE probe, the rate-limiting step of lateral diffusion is visualized in the localization of the probe to the intercellular region and to the larger domains (‘pools’) of high probe intensity. The inherent hydrophobicity of the SC drives hydrophobic probe partitioning into the SC. The oleic acid-induced increase in this first
transdermal transport step (see Chapter 5, Section 5.1.2), however, is followed by the hydrophobic probe diffusional resistance of the tortuous lipid multilamellar region that is so well delineated in Figures 6-2(b) and 6-2(e), and further quantified in Figure 6-5(e).

The results presented in this chapter identify two different mechanisms of oleic acid enhancer action that further validate the lipoidal and the aqueous pore transdermal transport routes proposed for the hydrophobic and the hydrophilic permeants, respectively. The application of the intrinsic skin autofluorescence emission as a template to define the spatial positions of the SC structural features has enabled the quantification of transdermal probe spatial distributions with respect to the corneocytes and the lipid multilamellae that comprise the SC barrier. The methods introduced in this chapter to specifically illuminate the mechanisms of oleic acid chemical enhancer action for a model hydrophobic and a model hydrophilic probe provides an illustration of how the visual and quantitative analyses techniques presented in this chapter can be applied to elucidate the mechanisms of enhancer action for a multitude of chemical enhancer formulations. The intrinsic skin autofluorescence, as well as the quantitative characterization of the probe and of the autofluorescence intensity distributions using the autocorrelation function followed by the linear image deconstruction spectra, remain independent of the model drug and of the chemical enhancer system of interest. Given that the model probe emission spectrum can be separated from the skin autofluorescence spectrum using methods like the dual-channel detection scheme described in Section 6.2.2, the universal implementation of the methods introduced in this chapter to evaluate
an array of chemical enhancer-model probe formulations appears very promising, and
warrants further consideration.

As the work presented in this thesis has been motivated by the elucidation of the
mechanisms of chemical enhancer action in general, the specific findings presented in
this chapter with respect to the mechanisms of oleic acid enhancer action illustrate how
mechanistic insight expedites the development of effective chemical enhancer
formulations. The oleic acid-induced increase in the hydrophilic probe lipid to corneocyte
partitioning, coupled with the oleic acid-induced localization of the hydrophobic probe to
the intercellular region, propose two mechanisms by which transdermal transport is
enhanced. Although the extent of lipid to corneocyte partitioning depends, in part, on the
specific physicochemical properties of the hydrophobic and the hydrophilic probes
examined in this chapter, the mechanistic insight provided here poses the question of how
localizing the chemically induced-enhancement of transdermal permeant diffusion to the
intercellular region may further increase the skin permeabilities to hydrophilic permeants
currently achieved with well-known, effective chemical enhancer formulations. This, as
well as the other questions that arise from the results presented in this chapter, lay the
foundations for the future work proposed in Chapter 8.
6.5. References


Chapter 7
Chemically-Induced Permeability Enhancement of Model Drugs

7.1. Introduction

In Chapters 4 and 5, the application of High-Speed Two-Photon Microscopy (HTPM) to examine probe spatial distributions in the skin dramatically improved the efficiency of the Two-Photon Microscopy (TPM) methodology introduced in Chapter 3 (see also Yu et al., 2001). While the focus of this thesis so far has been devoted to understanding the mechanism of oleic acid chemical enhancer action, the introduction and the successful implementation of the analysis techniques presented in the previous chapters, in conjunction with the high throughput, automated capacity of HTPM, warrants additional scrutiny, with respect to the effects of a broader spectrum of chemical enhancer formulations on transdermal drug spatial distributions. With this in mind, this chapter summarizes the results of diffusion cell studies in which the transport of radiolabeled model permeants was examined in response to the application of different chemical enhancer techniques. More specifically, these studies include: (i) the dependence of the permeability on the molecular weight and hydrophobicity of the model permeants examined (see Section 7.2), (ii) the effect of oleic acid pretreatment on the permeability
enhancements (see Section 7.3), and (iii) the modification of protein physicochemical properties through the use of oppositely-charged surfactant-protein complexation, in an attempt to enhance the transdermal transport of the modified protein (see Section 7.4).

Evidence of the microscopic changes that occur in the skin structure as a result of the chemical enhancer treatment can be seen in the increased macroscopic conductivity values measured across the skin (Li et al., 1998), as well as in previous work done using more microscopic techniques such as differential scanning calorimetry (Golden et al., 1986, Golden et al., 1987, Potts and Francoeur, 1991), Fourier transform infrared spectroscopy (Naik et al., 1995, Ongpipattanakul et al., 1991), and nuclear magnetic resonance spectroscopy (Lazo et al., 1995). The increase in skin conductivity reflects the passing of more ionic species across the skin in response to the chemical enhancer action, and indicates the formation of a more permeable skin structure either through ‘pore’ formation or through uniform lipid fluidization (Tang et al., 2001).

In the case of PBS, the predominant ionic species are sodium and chloride ions (0.14M). As discussed in Chapter 3, the proposed pathway for hydrophobic molecules is lateral diffusion in the continuous phase of lipid bilayers (Johnson et al., 1997), while the ‘aqueous pore pathway’ through the same lipoidal intercellular region has been proposed for hydrophilic molecules (Li et al., 1998, Menon and Elias, 1997). The studies reported in this chapter aim to understand the role of chemical enhancers on these transport pathways. Whereas the HTPM studies presented in the previous chapters attribute oleic acid enhancer action to increased probe partitioning followed by an increased probe
concentration gradient through visualization techniques, the studies presented below rely on the responses of model radiolabeled drug permeabilities to different chemical enhancer formulations that are selected to isolate the confounded effects of the chemical enhancer action on different aspects of the transdermal system. These aspects include the interactions between: (i) the drug and the vehicle, (ii) the chemical enhancer and the SC lipid multilamellae, and (iii) the drug and the SC, in addition to the impact of the chemical enhancer action on weakening the inherent SC barrier properties.\(^5\)

7.2. Hydrophobic and Hydrophilic Model Permeant Permeabilities

7.2.1. Evaluation of Model Permeant Drug Permeabilities: Effect of the Model Permeant Physicochemical Properties on Chemical Enhancement

Table 7-1 lists the molecular weights (MW) and the octanol-water partition coefficients (\(\log K_{ow}\)) of the model permeants examined. These model permeants were selected based on their ability to span a broad range of molecular weights and octanol-water partition coefficients (a quantitative measure of the hydrophobic and the hydrophilic nature of the permeant). Note that the hydrophobic model permeants possess positive \(\log K_{ow}\) values, whereas the hydrophilic model permeants possess negative \(\log K_{ow}\) values. The hydrophobic molecules selected span the range of molecular weights from 158 Da (decyl alcohol) to 1000 Da (dolichol), whereas the hydrophilic molecules examined range from 182 Da (mannitol) to 5000 Da (inulin) in their molecular weights.

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\(^5\) A statistical analysis of the mean model drug permeabilities reported in this chapter is presented in Appendix A, where the corresponding 95% confidence intervals are tabulated.
Table 7-1: Physicochemical properties of model permeants.

<table>
<thead>
<tr>
<th>Hydrophobic Model Permeants</th>
<th>Estimated log $K_{ow}$</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decyl Alcohol</td>
<td>4.6</td>
<td>158</td>
</tr>
<tr>
<td>Estradiol</td>
<td>3.9</td>
<td>272</td>
</tr>
<tr>
<td>Duanomycin</td>
<td>0.38</td>
<td>528</td>
</tr>
<tr>
<td>Cholesteryl-Hexyl-Ether</td>
<td>1.5</td>
<td>611</td>
</tr>
<tr>
<td>L-α-Phosphatidylycholine</td>
<td>0.81</td>
<td>734</td>
</tr>
<tr>
<td>Dolicchoil</td>
<td>32</td>
<td>1000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrophilic Model Permeants</th>
<th>Estimated log $K_{ow}$</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>-3.1</td>
<td>182</td>
</tr>
<tr>
<td>PEG</td>
<td>-3.8</td>
<td>400</td>
</tr>
<tr>
<td>Ouabain</td>
<td>-1.8</td>
<td>584</td>
</tr>
<tr>
<td>PEG</td>
<td>-6.4</td>
<td>900</td>
</tr>
<tr>
<td>PEG</td>
<td>-25</td>
<td>4000</td>
</tr>
<tr>
<td>Inulin</td>
<td>-40</td>
<td>5000</td>
</tr>
</tbody>
</table>

Using the protocol described in Chapter 2, Section 2.2, the permeabilities of these model permeants were determined using full-thickness human cadaver skin, as the skin model, and three standard vehicles – PBS, PBS/ethanol (50/50 v/v), and PBS/EtOH with an additional 5% oleic acid by volume. Oleic acid was selected as the model chemical enhancer based on the extensive references characterizing its effects on altering the skin barrier (Garrison et al., 1994, Ongpipattanakul, et al., 1991, Pershing et al., 1993). Table 7-2 summarizes the permeabilities measured for each permeant-vehicle combination.
Table 7-2: Measured permeabilities of the model hydrophobic and hydrophilic permeants.

<table>
<thead>
<tr>
<th>Hydrophobic Permeant Permeabilities (10^6 cm/hr)</th>
<th>PBS</th>
<th>PBS/EtOH</th>
<th>PBS/EtOH and 5% Oleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decyl Alcohol</td>
<td>1880 ± 320</td>
<td>11.9 ± 4.81</td>
<td>10.9 ± 1.09</td>
</tr>
<tr>
<td>Estradiol</td>
<td>500 ± 158</td>
<td>35.0 ± 9.63</td>
<td>290 ± 84.0</td>
</tr>
<tr>
<td>Duanomycin</td>
<td>67.5 ± 9.76</td>
<td>494 ± 18.3</td>
<td>881 ± 384</td>
</tr>
<tr>
<td>Cholesteryl-Hexyl Decyl Ether</td>
<td>19.5 ± 2.66</td>
<td>131 ± 21.8</td>
<td>39.8 ± 5.16</td>
</tr>
<tr>
<td>L-α-Phosphatidyl-Choline</td>
<td>233 ± 210</td>
<td>198 ± 102</td>
<td>592 ± 2.79</td>
</tr>
<tr>
<td>Dolichol</td>
<td>159 ± 10.4</td>
<td>71.1 ± 15.4</td>
<td>1210 ± 249</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrophilic Permeant Permeabilities (10^6 cm/hr)</th>
<th>PBS</th>
<th>PBS/EtOH</th>
<th>PBS/EtOH and 5% Oleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>7.14 ± 7.41</td>
<td>34.6 ± 10.0</td>
<td>58.3 ± 25.7</td>
</tr>
<tr>
<td>PEG (400 Da)</td>
<td>19.4 ± 9.80</td>
<td>12.8 ± 6.46</td>
<td>258 ± 53.0</td>
</tr>
<tr>
<td>Ouabain</td>
<td>14.5 ± 1.05</td>
<td>43.1 ± 4.62</td>
<td>73.6 ± 7.83</td>
</tr>
<tr>
<td>PEG (900 Da)</td>
<td>10.8 ± 4.74</td>
<td>83.2 ± 28.9</td>
<td>183 ± 12.1</td>
</tr>
<tr>
<td>PEG (4000 Da)</td>
<td>9.94 ± 1.05</td>
<td>31.5 ± 2.97</td>
<td>94.1 ± 29.0</td>
</tr>
<tr>
<td>Inulin</td>
<td>32.2 ± 6.60</td>
<td>290 ± 48.6</td>
<td>766 ± 46.0</td>
</tr>
</tbody>
</table>

A comparison of the permeabilities attained by the hydrophobic and the hydrophilic permeants in PBS (the control vehicle) reveals that, in general, the SC is inherently more
permeable to the hydrophobic molecules. From Table 7-2, the control permeabilities for the hydrophobic probes examined range from \((1.88 \pm 0.32) \times 10^{-3}\) cm/hr for decyl alcohol, the smallest hydrophobic model permeant (158Da, \(\log K_{o/w} = 4.6\)) examined, to \((19.5 \pm 2.66) \times 10^{-6}\) cm/hr for cholesteryl hexyl ester (611 Da, \(\log K_{o/w} = 1.5\)). In general, the hydrophilic probes, however, display lower permeabilities for the control PBS case, with values ranging from \((7.14 \pm 7.41) \times 10^{-6}\) cm/hr for mannitol (182 Da, \(\log K_{o/w} = -3.1\)) to \((32.2 \pm 6.6) \times 10^{-6}\) cm/hr measured for inulin (5000 Da, \(\log K_{o/w} = -40\)). Since the primary permeant pathways for both the hydrophobic and the hydrophilic permeants have been described to lie within the SC intercellular region (see Chapter 5, Section 5.1.2), the lower permeabilities reported for the hydrophilic permeants in the control PBS case reflect the inherently low affinity of this class of permeants for an oily environment like the SC lipid multilamellae.

While the differences in the permeabilities between these two classes of molecules in the PBS control case is apparent in Table 7-2, the existence of pronounced trends within each class remains more difficult to characterize, and can be attributed to the dependence of permeability on a number of permeant physicochemical properties that include the size (or MW), hydrophobicity, functional groups, and geometry. Although dolichol is the largest hydrophobic permeant examined (1000Da), it exhibits higher permeabilities than both duanomycin (528 Da) and cholesteryl hexyl decyl ether (611 Da). This suggests that the inherently high hydrophobicity of dolichol, reflected in the estimated \(\log K_{o/w}\) value of 32, offsets the size-restricted diffusion of a large molecule such as dolichol into the tightly-packed SC lipid multilamellae. The implications of this finding on the delivery of
macromolecules will be discussed further in Section 7.4. Although specific trends were not detected for the hydrophobic permeants examined, the examination of the hydrophilic PEG molecules at three different molecular weights (400 Da, 900 Da, and 4000 Da) reveals a decrease in permeability ((19.4±9.80)x10^{-6} cm/hr, (10.8 ±4.74)x10^{-6} cm/hr, and (9.94 ±1.05)x10^{-6} cm/hr, respectively) associated with an increase in permeant size. A detailed examination of the chemically-induced enhancement of PEG transport will be discussed in detail in Sections 7.2 and 7.3, where the effects of increasing the PEG molecular weight through an increase in the number of monomer units of this polymer will be evaluated.

Figures 7-1 and 7-2 illustrate the permeability enhancements achieved with the two chemical enhancer vehicles (PBS/EtOH and PBS/EtOH with 5% oleic acid) for the hydrophobic and the hydrophilic permeants, respectively, where the permeability enhancement is the ratio of the permeability measured for an enhancer vehicle and the permeability in the control case (PBS only). The permeants shown on the x-axis of Figures 7-1 and 7-2 are arranged in the order of increasing molecular weight, with permeants at the origin possessing the lowest molecular weight (see Table 7-1). The results presented in Figures 7-1 and 7-2 will be discussed in Sections 7.2.1.1 and 7.2.1.2, respectively.

7.2.1.1. Hydrophobic Model Probe Permeability Enhancement

For the hydrophobic model permeants (see Figure 7-1), the addition of EtOH to PBS increases the permeabilities of duanomycin and cholesteryl hexyl decyl ether by 7.31 ± 1.09 and 6.70±1.45 times, respectively. The permeabilities of the other hydrophobic
molecules, however, reveal lower values compared to the passive case (PBS only), as the enhancements shown in Figure 7-1 for these permeants have values less than unity. The addition of 5% oleic acid to the PBS/EtOH increases the permeabilities for a majority of the permeants with respect to the control PBS case, with the exception of the two smallest hydrophobic model permeants, decyl alcohol (D-OH) and estradiol, which possess higher passive permeabilities for both the ethanol and the oleic acid cases examined.

![Figure 7-1: Hydrophobic model probe permeability enhancements. The white bars and the gray bars represent the permeability enhancements achieved using the vehicles, PBS/EtOH and PBS/EtOH with 5% oleic acid, respectively. The permeability enhancements for decyl alcohol (D-OH), estradiol, duanomycin, Cholesteryl-Hexadecyl Ether (CHDE), L-α-Phosphatidyl-Choline (L-a-PC), and dolichol are (6.33 ± 2.78)x10^{-3}, (7.00 ± 2.93)x10^{-2}, 7.31 ± 1.09, 6.70 ± 1.45, (8.47 ± 8.79)x10^{-1}, and (4.47 ±1.01)x10^{-1}, respectively, with the PBS/EtOH vehicle. For the PBS/EtOH with 5% oleic acid vehicle, the permeability enhancement values are (5.79 ± 1.15)x10^{-3}, 0.58 ± 0.25, 13.1 ± 5.99, 2.05 ± 0.38, 2.54 ± 2.28, and 7.61 ± 1.64, respectively. The error bars represent one standard deviation from the mean permeability enhancement."
Duanomycin and dolichol exhibited the two greatest oleic acid-induced permeability enhancements, with increases in permeability of 13.1 ± 5.99 and 7.61 ± 1.64, respectively. Note that duanomycin is the most hydrophilic (log $K_{o/w} = 0.38$, see Table 7-1) of the hydrophobic model permeants, while dolichol is the largest in molecular weight (1000Da). These two attributes, low hydrophobicity and large molecular weight, may contribute to the sensitivity that these two molecules show in response to the oleic acid-induced changes in the SC barrier function. Hence, the combined effect of both the hydrophobicity and the molecular weight of the permeant on transdermal transport becomes evident, as the high hydrophobicity of dolichol (log $K_{o/w} = 32$) and the relatively small duanomycin molecular weight (528 Da), two attributes promoting passive transdermal transport, are offset by their correspondingly large size and low hydrophobicity, respectively. For duanomycin, partitioning into the inherently oily SC becomes more favorable as a result of oleic acid-induced SC lipid multilamellar fluidization. For the large hydrophobic molecule dolichol, the diffusion constraints resulting from the inherent large size of the molecule become alleviated due to the oleic acid-induced fluidization of the SC multilamellae.

Furthermore, the observed difference in permeabilities between dolichol (1000 Da) and decyl alcohol (158 Da), would suggest that, as the size of the molecule increases, passive transport decreases and the presence of chemical enhancers takes an effect on increasing the transport of the larger hydrophobic molecule. Figure 7-1 shows an increase in the enhancement ratio for estradiol from 0.07 to 0.50 when the PBS/EtOH and 5% oleic acid
vehicle is used in place of PBS/EtOH, whereas no change in enhancement is observed for decyl alcohol. Because the larger molecules are hindered by the volume that they require to diffuse through the lipid bilayer, fluidization of the lipid bilayer increases the free volume available to the permeants and allows their entry into the skin (Mitragotri et al., 1999). The tightly-packed nature of the lipid multilamellae structure places geometric constraints on the transport properties of the molecule despite its hydrophobicity (Norlén, 2001). Therefore, a larger hydrophobic molecule would require the additional assistance of a chemical enhancer to relax the rigidity of the ordered lipid multilamellar environment, and enable the molecule to overcome the geometric limitations of the original system. Upon elimination of these geometric constraints, the molecules can freely engage in hydrophobic interactions within the lipid multilamellae. The origin of these observed effects will be addressed in more detail in Section 7.3, where the PBS/EtOH and 5% oleic acid chemical enhancer formulation is used as a pretreatment solution to decouple the chemical enhancer-induced effects of increased partitioning into the SC and increased SC lipid fluidization on permeant transport.

In contrast to these two hydrophobic molecules whose physicochemical properties present barriers to passive transdermal transport, the small hydrophobic permeants such as decyl alcohol (158 Da) and estradiol (272 Da) possess inherently high passive permeabilities. The absence of permeability enhancements reported for these two molecules as a result of EtOH or oleic acid chemical enhancer actions can be attributed to the changes in the vehicle to skin partition coefficients, that result from the introduction of additional compounds to the vehicle. The effect of EtOH and oleic acid on the vehicle
to skin partitioning will be addressed in Section 7.3.

7.2.1.2. Hydrophilic Model Probe Permeability Enhancement

![Bar chart showing permeability enhancements for various substances.]

Figure 7-2: Hydrophilic model probe permeability enhancements. The PBS/EtOH induced permeability enhancements (white bars) for mannitol, PEG-400, ouabain, PEG-900, PEG-4000, and inulin are 4.85 ± 5.22, 0.66 ± 0.47, 2.97 ± 0.39, 7.69 ± 4.3, 3.17 ± 0.45, and 9.01± 2.38, respectively. In the presence of PBS/EtOH and 5% oleic acid, the permeability enhancements (gray bars) become 8.17 ± 9.21, 13.27± 7.23, 5.08± 0.65, 17.95 ± 7.51, 9.47 ± 3.09, and 23.79 ± 5.08, respectively. The permeability enhancements are reported as mean ± standard deviation (SD), where the error bars indicate 1 SD from the mean.

For the hydrophilic model permeants, the permeability enhancements reported in Figure 7-2 indicate that oleic acid is more effective than ethanol in increasing drug permeabilities. Unlike the hydrophobic probes, all the hydrophilic permeants, with the exception of PEG-400 in the PBS/EtOH vehicle, exhibit permeability enhancements (values greater than 1) for the two chemical enhancers examined. Figure 7-2 illustrates the general trend of increases in the measured permeability enhancement with increases in the hydrophilic permeant molecular weight as a result of the oleic acid chemical
enhancer action. The deviations from this trend that are observed for permeants such as ouabain and PEG-4000, further highlight the dependence of transdermal transport on additional physicochemical properties of the model permeant, such as hydrophobicity and geometry.

Inconsistencies observed in the reported inulin data, such as the higher passive permeability compared to that of mannitol (see Table 7-2), warrants additional investigation in future transdermal transport experiments.

7.3. Chemical Enhancer Pretreatment of the SC

The effects of EtOH and of oleic acid on the permeant vehicle to SC partitioning was examined further. Based on the permeability results reported in Table 7-2, the hydrophobic molecules—estradiol, dolichol, and decyl alcohol— all displayed decreases in their permeabilities across the skin by a factor of (6.33 ± 2.78)×10^3, (7.00± 2.93)×10^2, and (8.47± 8.79)×10^1, respectively, as a result of the ethanol chemical enhancer action. These three molecules contain polar alcohol groups, which may increase favorable interactions between the vehicle and the molecules leading to a decrease probe partitioning into the SC. Passively, the three model hydrophobic molecules exhibit permeabilities of (18.8± 3.24)×10^{-4} cm/hr, (5.00 ± 1.58)×10^{-4} cm/hr , and (1.59 ± 0.10)×10^{-4} cm/hr for decyl alcohol, estradiol, and dolichol, respectively. The trend in the permeabilities for these three hydrophobic molecules reflects the dependence of permeability on molecular weight, with dolichol, the largest molecule (1000Da), exhibiting the lowest passive permeability, and decyl alcohol, the smallest molecule (158 Da), exhibiting the highest passive permeability. Estradiol, with a molecular weight (272
Da) between decyl alcohol and dolichol, exhibits a permeability value bounded by the permeabilities of these two permeants. In the presence of oleic acid, a permeability enhancement is only observed for dolichol, the largest hydrophobic molecule examined in this set. This result suggests that the fluidization of the lipid bilayers induced by oleic acid does not induce a significant decrease in the free energy of permeant partitioning into the SC (see Chapter 5, Section 5.3.2) to overcome the favorable interactions between the vehicle and the drug for the smaller hydrophobic permeants.

Pretreatment of human cadaver skin samples with solutions of PBS/EtOH and of PBS/EtOH and 5% oleic acid, followed by skin exposure to the vehicle of interest, was examined to isolate the effects of the changes in the vehicle to skin partition coefficient from the effects of the SC barrier function reduction on the measured permeability enhancements of the model hydrophobic and hydrophilic drugs examined. The preparation of the human cadaver skin samples is described in Chapter 2, Section 2.2, In evaluating the permeabilities of the model permeants as a result of the pretreatment procedure, one modification to the previously described protocol (see Chapter 2, Sections 2.2 and 2.3) was introduced, in which the skin samples were exposed to each of the two vehicles for 3 hours following the one hour of skin hydration in PBS. After the 3-hour pretreatment, the skin samples were exposed to a solution of either PBS or PBS/EtOH and the radiolabeled permeant of interest. The permeabilities resulting from the pretreatment procedure were determined using the scintillation counter method described earlier (see Chapter 2, Section 2.2). The selection of the 3-hour pretreatment duration will become apparent in Section 7.3, where the mechanism of chemical pretreatment enhancer
action is discussed.

7.3.1. Effects of Skin Pretreatment on the Transdermal Permeabilities of Mannitol, Estradiol, Inulin, and Dolichol

Mannitol, estradiol, inulin, and dolichol were selected for the pretreatment studies because these permeants spanned a broad range of molecular weights for the hydrophilic and the hydrophobic model permeants examined. Note that estradiol was selected as the low molecular weight, model hydrophobic permeant rather than decyl alcohol, which does not display an oleic acid-induced permeability enhancement, and hence, prevents the subsequent evaluation of chemical enhancer action. For mannitol and estradiol, three pretreatment conditions were examined: (i) a 3-hour pretreatment with PBS/EtOH and 5% oleic acid followed by exposure to PBS and the radiolabeled model permeant, (ii) a 3-hour pretreatment with PBS/EtOH and 5% oleic acid followed by exposure to PBS/EtOH and the radiolabeled model permeant, and (iii) a 3-hour pretreatment with PBS/EtOH followed by exposure to PBS and the radiolabeled model permeant. For inulin and dolichol, cases (i) and (ii) were examined. Figure 7-3 shows the permeability enhancements attained using the three pretreatment cases described.

The permeability enhancements attained using the three pretreatment conditions for estradiol are considerably higher than those attained in which the skin was left in continuous contact with PBS/EtOH (enhancement=$(7.00\pm2.93)\times10^{-2}$) and with PBS/EtOH and 5% oleic acid (enhancement=$0.58\pm0.25$), although the permeability enhancement values are still on the order of unity. Because of the inherently high estradiol passive permeability, the effects of pretreatment on permeability are more
difficult to detect.

Figure 7-3: Permeability enhancements of mannitol, estradiol, inulin, and dolichol, resulting from various pretreatment cases. The white and gray bars correspond to skin contact with PBS (pretreatment case (i)) and PBS/EtOH (pretreatment case (ii)) following chemical enhancer pretreatment with PBS/EtOH and 5% oleic acid, respectively. For mannitol, these enhancement values are 18 ± 20 and 46 ± 54, for case (i) and case (ii), respectively. For estradiol, the enhancements values are 1.44 ± 0.59 and 1.30 ± 1.05, respectively. The permeability enhancements for cases (i) and (ii) of inulin are 10.71 ± 2.23 and 10.9 ± 9.79, respectively, while for dolichol, these values are 6.79 ± 2.10 and 11.45 ± 5.73, respectively. The black bars correspond to pretreatment case (iii), where only mannitol and estradiol permeability enhancements (33±37 and 1.23± 0.46, respectively) were measured using a pretreatment with PBS/EtOH, followed by continuous contact with PBS. The error bars represent 1 SD from the average permeability enhancement.

The application of case (iii) to estradiol evaluates the role of ethanol in decreasing the estradiol vehicle to skin partitioning. The permeability enhancement of order unity that results from pretreating the skin with PBS/EtOH, followed by the radiolabeled estradiol in PBS only, suggests that the continuous application of the PBS/EtOH vehicle with the
model permeant hinders transport as a result of increased estradiol solubility in the vehicle. When the skin is pretreated with PBS/EtOH and 5% oleic acid, and subsequently exposed to the PBS/EtOH vehicle with estradiol, however, the transdermal transport enhancement returns to unity. Therefore, the presence of ethanol in the vehicle solution decreases the estradiol vehicle to SC partitioning in the absence of skin pretreatment with oleic acid, which decreases the free energy of partitioning, as discussed in Chapter 5, Section 5.3.2.

Figure 7-3 shows a comparable permeability enhancement for cases (i) and (ii), for which an oleic acid vehicle pretreatment for 3 hours was followed by the application of radiolabeled dolichol in PBS and in PBS/EtOH, respectively. For dolichol, the effect of the oleic acid pretreatment on fluidizing the SC lipid multilamellar barrier is vividly observed in the drastically increased permeability enhancement for case (ii) compared with the case when PBS/EtOH is continuously applied without the pretreatment of oleic acid (11.45±5.73 versus (4.47±1.01)x10^{-1}). Furthermore, case (i) (6.79±2.10) yields permeability enhancement values which are much closer to the case when oleic acid is continuously applied (7.61±1.64). This suggests that the primary effect of oleic acid is to weaken the SC barrier, and that the oleic acid interactions with the permeant in the vehicle are not crucial to permeability enhancement. Furthermore, the fluidizing action of the oleic acid pretreatment decreases the free energy of probe partitioning (see Chapter 5, Section 5.3.2) by creating a more favorable SC lipid multilamellar enviroment such that dolichol partitioning from the subsequent PBS/EtOH vehicle now results in permeability enhancements greater than unity. Hence, perturbation of the SC with oleic acid can be
achieved in 3 hours, after which a milder vehicle such as ethanol or PBS can be utilized to facilitate drug transport.

For the hydrophilic molecules, however, skin pretreatment did not lead to similar increased drug permeabilities. The continuous application of oleic acid to the skin yielded the highest inulin permeability (compare Figures 7-2 and 7-3), suggesting that a greater degree of SC disorder is required for permeants with inherently low passive permeabilities at the high molecular weight and high hydrophilicity extremes of the model permeants examined. Note that for mannitol, the mean permeability enhancement values for all three cases (18 ±20, 46±54, and 33±37 for cases (i), (ii), and (iii), respectively, see Figure 7-3) are greater than in the case when the oleic acid chemical enhancer is continuously left in contact with the skin (enhancement of 8.17 ± 9.21 in Figure 7-2). The error bars associated with these mannitol permeability enhancement values exceed 100%, and require additional validation.

7.3.2. Dependence of PEG Permeabilities on the Molecular Weight of PEG and on the PEG Vehicle

To further investigate the size dependence of hydrophilic permeant permeabilities resulting from the previously described pretreatment protocols, tritium-labeled polyethylene glycol (PEG) of molecular weights 400, 900, and 4000 were examined. In these diffusion cell experiments, five cases of chemical enhancer action were investigated (refer to Table 7-3). The first three cases involved the continuous application of the radiolabeled permeant in a vehicle of (I) PBS, (II) PBS/EtOH, and (III) PBS/EtOH and 5% oleic acid. The remaining two cases involved skin pretreatment with a solution of
PBS/EtOH and 5% oleic acid followed by either a (IV) PBS/EtOH vehicle or a (V) PBS vehicle containing the radiolabeled PEG of interest. Table 7-3 summarizes the 5 cases examined. The permeability measurements were performed using the previously described methods (see Chapter 2, Section 2.2).

<table>
<thead>
<tr>
<th>Case</th>
<th>Donor Solution</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>PBS</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>PBS/EtOH</td>
<td>None</td>
</tr>
<tr>
<td>III</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
<td>None</td>
</tr>
<tr>
<td>IV</td>
<td>PBS/EtOH</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
</tr>
<tr>
<td>V</td>
<td>PBS</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
</tr>
</tbody>
</table>

7.3.2.1. Effect of Chemical Enhancer Pretreatment on PEG Permeability as a Function of PEG Molecular Weight

Figure 7-4 illustrates the dependence of the permeability of PEG on molecular weight for each vehicle or pretreatment procedure applied. The passive permeabilities of PEG in PBS are denoted by the solid diamonds. The PEG permeabilities for PBS/EtOH (case II) and for PBS/EtOH with 5% oleic acid (case III) are denoted by the solid triangles and the open diamonds, respectively. The application of the pretreatment protocol for case IV, which involves a 3-hour contact period with PBS/EtOH and 5% oleic acid followed by contact with the radiolabeled PEG in PBS/EtOH (case(iv)), yielded permeabilities
denoted by the open triangles. The permeabilities for case V (pretreatment with PBS/EtOH and 5% oleic acid followed by the PBS vehicle) are denoted by the open diamonds in Figure 7-4.

![Graph of permeability vs MW](image)

**Figure 7-4:** Dependence of the PEG permeability on PEG molecular weight (MW). At the molecular weight indicated by the x-axis, and for the vehicles described in Table 7-3, the solid diamonds, the solid triangles, and the solid squares denote the measured PEG permeabilities for vehicles I, II, and III, respectively, while the open diamonds and the open triangles represent the measured PEG permeabilities for vehicles IV and V, respectively. The solid lines are used to guide the eye and to connect the PEG permeabilities corresponding to a given vehicle. The error bars depict 1 SD from the mean. The permeability of each permeant-vehicle pair is also reported in Table 5-4.
As expected, the passive permeabilities are the lowest among the five treatments examined for the hydrophilic PEG permeants. The largest permeabilities were attained with case IV, shown by the open triangles in Figure 7-4. The similar trends observed for cases II and IV suggests that the oleic acid pretreatment solution in case IV increases the subsequent partitioning of the PEG from the PBS/EtOH solution into the SC, while not

Table 7-4: PEG permeabilities measured for cases listed in Table 7-3 (see Figure 7-4 for a graphical representation).

<table>
<thead>
<tr>
<th>PEG MW</th>
<th>PBS (I)</th>
<th>PBS/EtOH (II)</th>
<th>PBS/EtOH and 5% Oleic Acid (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>19.4 ± 9.80</td>
<td>12.8 ± 6.46</td>
<td>258 ± 53.0</td>
</tr>
<tr>
<td>900</td>
<td>10.8 ± 4.74</td>
<td>83.1 ± 28.9</td>
<td>183 ± 12.1</td>
</tr>
<tr>
<td>4000</td>
<td>9.94 ± 1.05</td>
<td>31.5 ± 2.97</td>
<td>94.1 ± 29.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PT PBS/EtOH and 5% Oleic Acid, PBS/EtOH (IV)</th>
<th>PT PBS/EtOH and 5% Oleic Acid, PBS (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>373 ± 65.8</td>
</tr>
<tr>
<td>900</td>
<td>817 ± 448</td>
</tr>
<tr>
<td>4000</td>
<td>215 ± 77.5</td>
</tr>
</tbody>
</table>

changing the mechanism of hydrophilic permeant transport. This increase in permeant partitioning is revealed in the translation of the PEG permeabilities along the positive
direction. Whereas the closed triangles depict permeabilities that are $10^5$ cm/hr in magnitude, the open triangles reveal that pretreatment of the skin with oleic acid for 3 hours (case IV), followed by an application of the same vehicle used in case II, results in permeabilities that are now one order of magnitude larger (see Figure 7-4 and the corresponding permeabilities reported in Table 7-4). This finding agrees well with our HTPM studies, in which the proposed rate-limiting step for the hydrophilic model probe SRB was found to be probe partitioning from the vehicle into the SC (see Chapter 5, Section 5.3.5). The oleic acid pretreatment induces a decrease in the free energy of probe partitioning into the SC (See Chapter 5, Section 5.3.2), and results in enhanced permeabilities for all the three hydrophilic PEG molecules examined.

Table 7-5: PEG Permeability Enhancements Relative to Case I (see Table 7-4).

<table>
<thead>
<tr>
<th>PEG MW</th>
<th>Case II</th>
<th>Case III</th>
<th>Case IV</th>
<th>Case V</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>0.66 ± 0.47</td>
<td>13.27 ± 7.23</td>
<td>19.22 ± 10.27</td>
<td>1.73 ± 0.99</td>
</tr>
<tr>
<td>900</td>
<td>7.69 ± 4.30</td>
<td>16.95 ± 7.51</td>
<td>75.50 ± 52.96</td>
<td>1.64 ± 0.62</td>
</tr>
<tr>
<td>4000</td>
<td>3.17 ± 0.45</td>
<td>9.47 ± 3.09</td>
<td>21.64 ± 8.13</td>
<td>2.47 ± 1.86</td>
</tr>
</tbody>
</table>

A similar positive shift in the permeabilities is observed when the oleic acid pretreatment is used with the PBS vehicle. Indeed, a comparison of case I (PBS vehicle) and case V (oleic acid pretreatment followed by the PBS vehicle) denoted by the solid and the open
diamonds in Figure 7-4, respectively, reveals that the oleic acid pretreatment induced permeability increases for the three PEG molecular weights examined. Moreover, the permeability enhancements obtained for case V, maintained the trend in permeability versus the PEG molecular weight observed for the passive case (case I). The similar permeability enhancements for PEG-400, PEG-900, and PEG-4000 of 1.73±0.99, 1.64±0.62, and 2.47±1.86 for case V, respectively (see Table 7-5), further corroborates the role of the oleic acid pretreatment on increasing the hydrophilic permeant partitioning into the SC, while maintaining the transport mechanism controlling passive diffusion across the SC. This result for cases I and V is analogous to the findings discussed earlier for cases II and IV, where hydrophilic permeant partitioning was implicated as the rate-limiting step in transdermal transport.

As the permeant molecular weight increases, its permeability is expected to decrease (Potts and Guy, 1995). The trends observed for the PEG permeabilities as a function of PEG molecular weight in Figure 7-4 reveal that cases I, III, and V (group I) agree well with these expected trends (that is, that permeability should decrease with a corresponding increase in the PEG molecular weight). For cases II and IV (group II), however, the measured PEG-400 permeabilities are less than the PEG-900 permeabilities measured, which deviates from the expected relationship between permeability and molecular weight. A comparison of group I and group II suggests that the PBS/EtOH vehicles used in both case II and case IV contribute to the deviation of the PEG-400 permeabilities from the expected trend, and that the presence of ethanol in the vehicle may alter the PEG-400 vehicle to SC partition coefficient such the free energy of
partitioning is increased (see Chapter 5, Section 5.3.2). The subsequent decrease in PEG-400 partitioning from the vehicle into the SC would result from an increased PEG-400 solubility in the donor vehicle solution. The increased PEG-400 solubility in the vehicle solution is reflected in the permeabilities of \((1.28 \pm 0.646) \times 10^{-5} \text{ cm/hr}\) and \((3.73 \pm 0.658) \times 10^{-4} \text{ cm/hr}\) for cases II and IV, respectively, which are lower than the corresponding permeabilities of \((8.32 \pm 2.89) \times 10^{-5} \text{ cm/hr}\) and \((8.32 \pm 2.89) \times 10^{-5} \text{ cm/hr}\), belonging to the larger PEG-900 permeant (see Table 7-4).

The significance of PEG partitioning as the rate-limiting step in transdermal transport for hydrophilic compounds becomes increasingly evident upon considering the trends in PEG permeabilities for case III, in which the skin is continuously exposed to the oleic acid solution. In spite of the presence of ethanol in this vehicle, the SC barrier alterations that result from the continuous exposure to oleic acid induce decreases in the free energy of partitioning (see Chapter 5, Section 5.3.2) such that, for case III, the trend in permeability as a function of PEG molecular weight follows the expected relationship. Furthermore, the PEG-400 permeabilities of \((2.58 \pm 5.30) \times 10^{-4} \text{ cm/hr}\) and \((3.73 \pm 0.658) \times 10^{-5} \text{ cm/hr}\) for case III and case IV, respectively, indicate that continuous application of oleic acid to the skin and the 3-hour oleic acid pretreatment of the skin yield comparable permeabilities such that the presence of ethanol in the vehicle (case III) does not significantly contribute to changes in the PEG-400 vehicle to SC partitioning. The specific effect of ethanol on decreasing the PEG-400 partitioning into the SC becomes apparent upon examining cases III and IV. Whereas PEG-400 exhibits similar permeabilities for these two cases, PEG-900 displays significantly different values, with
the pretreatment followed by the PBS/EtOH vehicle yielding a permeability of $(8.17 \pm 4.48) \times 10^{-4}$ cm/hr (case IV) compared to $(1.83 \pm 0.121) \times 10^{-5}$ cm/hr for case III. This behavior is unexpected, because PEG-900 is more than twice the size of the PEG-400, and yet the permeability PEG-900 achieved for case IV is greater than that of the smaller molecule. This trend is also observed in case II where the PEG-900 permeability exceeded that measured for PEG-400. Considering that both case II and case IV expose PEG-400 to a PBS/EtOH vehicle, the inconsistencies observed in these results can be attributed to a reduction in the PEG-400 vehicle to probe partitioning due to the presence of ethanol in the vehicle. The increased interactions between PEG-400 and the PBS/EtOH vehicle subsequently hinder PEG-400 transdermal transport, and account for the lower than expected permeabilities observed for cases II and IV.

### 7.3.3. Elucidation of the Mechanism of Chemical Enhancer Action with Oleic Acid Skin Pretreatment

Based on the results described in Section 7.3.2 which indicated that the 3-hour PBS/EtOH and 5% oleic acid skin pretreatment, followed by the application of a model drug in a vehicle of PBS/EtOH, yields permeabilities comparable to those obtained when the skin is in continuous contact with oleic acid, the mechanism of chemical enhancer pretreatment action was investigated further. In these studies, emphasis was placed on the effect of the chemical enhancer oleic acid on the epidermal barrier. Rather than examining the permeability enhancements of model permeants, the transport of oleic acid across the epidermis was evaluated in the experiments described below.

#### 7.3.3.1. Materials and Methods

Table 7-6 lists the conditions under which the oleic acid transport measurements were
performed, using the methodology described in Chapter 2, Section 2.3.

Table 7-6: Summary of the pretreatment procedures for the $^{14}$C-radiolabeled oleic acid transport experiments.

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Pretreatment Solution (w/ $^{14}$C-Oleic Acid Label)</th>
<th>PT Time (Hours)</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
<td>3</td>
<td>PBS/EtOH</td>
</tr>
<tr>
<td>2</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
<td>3</td>
<td>14 hr PBS/EtOH, PBS</td>
</tr>
<tr>
<td>3*</td>
<td>PBS/EtOH and 5% Oleic Acid</td>
<td>3</td>
<td>None</td>
</tr>
</tbody>
</table>

*The skin samples for this set were dissolved in Soluene solvent after the PT.

Figure 7-5 illustrates the dependence of the heat-stripped skin conductivity on the time of exposure to three different pretreatment conditions. Oleic acid pretreatment durations of 3, 14, and 18 hours were examined, and the corresponding measured skin conductivities are depicted by the squares, circles, and triangles, respectively, in Figure 7-5. The skin conductivities for the three conditions examined increase over the first 20 hours, and then plateau at skin conductivities approaching $47$ (kΩ·cm$^{-2}$)$^{-1}$. The independence of the final skin conductivity on the pretreatment time indicates that pretreating the skin with PBS/EtOH and 5% oleic acid for 3 hours followed by a PBS/EtOH vehicle imparts similar perturbations to the skin barrier as that attained with the longer pretreatment times. Therefore, for the pretreatment studies, 3 hours was selected as the optimum skin pretreatment time, and is the basis for the diffusion cell experiments discussed in this section.
Figure 7-5: Skin conductivities resulting from three different oleic acid pretreatment times. The solid squares, the open circles, and the open triangles correspond denote exposure times of 3, 14, and 18 hours, respectively, followed by skin contact with the PBS/EtOH vehicle for the duration of the experiment. The various lines are shown to guide the eye. The error bars represent 1 SD from the mean.

Because ethanol increases the solubility of oleic acid in the PBS/EtOH and 5% oleic acid vehicle, its subsequent removal from the solution of PBS/EtOH in these oleic acid transport experiments (Set 2, Table 7-5) was anticipated to decrease the amount of oleic acid detected in the donor compartment.

7.3.3.2. Oleic Acid Transdermal Transport Detected in the Donor and the Receiver Diffusion Cell Compartments

The transport of radiolabeled oleic acid following the skin pretreatment process, summarized in Table 7-6 (see Chapter 2, Section 2.3 for a discussion of the experimental protocol), is illustrated in Figure 7-6. The right y-axis corresponds to the amount of $^{14}$C-
radiolabeled oleic acid (DPM) detected in the donor compartment following the removal of the radiolabeled pretreatment solution, and corresponds to the solid squares and the solid diamonds that represent the cumulative amount of oleic acid extracted from the skin for Set 1 and Set 2, respectively. The left y-axis indicates the amount of oleic acid detected in the receiver compartment, represented by the open squares and the open diamonds depicting the cumulative transport of oleic acid across the skin, into the receiver diffusion cell compartment for Set 1 and Set 2, respectively.

The transport of oleic acid across the skin for Set Nos. 1 and 2 are represented by the open squares and the open diamonds, respectively, in Figure 7-6 (see left-hand axis). The cumulative transport of oleic acid across the skin is higher for Set No. 1 (open squares), although the shapes of the oleic acid transport curves are similar, reflecting similar skin permeabilities to oleic acid, since the permeability is directly related to the slope of the cumulative transport curve (see Chapter 2, Eq.(2.1)). The differences observed between the cumulative transport for Set Nos. 1 and 2, hence, can be attributed to variations in the oleic acid concentration gradient from skin sample to skin sample that was established from the radiolabeled oleic acid pretreatment procedure. Note that the similarity in the cumulative transport into the receiver compartments for Set Nos. 1 and 2 is also seen in the overlapping error bars that represent one standard deviation from the mean (compare error bars for the open squares with those for the open diamonds).
Figure 7-6: Normalized quantity of $^{14}$C-radiolabeled oleic acid, detected in the donor and the receiver compartments. The quantity of $^{14}$C-radiolabeled oleic acid, measured in disintegrations per minute (DPM), is normalized by the quantity of $^{14}$C-radiolabeled oleic acid in the donor solution, also in DPM. Gray arrows for each curve point to the corresponding y-axis. The various lines are shown to guide the eye. The solid and the open squares correspond to the amount of $^{14}$C- radiolabeled oleic acid detected in the donor and the receiver compartments, respectively, for Set No. 1 (see Table 7-6). The solid and the open diamonds correspond to the amount of $^{14}$C- radiolabeled oleic acid detected in the donor and the receiver compartments, respectively, for Set No. 2 (see Table 7-6). The error bars indicate 1 SD from the mean.
While the skin permeabilities to oleic acid remain similar for the Set Nos. 1 and 2, the transport of the \(^{14}\)C-radiolabeled oleic acid from the skin into the donor compartment varies for Set Nos. 1 and 2 (see solid squares and solid diamonds in Figure 7-6). At 3 hours and at 17 hours, oleic acid transport back into the donor compartment remains the same for Set Nos. 1 and 2, as observed in the overlapping solid squares and solid diamonds, respectively, in Figure 7-6. This is expected because the Set Nos. 1 and 2 (see Table 7-6) are identical until 17 hours, when the PBS/EtOH vehicle in the donor compartment for Set No. 2 is replaced by PBS only. As reflected in the decreased transport of the \(^{14}\)C-radiolabeled oleic acid back into the receiver compartment after the 17-hour timepoint, the PBS hinders the extraction of oleic acid introduced into the SC from the pretreatment step (see solid diamonds in Figure 7-6). In contrast Set No. 2, the solid squares in Figure 7-6 indicate continued extraction of the \(^{14}\)C-radiolabeled oleic acid from the skin after the 17 hour timepoint when the donor PBS/EtOH vehicle is maintained in the donor compartment for Set 1. These findings indicate that in addition to the oleic acid-induced weakening of the SC barrier described in Chapter 5, the measured permeability enhancements shown in Table 7-5 also result from a PBS/EtOH vehicle-induced extraction of oleic acid from the SC. The presence of the radiolabeled oleic acid in the donor compartment correlates well with the continued application of the PBS/EtOH vehicle following the skin exposure to the oleic acid pretreatment. The evidence of oleic acid extraction from the skin supports additional studies evaluating the PBS/EtOH vehicle-induced extraction of endogenous SC lipids as a consequence of the oleic acid pretreatment induced alteration of the skin barrier. The skin conductivity
results presented in Section 7.3.3.3 will provide additional evidence for the oleic acid pretreatment induced alteration of the skin barrier properties. Future research in this direction warrants the development of chromatography analysis techniques that will enable the separation and the detection of the different lipid components present in the donor solution.

Set No. 3 provided the average amount of oleic acid retained in the skin after a pretreatment of 3 hours with PBS/Ethanol and 5% oleic acid. Assuming that the proportion of nonradiolabeled oleic acid entering the skin from the donor compartment is well represented by the proportion of $^{14}$C-radiolabeled oleic acid detected in the skin following the pretreatment, the scintillation counts of the dissolved skin samples (see Chapter 2, Section 2.3) provides an estimate indicating that 9% of the total amount of oleic acid present in the donor compartment enters the skin. Assuming that the epidermis is 200 µm thick (see Chapter 1, Section 1.2) and that the area exposed to the donor solution is 0.636 cm$^2$, the percent volume occupied by oleic acid is 50%, a majority of the total skin volume. A mass balance of Set No. 2 shows that 9% of the oleic acid enters the skin, supporting the value deduced from Set No. 3. However, a mass balance performed on Set No. 1 showed discrepancies with the data, in that the deduced percentage of oleic acid entering the skin was 20%. The calculated value of 20% suggests that the volume fraction of oleic acid entering the skin is 120% that of the original skin piece. This can only be possible if the skin expands to accommodate the oleic acid, or if a fraction of the $^{14}$C-radiolabeled oleic acid that was counted as being in the skin was actually on the skin surface. The additional work proposed to resolve this issue will be discussed in
Chapter 8.

7.3.3.3. Examination of Oleic Acid-Induced Changes in Skin Conductivities as an Indicator of the SC Barrier Alterations

An examination of the measured skin conductivities shown in Figure 7-7 reveals that the initial average skin conductivities of the samples in Set No. 1 (white bars) are slightly higher than those in Set No. 2 (black bars). This may contribute to the higher amounts of oleic acid measured in Set No. 1, because the skin conductivities reflect the “porosity” of the epidermis. Higher skin conductivities imply that the NaCl ions in the buffer solution are crossing the epidermis in greater quantities as a result of a the greater availability of a greater number of pathways or pores (Tang, et al., 2001). These pores, in turn, could also allow a greater amount of oleic acid to enter the skin, and help explain the higher fraction of oleic acid found in Set No. 1. In fact, from Figure 7-7, the average skin conductivity for Set No. 1 (white bars) is the highest of the three, suggesting that Set No.1 should also contain the largest amount of oleic acid, a result that agrees well with the findings presented in Section 7.3.3.2.

The 3-hour timepoint in Figure 7-7, corresponds to the replacement of the pretreatment solution with PBS/EtOH for both Set Nos. 1 and 2 (white and black bars, respectively). The 17-hour time-point indicates when the PBS/EtOH is removed from the donor cells of Set No. 2 (black bars) and replaced by PBS. The decrease in the skin conductivity observed at the end of the 3-hour pretreatment period was not expected, because the skin pretreatment should permeabilize the skin and thus increase the skin conductivity.
Figure 7-7: Skin conductivity at (a) 10Hz and (b) 1kHz at each time point for the experiments listed in Table 7-7. The white bars represent the skin conductivities resulting from the 3-hour oleic acid pretreatment followed by skin exposure to the PBS/EtOH vehicle (Set No. 1, Table 7-6). The black bars represent the skin conductivities resulting from the 3-hour oleic acid pretreatment, followed by the 14-hour PBS/EtOH pretreatment, and the final skin exposure to PBS vehicle (Set No. 2, Table 7-6). The error bars represent 1 SD from the mean.
This decrease in skin conductivity has also been observed by Konturri et al. (1993) when chemical enhancers were used to enhance skin permeability, and was attributed to the creation of pores which are not accessible to the large, charged ions, but are accessible to water and other molecules.

As seen in the oleic acid volume fraction estimates (see Section 7.2.2.1), the skin contains 60% oleic acid after the 3-hour pretreatment period (Set No. 1). As a result, the observed decrease in skin conductivity may result from the reduced number of ions capable of crossing the oleic acid saturated skin lipid bilayers (Kalia and Guy, 1995). The fluidization of the lipid bilayers by oleic acid may reduce the ‘aqueous’ pathways initially available to the ions by creating an oily phase which, in incorporating the oleic acid, has occupied the volume originally available to the ion channels. This initial decrease in skin conductivity is consistent with the proposed mechanism of oleic acid enhancer action, where phase separated domains of oleic acid exist in the SC (Naik, et al., 1995, Tanojo et al., 1997). Moreover, in Chapters 4 and 6, ‘pools’ of high fluorescent probe intensity were also visualized as a result of oleic acid chemical enhancer action (see Figure 4-1 in Chapter 4 and Figures 6-3 and 6-3 in Chapter 6). Note, however, that the conductivity of the skin eventually increases after the pretreatment solution is removed and replaced by either PBS or PBS/EtOH. These increases in skin conductivity could possibly result from extraction of the oleic acid from the SC resulting from the skin exposure to the PBS/EtOH solution, which would promote the transport of charged species across the skin. Furthermore, the gradual extraction of oleic acid from the skin, as seen in Figure 7-6 (solid squares), could be an indication of the role of PBS/EtOH in creating ‘aqueous’
pathways by replacing the original oleic acid paved paths.

7.3.3.4. Evaluation of Skin Conductivities Resulting from Additional Oleic Acid Pretreatment Time Durations

Figure 7-8: The measured skin conductivities for the additional skin pretreatments and vehicles examined. The open diamonds, the open squares, and the open triangles correspond to skin conductivities resulting from a continuous exposure to PBS, PBS/EtOH, and PBS/EtOH and 5% oleic acid, respectively, in (a). The solid squares and the solid circles represent measured skin conductivities resulting from a PBS/EtOH pretreatment for 3 hours and 18 hours, respectively, followed by exposure to PBS, in (b). The solid triangles show the skin conductivities for 18 hours of oleic acid pretreatment followed by PBS, in (b). The various lines are shown to guide the eye. The error bars represent 1 SD from the mean.
The skin conductivities achieved with additional chemical enhancer systems are shown in Figures 7-8(a) and 7-8(b). The open diamonds, open squares, and open triangles in Figure 7-8(a) correspond to the skin conductivities measured at the time indicated on the x-axis for vehicles of PBS, PBS/\text{EtOH}, and PBS/\text{EtOH} and 5\% oleic acid, respectively. The additional pretreatment conditions examined are shown in Figure 7-8(b) by the solid squares and the solid circles which correspond to PBS/\text{EtOH} pretreatments for 3 hours and for 18 hours, respectively, followed by the application of PBS. The solid triangle in Figure 7-8 (b) corresponds to skin conductivities resulting from an 18-hour oleic acid skin pretreatment followed by PBS. In PBS, no change in the skin conductivity is detected, as shown by the open diamonds. This is consistent with the use of PBS as a control vehicle, relative to which chemical enhancer-induced changes in skin conductivity are compared. Treatment of the skin with PBS/\text{EtOH} for 3 hours (solid squares) and 18 hours (solid circles), both followed by PBS, exhibit an increase in skin conductivity to about 20 (\text{k}\Omega\cdot\text{cm}^2)^{-1} for the duration of the pretreatment period. The replacement of these two pretreatment solutions by PBS results in skin conductivity decreases over the duration of the experiment. The final skin conductivity of the samples exposed to the 18-hour PBS/\text{EtOH} pretreatment (solid circles in Figure 7-8(b)) is higher than the skin conductivities measured for the 3-hour PBS/\text{EtOH} pretreatment (solid squares in Figure 7-8(b)). Both curves follow the same trend, with the peak in skin conductivity occurring at the 3-hour timepoint when the pretreatment solution was removed. The average skin conductivity of the samples pretreated for a longer period of time never recover their original skin conductivity. This suggests that a longer pretreatment time causes changes in the skin structure that are less reversible. For the
skin samples exposed to PBS/EtOH for the entire duration of the experiment (open squares in Figure 7-8(a)), the fairly constant skin conductivity of \(17 \text{ (k}\Omega\text{-cm}^2)^{-1}\) established after 3 hours of skin exposure to PBS/EtOH suggests the existence of a pretreatment time threshold, after which the skin structure is no longer altered. This threshold in the pretreatment time, after which the effect of skin exposure to a chemical enhancer formulation is not important, can be further verified by performing the skin conductivity measurements for multiple sets of diffusion cell experiments, in which shorter pretreatment time intervals are examined.

7.3.3.5. Additional Mechanistic Insight Provided by the Skin Conductivity Studies

From Figure 7-5, skin exposure to the PBS/EtOH vehicle following the oleic acid pretreatment yielded the highest skin conductivity values. This suggests that the absence of oleic acid in the donor vehicle following SC barrier disruption by the oleic acid pretreatment enables increased transport of the NaCl ions reflecting the skin conductivity measurements, and hence, increased transport of hydrophilic permeants such as PEG, mannitol, and inulin. This finding is consistent with the correlation described between the skin conductivity and the transdermal drug permeability for hydrophilic model drugs (Inamori et al., 1994, Peck et al., 1994, Tang, et al., 2001), as well as with Figure 7-6, in which the addition of the PBS/EtOH vehicle to the oleic acid pretreated skin sample led to increased detection of \(^{14}\text{C}-\text{radiolabeled oleic acid in the donor compartment. This suggests that the mechanism of the pretreatment action is to first increase drug partitioning by disrupting the SC barrier, and then to increase drug diffusion by removing the oleic acid from the weakened SC barrier upon the introduction of the PBS/EtOH}
vehicle.

While PBS/Ethanol as a pretreatment solution provides evidence of barrier recovery depending on the time of pretreatment, its use following an oleic acid pretreatment solution suggests that it further contributes to a weakening of the skin barrier. The only samples showing full recovery of skin barrier properties are those pretreated with PBS/ethanol for 3 hours followed by PBS. Partial recovery is seen in skin samples pretreated with PBS/ethanol for 14 hours and with PBS/ethanol and oleic acid for 14 hours, both followed by contact with a replacement solution of PBS. The lack of recovery observed for skin samples pretreated with the oleic acid solution and subsequently exposed to PBS/ethanol suggests that the ethanol prevents skin barrier recovery since partial skin barrier recovery is observed when PBS is the replacement solution. Ethanol then acts synergistically with oleic acid in disturbing the lipid barrier in addition to further altering the skin barrier structure after application of the oleic acid pretreatment solution.

Based upon the oleic acid transport measurements shown in Figures 7-6 and the variations in the skin conductivities results from the different skin pretreatment protocols, shown in Figure 7-8, the time of pretreatment and the order in which the donor solutions are applied have a significant impact on the skin barrier properties. After a threshold time of pretreatment, the conductivity of the skin no longer changes. This threshold time may correspond to the chemical enhancer-induced irreversible alteration of the SC barrier properties, that may be specific to the in vitro system examined in this chapter. Because
the experiments are performed in vitro, a fair representation of the skin barrier recovery in response to the application of the chemical enhancers is not observed, but the extent to which a chemical enhancer solution disturbs the skin is revealed. The conductivity data show that a minimum of 3-hour pretreatment time with the PBS/ethanol and 5% oleic acid solution followed by a vehicle solution of PBS/ethanol provides the highest increases in skin conductivity.

7.3.3.6. Application of Skin Conductivity Measurements to Evaluate Real-Time Skin Barrier Function: Circumvention of Transdermal Transport Lag-Time Effects

The chemical enhancer-induced transdermal transport measurements of model permeant permeabilities presented in this chapter have been substantiated further with skin conductivity measurements that indicate the extent of skin barrier perturbation resulting from the chemical enhancer action. Increases in skin conductivity reflect the increases in ion transport across the skin, which in turn serves as an indicator of the chemical enhancer-induced alterations of the skin barrier integrity. The increased ion transport across the skin that is captured by the skin conductivity measurements can be attributed to changes which occur in the skin structure, such as, the formation of aqueous channels or an increase in free volume within the lipid multilamellae resulting from the chemical enhancer action.

Comparing the skin conductivity measurements in Figure 7-7 with the corresponding oleic acid transport data in Figure 7-6, the increase in transport in the receiver compartments from 36 to 64 hours corresponds to the increase in conductivity for Set No. 1 and Set No. 2 shown in Figure 7-7 from 3 to 17 hours. The time difference between the
observed skin conductivity increases, where oleic acid transport increases, can be attributed to the instantaneous nature of the skin conductivity measurement, where changes in skin conductivity reflect the real-time changes in the skin barrier. This time difference corresponds to the transport lag time, during which transport processes approach steady state. Based on Figure 7-6, the lag time corresponds to the period spanning the first 36 hours (see open squares and open diamonds corresponding to the oleic acid transport), before changes in the slope of the cumulative transport curve approach a constant value. This period of 36 hours is consistent with the 40-hour time difference between the time-periods over which the greatest increases in the skin conductivity and the oleic acid transport were detected. The use of skin conductivity measurements to monitor the real-time changes in skin barrier properties resulting from the chemical enhancer action deserves further consideration in supplementing the future work proposed in Chapter 8, Section 8.2.2, where the evaluation of transient transdermal transport is proposed.

7.4. Preliminary Examination of Chemically-Induced Transdermal Protein Transport

7.4.1. Background

The pharmaceutical relevance of proteins makes their transdermal delivery highly desirable. The low permeability of these large hydrophilic molecules across the skin, however, has been overcome by mechanical perturbation of the SC barrier using methods which include sonophorosesis, iontophoresis, and electroporation (Chen et al., 1998, Mitragotri et al., 1995, Prausnitz et al., 1993). While the passive transport of polypeptides and proteins is nowhere near the rate required for therapeutic drug delivery,
the studies presented next represent a preliminary investigation of the feasibility of chemically-induced transdermal protein delivery which exploit protein-surfactant interactions.

Chemical enhancement of transdermal protein delivery is examined below by: 1) SC barrier perturbation, and 2) protein-surfactant complex formation. The pretreatment method of applying PBS/EtOH and 5% oleic acid for 3 hours followed by PBS/EtOH with the permeant for the remainder of the experiment (presented in Section 7.2) was employed to determine the effect of the skin permeabilization by chemical enhancers on protein transport. To examine the drug complexation mechanism, two anionic surfactants were examined (aerosol-OT (AOT) and sodium dodecyl sulfate (SDS)) with oxytocin selected as the model protein.

7.4.2. Procedure

7.4.2.1. Chemical Enhancer Pretreatment

Heat-stripped human cadaver skin was prepared following the method described in Chapter 2, Section 2.2, and the diffusion cell experiments performed using the pretreatment procedure described in Chapter 2, Section 2.3.

7.4.2.2. Protein-Surfactant Complexation

The interaction of a protein with an oppositely-charged surfactant to produce a hydrophobic complex has been investigated, in an effort to increase protein partitioning from an aqueous phase into an organic phase (Bromberg and Klibinov, 1994). Through such protein-surfactant pairings, the transdermal transport of proteins will be evaluated
using a model protein, oxytocin (1000 Da), and two different negatively charged surfactants: Aerosol-OT (AOT) and sodium-dodecyl sulfate (SDS), following the methodology introduced in Chapter 2, Section 2.5. At a pH of 4.6 the oxytocin takes on an overall negative charge as a result of changes in the protein conformation to allow its pairing with the negatively-charged surfactants selected. The examination of this approach was motivated by the potential for increasing protein hydrophobicity as a result of the complexation, which in turn may promote protein partitioning into the inherently hydrophobic SC. Evidence of high permeabilities achieved by the large hydrophobic model permeant dolichol (see section 7.2.1) further motivated the exploitation of permeant hydrophobicity in increasing transdermal transport of macromolecules.

Table 7-7 describes the conditions investigated in these experiments. AOT is a negatively-charged, double-tailed surfactant (also referred to as dioctyl sulfosuccinate sodium). The cost of oxytocin remains the limiting factor in the number of experiments performed and the concentrations chosen for the protein and the surfactant. The effects of using higher concentrations of AOT above the critical micelle concentration are not addressed here.

7.4.3. Results

Figures 7-9 and 7-10 illustrate the permeabilities and the permeability enhancements attained, respectively, for each experiment set listed in Table 7-7. Set Nos. 1, 2, and 3 yield permeability enhancements of 0.1, 0.07, and 0.3, respectively. The absence of increased oxytocin transport resulting from these three experiment sets (see Table 7-7), indicate that the use of citrate buffer (CB) alone, in conjunction with AOT, and with
Table 7-7: Summary of the \(^3\)H-radiolabeled oxytocin experiments.

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Pretreatment</th>
<th>Buffer</th>
<th>Protein</th>
<th>Surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>CB</td>
<td>Oxytocin</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>CB</td>
<td>Oxytocin</td>
<td>AOT</td>
</tr>
<tr>
<td></td>
<td>PBS/EtOH and 5% OA (5hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PBS/EtOH (12 hr)</td>
<td>CB</td>
<td>Oxytocin</td>
<td>AOT</td>
</tr>
<tr>
<td></td>
<td>PBS/EtOH and 5% OA (5hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PBS/EtOH (12 hr)</td>
<td>CB</td>
<td>Oxytocin</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>PBS/EtOH and 5% OA (5hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PBS/EtOH (12 hr)</td>
<td>PBS</td>
<td>Oxytocin</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>PBS</td>
<td>Oxytocin</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>CB</td>
<td>Oxytocin</td>
<td>SDS</td>
</tr>
</tbody>
</table>

AOT following the oleic acid pretreatment, are not sufficient means of inducing increased protein transport relative to the passive case, in which PBS is the vehicle used (Set No. 6).

Set Nos. 4, 5, and 7, however, all displayed enhanced oxytocin transdermal transport as reflected in the permeability enhancement values of 3.20, 2.49, and 2.01, respectively. For Sets Nos. 4 and 5, the permeability enhancements resulted from skin pretreatment with oleic acid followed by the citrate buffer (CB) and the PBS vehicles, respectively. As
Figure 7-9: Oxytocin permeabilities corresponding to Set Nos. 1 through 7 listed in Table 7-7.

observed for the hydrophilic model permeants in Section 7.2, the pretreatment procedure increases skin conductivity, a quantitative indicator of increased skin porosity. Skin pretreatment, for the case of oxytocin, also alters the SC barrier properties such that hydrophilic permeant partitioning into the skin is increased. Although Set No. 2 indicates that the 1:1 complexation of oxytocin with AOT does not induce permeability enhancement, a comparison of Set No. 3, in which AOT was applied to oxytocin, and Set No. 4, in which only oxytocin was present, suggests that the application of the AOT in CB following skin pretreatment actually hinders transport. This may result from the sterically unfavorable double-tail geometry of AOT, which, when paired with oxytocin, prevents their partitioning into the SC.

In contrast to the absence of an enhancement effect observed with the surfactant AOT, the 2-fold increase in oxytocin permeability exhibited by Set No. 7, in which a 1:1 ratio
of oxytocin: SDS was present in CB, indicates the potential of using protein-surfactant pairs to increase protein transdermal transport. While SDS is also a well-known chemical enhancer, the negligible changes measured in pig skin conductivities for samples exposed to SDS in citrate buffer (not shown) indicated that the skin barrier properties remained intact at the concentrations used (0.3 mM).

![Permeability Enhancement Chart](image)

**Figure 7-10: Permeability enhancements of oxytocin for each chemical treatment procedure listed in Table 7-7, calculated relative to Set No. 6 (oxytocin and PBS only).**

The presence of skin barrier functionality revealed by the conductivity measurements suggest that SDS may facilitate oxytocin transport by complexation, rather than by directly disrupting the skin barrier. In light of these enhancement results, however, the permeability enhancements attained using the pretreatment protocols listed in Table 7-7 for Set Nos. 4 and 5 yielded the largest oxytocin permeability enhancements (3.2±0.75 and 2.49±0.966, respectively). The two to three-fold permeability enhancements achieved for oxytocin suggests that chemical enhancer formulations alone will not
provide sufficient increases in protein transdermal transport, although the application of the chemical enhancer combinations examined in these studies should be considered in conjunction with other transdermal enhancement techniques that induce greater perturbations to the skin barrier, including ultrasound, electroporation, and iontophoresis.

7.5. Conclusions

A variety of chemical enhancement conditions were investigated to understand the mechanisms of chemical enhancer action, as well as to explore opportunities for enhancing the transdermal transport of permeants having inherently low passive permeabilities, such as, macromolecules and hydrophilic molecules. The investigation of the basic vehicles –PBS, PBS/EtOH, and PBS/EtOH and 5% oleic acid, – yielded expected trends in permeability enhancements for the hydrophobic and the hydrophilic model permeants examined, with oleic acid inducing greater permeability enhancements for each model permeant examined relative to ethanol. To further understand the lack of permeability enhancements attained with PBS/EtOH relative to PBS for the model permeants decyl-alcohol, estradiol, dolichol, and PEG-400, the oleic acid skin pretreatment procedure was introduced to isolate the effects of oleic acid on the SC barrier alteration and on the vehicle to SC partitioning.

Skin conductivity measurements indicated that the oleic acid pretreatment followed by the application of the PBS/EtOH vehicle yields the largest increases in skin conductivity relative to the other chemical enhancers examined. These skin conductivity results, along with the $^{14}$C-radiolabeled oleic acid skin pretreatment experiments suggesting that PBS/EtOH extracts oleic acid from the SC, are consistent with the chemical enhancer
induced increases in SC ‘porosity’ that contribute to the measured permeability enhancements.

The changes in the SC barrier properties resulting from the oleic acid skin pretreatment followed by skin exposure to the model permeant in a PBS/EtOH vehicle, reveal different mechanisms of action for the hydrophobic and the hydrophilic permeants. For the hydrophobic permeants, the chemical enhancer pretreatment induced permeability enhancement is attributed to decreases in the free energy of partitioning (see Chapter 5, Section 5.3.2) as a result of the SC lipid multilamellae fluidization. In light of the skin conductivity increases resulting from the skin pretreatment, permeability increases for the hydrophobic permeants estradiol and dolichol are not as significant as those revealed for the hydrophilic permeants, mannitol, PEG, and inulin. This suggests that interactions between the hydrophobic permeant and the lipoidal pathway remain a significant barrier, despite the overall increase in SC ‘porosity’. This result is consistent with our previous finding that lateral diffusion is the rate-limiting step in transport, determined from an analysis of HTPM imaging of the model probe RBHE presented in Chapter 5.

The largest increases in the hydrophilic model permeant permeabilities resulting from the oleic acid pretreatment are attributed to both the increased ‘porosity’ of the SC, as well as to the decrease in the free energy of probe partitioning (see Chapter 5, Section 5.3.2). The chemical enhancer-induced increases in skin conductivity reflect an increase in the aqueous pore pathways that dictate the transdermal transport of the hydrophilic permeants. The increase in the accessibility of the SC to the hydrophilic permeants is
observed in the overall positive translation of permeabilities due to the oleic acid pretreatment relative to the trend attained without the oleic acid pretreatment. This positive translation in the PEG permeabilities suggests that increased permeant partitioning accounts for the subsequent enhanced diffusion through the aqueous pore pathways. The oleic acid pretreatment also induced permeability enhancements for the model protein oxytocin, a hydrophilic permeant, in preliminary investigations of chemical enhancer-induced protein transdermal transport. For the hydrophilic model permeants, probe partitioning is the rate-limiting step. This finding remains consistent with the results obtained through HTPM analysis of the model hydrophilic probe SRB (see Chapter 5, Section 5.3.5).

Elucidation of the mechanisms underlying the permeability enhancements achieved with each chemical enhancement technique discussed in this chapter warrants further investigation using the HTPM analyses introduced in the previous chapters. Furthermore, the increased skin sample analysis times associated with HTPM can now be exploited to further understand the mechanisms of a myriad additional chemical enhancer formulations. With this in mind, future HTPM experiments facilitating the elucidation of chemical enhancer action mechanisms are proposed in the next chapter.
7.6. References


Chapter 8

Conclusions and Future Work

8.1. Summary of Thesis

The inherent impermeability of the skin barrier to a broad range of pharmaceutically relevant compounds has fostered interest in the development of effective chemical enhancer formulations that reversibly alter the skin barrier properties to enable transdermal drug administration. The work described in the previous chapters aimed to elucidate the mechanisms of chemical enhancer action using the well-known chemical enhancer, oleic acid. The introduction and development of the Two-Photon Microscopy (TPM) methodology in Chapter 3 enabled the visualization and quantification of the oleic acid-induced microscale changes in transdermal transport properties. The transport properties examined included the vehicle to skin partition coefficient, the skin concentration gradient, the skin diffusion coefficient, and the skin barrier diffusion path length. The evaluation of the relative changes in these transdermal transport properties, using a model hydrophobic probe (RBHE) and a model hydrophilic probe (SRB), indicated that increased probe partitioning was the primary action induced by oleic acid.
Furthermore, the quantitation of these changes over the 4 to 6 skin sites examined drew attention to the inherent skin morphological heterogeneity, and motivated the need to increase the number of skin sites sampled to ascertain the valid number of skin sites that are representative of the global changes in transdermal transport.

With this in mind, Chapter 4 addressed the question of how sample size contributes to the measured changes in tranadermal transport properties. Coupled with the recent modifications to the TPM microscope implemented by Professor Peter So and his group in the Department of Mechanical Engineering at MIT, the increased sampling efficiency associated with the High-Speed Two-Photon Microscope allowed for the sampling of macroscale skin areas. In Chapter 4, 400 consecutive skin sites were examined using the same model probes and chemical enhancer systems that were evaluated in Chapter 3. The 400 individual skin sites spanned a total skin area of 2 mm by 2 mm, which is a larger, and more pharmaceutically relevant skin area. From these studies, the variations in probe spatial distribution over a wide area were visualized, and the optimum number of skin sites that need to be sampled were determined. For the hydrophobic probe, a limited sampling of 6 skin sites sufficiently reflected the global changes in transdermal transport, whereas for the hydrophilic probe, a sampling size between 12-24 skin sites was suggested. In addition to the establishment of these skin sampling criteria, the oleic acid-induced variations in probe spatial distributions, as reflected in two pertinent transdermal transport parameters – the probe vehicle to skin partition coefficient and the probe concentration gradient – were evaluated to gain insight on the rate-limiting steps in transdermal transport for the hydrophobic and the hydrophilic probes examined.
Chapter 5 described the statistical analyses performed for the two relevant transdermal transport parameters – the vehicle to skin partition coefficient and the probe concentration gradient. The insensitivity of the changes in the statistical distribution of the probe intensity gradient over the 400 skin sites evaluated in response to the oleic acid-induced changes in probe partitioning for the hydrophobic probe suggested that lateral diffusion is the rate-limiting step in transdermal transport. On the other hand, for the hydrophilic probe, the correlation observed between the oleic acid-induced changes in the 400 skin site distribution of the vehicle to skin partition coefficients and of the probe intensity gradient suggested that probe partitioning into the skin is the rate-limiting step. The determination of these two different rate-limiting steps in transdermal transport for the hydrophobic and the hydrophilic probes, again points to the existence of the two proposed permeant pathways described by the lipoidal pathway for the hydrophobic permeants and by the aqueous pore pathway for the hydrophilic permeants.

In Chapter 6, the variations in the wide-area probe spatial distributions, visualized using High-Speed Two-Photon Microscopy (HTPM), were further characterized to elucidate the mechanisms of oleic acid-induced changes with respect to the SC structural features. Using dual-channel HTPM, the skin autofluorescence signal was isolated from the rhodamine-based model probe fluorescence signal. In this novel application, introduced in this thesis for the first time, the skin autofluorescence served as a template which delineates the boundaries of the corneocyte-lipid multilamellae interfaces. Relative to the correlation length obtained from this template, the corresponding image of the fluorescent
probe spatial distribution revealed a greater average correlation length for the four samples examined – RBHE-control, RBHE-enhancer, SRB-control, and SRB-enhancer--, suggesting that probe diffusion into the corneocytes may exist as a secondary transdermal transport pathway. The effects of enhanced corneocyte diffusion on transdermal transport remains to be elucidated. For now, the increased probe partitioning into the corneocytes may possibly contribute to decreasing the lag-time measured prior to the establishment of steady state diffusion. Based on this hypothesis, the corneocytes act as diffusion sinks within the SC, and hence, increasing the rate of probe diffusion into the corneocytes decreases the time required for the intercellular hydrophilic probe concentration to equilibriate with the probe concentration in the corneocytes, which, in turn, decreases the time to establish steady state. The absence of the sink conditions presented by the corneocytes within the SC, at steady state, eliminates the driving force promoting probe diffusion within the SC, and establishes the SC probe concentration gradient as the only driving force contributing to transdermal transport. This proposed mechanism of action also supports the localization of probe diffusion to the intercellular region as a method of reducing transdermal transport lag-times, such that probe diffusion into the corneocyte transport sinks is avoided entirely.

A more detailed analysis based on the decomposition of representative image sections of 150 pixels by 150 pixels revealed the oleic acid-induced increases in intracorneocyte penetration for the hydrophilic probe, whereas for the hydrophobic probe, localization of the probe to the lipid intercellular region was observed. The intracorneocyte diffusion visualized for the hydrophilic probe indicates that the hydrophilic probe transport is not
purely constrained to the tortuous multilamellar pathway specific to lateral diffusion. The oleic acid-induced increase in partitioning of the SRB hydrophilic probe into the SC intercellular region contributes to the increased driving force for SRB diffusion from the intercellular region into the corneocytes. Hence, the hydrophilic probe diffusion is no longer solely confined to the intercellular region, as seen in the increased corneocyte probe intensities in Chapter 6, Figure 6-6(f). The increased SRB diffusion into the corneocytes signals the relaxation of transport constraints to the pathway defined by lateral diffusion. These observations remain consistent with the finding that hydrophilic probe partitioning into the SC is the rate-limiting step.

The visualization of the RBHE hydrophobic probe spatial distributions, in combination with the image analyses performed, suggest that in addition to localization within the intercellular region, domains of high RBHE intensity also result from the oleic acid chemical enhancer action. Moreover, these domains contribute to the large value of the average correlation length calculated for RBHE in the presence of the oleic acid chemical enhancer. From Chapter 5, RBHE lateral diffusion through the intercellular space was found to be the rate-limiting step in transdermal transport, a finding that is consistent with the visualization and quantification of the oleic acid-induced RBHE probe localization to the lipid multilamellar region. Moreover, the localization of the hydrophobic probe to the intercellular space also contributes to decreasing the transdermal transport lag-time by circumventing probe diffusion into the corneocytes and, hence, eliminating diffusion sinks that would contribute to slowing down the transport process. The increased probe
localization to the intercellular region may result from an increase in the probe intercellular region solubility that decreases its lipid-corneocyte partition coefficient.

The findings that arise from the dual-channel HTPM analyses of transdermal fluorescent probe distributions suggest that the corneocytes behave as diffusion sinks that contribute to the measured permeant transdermal transport lag-times. The hydrophilic nature of the corneocytes, in part, influences the mechanism of oleic acid enhancer action with respect to the physicochemical properties of the permeant examined. While the underlying action of oleic acid is to increase transdermal transport by reducing intracorneocyte diffusion, the mechanisms are visually manifested in different ways. Oleic acid eliminates the diffusion sink action of the corneocytes by essentially ‘filling’ up the sink with the SRB hydrophilic probe, as observed in the oleic acid-induced increased intracorneocyte hydrophilic probe penetration (see Chapter 6, Figure 6-6). For the RBHE hydrophobic probe, these diffusion sinks are avoided altogether, as observed in the oleic acid-induced increased RBHE localization to the intercellular region (see Chapter 6, Figure 6-5). The potential applications of dual-channel HTPM to examine transdermal probe spatial distributions with respect to the inherent skin autofluorescence remain to be explored and will be discussed in Section 8.2.1.

In Chapters 3, 4, 5, and 6, the application of two-photon microscopy to examine transdermal transport has enabled the visualization of changes in the SC probe spatial distributions that result from the chemical enhancer (oleic acid) action. More importantly, the quantitative analyses presented in these chapters illustrate that additional insightful
information pertaining to the mechanisms of chemical enhancer action can be further extracted from the visualization of transdermal transport.

The increased throughput provided by HTPM enables the examination of an array of chemical enhancer formulations in shorter time periods. Chapter 7 summarizes the transdermal transport experiments using radiolabeled model drugs in side-by-side diffusion cells that were performed to evaluate the permeability enhancing effects of additional chemical enhancer formulations. Evidence based on the transdermal transport of additional hydrophilic as well as hydrophobic compounds resulting from the chemical enhancer pretreatment procedure has further substantiated the rate-limiting transdermal transport steps described in Chapter 5. Furthermore, the preliminary experiments investigating the use of oppositely-charged protein-surfactant pairs did not indicate the feasibility of enhancing transdermal protein delivery to pharmaceutically relevant permeability values solely from the application of chemical enhancer formulations. The evaluation of the changes in probe spatial distributions induced by the application of these and additional chemical enhancer formulations will provide further insight into the mechanisms of chemical enhancer action and warrants further consideration, especially in light of the high-throughput capability of HTPM. The proposed work for future development of this research is described next.
8.2. Future Experiments

8.2.1. Dual-Channel HTPM

The application of dual-channel HTPM to separate fluorescence emissions of different wavelengths can also be applied to simultaneously evaluate the spatial distributions of two probes having different physicochemical properties and fluorescence emission ranges. For example, a model hydrophobic probe, with a fluorescence emission in the red range, and a model hydrophilic probe, with a fluorescence emission in the green range, could be simultaneously exposed to skin to study their transdermal transport. Due to the different emission spectra associated with each probe, a direct comparison of the probe spatial distributions resulting from the probe physicochemical properties can be made. In addition to hydrophobicity, the effects of molecular weight can be examined by the selection of the appropriate probes in a similar manner, based on their emission spectra. Finally, these experiments could be repeated with a variety of chemical enhancers to elucidate the chemical enhancer-induced changes with respect to the transport pathways delineated by each fluorescent probe selected.

In addition to these proposed experiments, the feasibility of utilizing dual-channel HTPM to simultaneously evaluate the chemical enhancer-induced changes in transdermal transport properties for multiple fluorescent probes possessing different emission spectra, *within the same skin sample*, following the analyses presented in Chapters 3, 4, and 5, warrants additional investigation. The simultaneous examination of the model fluorescent probes in the same skin sample would eliminate variability due to intersample
differences, as well as reduce the time and material demands associated with preparing multiple skin samples.

8.2.2. Evaluation of Transient Transdermal Transport

The study of the mechanisms of chemical enhancer action presented in this thesis have been based on the steady-state, one-dimensional diffusion model. The increased throughput imaging capabilities of HTPM (video-rate) (Kim et al., 1999) offers the potential for examining probe spatial distributions over the same skin area as a function of time, over shorter time intervals. These time intervals, of course, would be limited by the HTPM instrumentation, where the current scan rate is approximately 15 seconds to acquire an image stack consisting of 100 consecutive z-positions. To visualize probe diffusion at one skin site, however, an imaging chamber representing the in vitro conditions of the side-by-side diffusion cells would need to be constructed such that the skin sample remains situated on the microscope stage. Aside from the geometric limitations placed on the size and shape of the imaging chamber, the design and successful implementation of the device is anticipated to require 3-4 months. The machining work would require the assistance of a machine shop.
The changes in the probe intensity profiles over time could be fit to the transient diffusion equation, shown below in Eq.(8.1), to evaluate the effects of chemical enhancers on transport parameters like the probe skin diffusion coefficient and the lag-time:

\[
\frac{C(x)}{KC_{veh}} = \left(1 - \frac{x}{L}\right) - \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{\sin \left(\frac{n\pi x}{L}\right)}{n} \cdot \exp \left[\frac{-Dn^2\pi^2 t}{L^2}\right]
\]  

(8.1)

where \(C(x)\) is the permeant concentration in the skin, \(x\) is the distance of drug diffusion into the skin (\(x=0\) corresponds to the skin surface), \(K\) is the probe partition coefficient from the vehicle into the SC, \(C_{veh}\) is the concentration of the probe in the donor vehicle, \(L\) is the diffusion pathlength of the permeant across the SC, \(D\) is the permeant diffusion coefficient, and \(t\) is the time. Note that \(KC_{veh} = C(x=0)\), and therefore, from the fluorescent probe intensity profile, \(I(x)\), the nondimensional value of \(C(x)/C(x=0)\) can be related to \(I(x)/I(x=0)\). For a given imaging timepoint, \(t\), and the measured normalized probe intensity profile ratio, \(I(x)/I(x=0)\), the time-dependent permeant diffusion coefficient, \(D(t)\), can be determined from a curve fit to Eq.(8.1), using the known value of the parameter, \(L\). Recall that \(L\) is the skin barrier diffusion thickness, and can be approximated by the known thickness of the SC (~15 \(\mu m\)), described in Chapter 1, Section 1.2. The application of Eq.(8.1) assumes an infinite probe concentration source at the skin surface (\(x=0\)), and that the probe concentration at \(x=L\), the thickness of the skin barrier, is close to negligible. Under the diffusion cell conditions implemented in standard transport studies, these assumptions hold, as the probe concentration in the donor compartment (the compartment exposed to the SC surface) is always orders of
magnitude greater than the probe concentration in the receiver compartment, due to the low skin permeability, to permeant transport. In fact, the calculation of the skin permeability, described in Chapter 2, Section 2.2.2.2, assumes that $C_D$, the donor compartment concentration, remains constant, and that $C_R$, the receiver compartment concentration, is well approximated by zero. In addition to those boundary conditions, the probe concentration in the skin at $t=0$ is assumed to be zero, which is consistent with the skin exposure to the probe commonly at $t=0$. Due to the heterogeneity of the skin structure, the value of $D(t)$ has not yet been measured noninvasively, although Pirot et al. have constructed concentration profiles of the permeant 4-hydroxybenzonitrile from sequential tape-stripped layers of SC exposed to the permeant (Pirot et al., 1997).

8.2.3. The Effects of Intracorneocyte Permeant Diffusion on Transdermal Transport

To further investigate the role of intracorneocyte diffusion in transdermal transport, the effect of surfactants, such as octyl glucoside, that are known to disrupt the corneocyte-lipid envelope can be compared with those of milder chemical enhancers that do not alter the corneocyte structure. These experiments could be performed in a manner similar to the methodology described in Chapter 3, where the model fluorescent probe spatial distributions in the skin, with and without the chemical enhancer treatment, are evaluated using the quantitative analyses presented in Chapters 5 and 6. For the hydrophilic probes, the increased partitioning that contributes to the increased probe concentration gradient from the intercellular region into the corneocyte region has been described to drive hydrophilic probe diffusion into the corneocytes (see Chapter 6, Section 6.3.4). Increased intracorneocyte probe penetration is expected for the hydrophilic probes due to the
hydrophilic nature of the corneocyte region. The inherent hydrophilic nature of the corneocytes would, hence, result in a larger probe lipid to corneocyte partition coefficient value for the hydrophilic fluorescent probe compared to the hydrophobic fluorescent probe.

The hydrophobic model probe, however, may not prefer to partition into the corneocytes even after corneocyte-lipid envelope disruption induced by the surfactant. For oleic acid, a fatty acid proposed to induce phase separation in the intercellular lipids, localization of the hydrophobic probe in the intercellular space may result from the increased probe solubility in the intercellular region resulting from the oleic acid-induced fluidization of the lipid bilayers. The skin exposure to RBHE and oleic acid, following treatment with octyl glucoside, would further elucidate the effects of oleic acid on restricting intracorneocyte diffusion due to increasing the hydrophobic probe solubility in the intercellular region. Moreover, through the examination of chemical enhancers with well-known effects on disrupting the corneocyte structure, such as, chloroform, octyl glucoside, and sodium dodecyl sulfate (Denda et al., 1994, Faucher and Goddard, 1978, Lopez et al., 2000, Lopez et al., 2000), the significance of the probe lipid to corneocyte partition coefficient, as well as of the probe intracorneocyte concentration gradient, with respect to transdermal transport enhancement can be understood.

Finally, the effects of hydrophilic probe localization to the intercellular region on hydrophilic probe transdermal transport enhancement may be investigated through the HTPM screening of potential chemical enhancer formulations. Drawing from the findings
for the hydrophobic permeants, the oleic acid-induced permeability enhancement is attributed to increased probe localization to the intercellular space relative to the control sample, which displayed probe diffusion beyond the intercellular space defined by the skin autofluorescence. The effects of inhibiting probe penetration into the corneocyte region for a hydrophilic permeant may similarly promote increased transdermal transport, by eliminating the role of the corneocytes as a probe diffusion sink, and hence, promoting hydrophilic probe transport directly across the SC through the chemical enhancer fluidized lipid multilamellar region. With this proposed transdermal transport enhancement mechanism in mind, considerable effort would be required to develop a chemical enhancer formulation inducing the desired probe spatial distribution characteristics, where these development efforts are now facilitated by the high-throughput capability of the HTPM. Through the analyses of the probe spatial distributions resulting from the chemical enhancer formulations evaluated, the impact of corneocyte diffusion on facilitating or hindering transdermal transport can be elucidated.

8.2.4. Evaluation of the Mechanisms of Chemical Enhancer Pretreatment and Transdermal Protein Transport

The chemical enhancer pretreatment formulations evaluated in Chapter 7 suggests that continuous skin exposure to chemical enhancer formulations may not be required to enhance transdermal drug permeabilities. The determination of a 3-hour threshold skin exposure time suggests the existence of a limit to the structural alterations that can be induced by the oleic acid formulation used. Moreover, the potential to reduce skin exposure times to harsher chemical enhancers that induce undesirable skin irritation, such as sodium dodecyl sulfate, while attaining transdermal transport enhancements,
comparable to that resulting from a continuous chemical enhancer exposure, requires additional investigation. The feasibility of using pretreatment formulations may enable the application of effective chemical compounds that are currently restricted due to the undesirable side effects that result from prolonged skin exposure. Finally, the applicability of the pretreatment threshold time to in vivo skin systems remains to be determined, as the regenerative processes that maintain the skin barrier function come into consideration.

The feasibility of achieving transdermal protein delivery using only chemical enhancers remains to be demonstrated. The skin barrier inherently prevents the transport of large, hydrophilic molecules such as proteins. Opportunities for transdermal protein delivery perhaps lie with the synergistic application of chemical enhancer formulations in combination with a technique capable of inducing greater mechanical alteration of the skin barrier, such as, ultrasound, electroporation, or iontophoresis.

8.3. Concluding Remarks

The abundance of chemical enhancer studies reported in the literature illustrates the relevance of elucidating the mechanisms of chemical enhancer action in the transdermal drug delivery field. The complex structure and composition of the stratum corneum, the primary barrier to transdermal transport, presents challenges to the efforts made to improve the current understanding of the effects of chemical enhancers on altering the permeant pathways in this heterogeneous medium. The visualization of transdermal transport processes described in this thesis, using TPM, HTPM, and dual-channel HTPM, provides tangible evidence of the oleic acid-induced changes in transdermal transport
through the three-dimensional images of probe spatial distributions. The extraction of mechanistic insight from the quantitative analysis of these visualized changes in transdermal transport has enabled us to more precisely describe the chemical enhancer-induced changes with respect to the relevant permeant transport parameters and rate-limiting steps that will facilitate the selection of potential chemical enhancer candidates. The application of the novel experimental methodologies introduced in this thesis to elucidate the mechanisms driving the multitude of other promising permeants across the skin paves the way for the future development of the research undertaken as part of this thesis and its potential impact on expediting the discovery of more effective chemical enhancer formulations.
8.4. References


Appendix A: Statistical Significance of Reported Model Drug Permeabilities

Table A-1: 95% Confidence intervals for mean permeability values of the model hydrophobic and hydrophilic permeants reported in Chapter 7.

<table>
<thead>
<tr>
<th>Hydrophobic</th>
<th>95% Confidence Interval (x10^5 cm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>Decyl Alcohol</td>
<td>146</td>
</tr>
<tr>
<td>Estradiol</td>
<td>39.2</td>
</tr>
<tr>
<td>Duanomycin</td>
<td>2.42</td>
</tr>
<tr>
<td>Cholesteryl-Hexyl Decyl Ether L-a-Phosphatidyl-Choline</td>
<td>0.66</td>
</tr>
<tr>
<td>Dolichol</td>
<td>52.2</td>
</tr>
<tr>
<td></td>
<td>3.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrophilic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1.84</td>
</tr>
<tr>
<td>PEG (400 Da)</td>
<td>2.43</td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.26</td>
</tr>
<tr>
<td>PEG (900 Da)</td>
<td>114</td>
</tr>
<tr>
<td>PEG (4000 Da)</td>
<td>0.26</td>
</tr>
<tr>
<td>Inulin</td>
<td>7.32</td>
</tr>
</tbody>
</table>
Table A-1 reports the 95% confidence intervals (cm/hr) about the mean permeability values for each model permeant that are reported in Chapter 7. Table A-2 lists the 95% confidence intervals for each Set No. corresponding to the oxytocin permeabilities shown in Figure 7-9. The fourth and fifth columns in Table A-1 refer to the confidence intervals corresponding to the permeabilities resulting from skin exposure to the PBS/EtOH and 5% oleic acid pretreatment (OA PT), followed by skin contact with the permeant in either PBS/EtOH or PBS, respectively. Experiments that were not performed are denoted by NA.

A confidence level of 95% reflects a statistical significance value of 0.05. The frequency that the mean permeability, for each case listed in Tables A-1 and A-2, lies within the corresponding 95% confidence interval about the reported mean value is 0.95.

<table>
<thead>
<tr>
<th>Set No.</th>
<th>(x10^5 cm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46.6</td>
</tr>
<tr>
<td>2</td>
<td>28.1</td>
</tr>
<tr>
<td>3</td>
<td>18.1</td>
</tr>
<tr>
<td>4</td>
<td>135</td>
</tr>
<tr>
<td>5</td>
<td>188</td>
</tr>
<tr>
<td>6</td>
<td>15.6</td>
</tr>
<tr>
<td>7</td>
<td>75.4</td>
</tr>
</tbody>
</table>