Interactions of Dendritic Nanomaterials with Skin Layers and Their
Applications in Topical Drug Delivery

By

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To my beloved parents, husband, and future children, who are the anchors of my life.
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<td>ddH$_2$O</td>
<td>Double Deionized Water</td>
</tr>
<tr>
<td>DE</td>
<td>Dermis / Dermal layers</td>
</tr>
<tr>
<td>DM</td>
<td>Dendron-based Micelle</td>
</tr>
<tr>
<td>DM-Ac</td>
<td>Acetyl-terminated Dendron Micelles</td>
</tr>
<tr>
<td>DM-COOH</td>
<td>Carboxyl-terminated Dendron Micelles</td>
</tr>
<tr>
<td>DM-NH$_2$</td>
<td>Amine-terminated Dendron Micelles</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethylammonium-propane</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol / Estrogen</td>
</tr>
<tr>
<td>EDC</td>
<td>(N)-(3-dimethylaminopropyl)-(N)'-ethy carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDX</td>
<td>Endoxifen, or 4-hydroxy-(N)-desmethyl Tamoxifen</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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</tbody>
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LIST OF ABBREVIATIONS (CONTINUED)

ER  Enhancement Ratio
EtOH  Ethanol
EVA  Ethylene Vinyl Acetate
FITC  Fluorescein Isothiocyanate
G2  Generation 2
G4  Generation 4
G2-RITC-Ac  Acetyl-terminated G2 Dendrimer-RITC Conjugates
G2-RITC-COOH  Carboxyl-terminated G2 Dendrimer-RITC Conjugates
G2-RITC-NH₂  Amine-terminated G2 Dendrimer-RITC Conjugates
G3.5-COOH  Carboxylated G3.5 Dendrimer
G4-NH₂  Amine-terminated G4 Dendrimer
G4-OH  Hydroxylated G4 Dendrimer
G4-RITC-NH₂  Amine-terminated G4 Dendrimer-RITC Conjugates
HLB  Hydrophilic-lipophilic Balance
I.S.  Internal Standard
IVIVC  In Vitro-In Vivo Correlation
J  Flux
KP  Ketoprofen
Kₚ  Permeability Coefficient
LBCs  Linear-block Copolymers
LC-MS  Liquid Chromatography-Mass Spectrometry
LLOQ  Lower Limit of Quantification
LMA  Lauryl Methacrylate
log P  1-octanol/PBS Partition Coefficients
MALDI-TOF  Matrix Assisted Laser Desorption Ionization-Time of Flight
Mass Spectrometer
**LIST OF ABBREVIATIONS (CONTINUED)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPEG</td>
<td>Methoxy-terminated PEG</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal Anti-inflammatory Drugs</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>OEGMA</td>
<td>Oligo(ethylene glycol) Methacrylate</td>
</tr>
<tr>
<td>P/PM</td>
<td>Plasmid-loaded Polymeric Micelles</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PDCs</td>
<td>PEGylated Dendron-based Copolymers</td>
</tr>
<tr>
<td>PE</td>
<td>Polyester</td>
</tr>
<tr>
<td>PEs</td>
<td>Penetration Enhancers</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEG400</td>
<td>Poly(ethylene glycol) 400</td>
</tr>
<tr>
<td>PEGylated</td>
<td>Poly(ethylene glycol)-conjugated</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic Acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic Acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PPE</td>
<td>Polymeric Penetration Enhancer</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS (CONTINUED)

PPG  Poly(propylene glycol)
QC  Quality Control
RHO  Rhodamine
RITC  Rhodamine B Isothiocyanate
RP-HPLC  Reversed-Phase High Performance Liquid Chromatography
RT  Room Temperature
SC  Stratum Corneum
SDS  Sodium Dodecyl Sulfate
SE  Solvent Evaporation
SP  Spantide II
TAM  Tamoxifen
TDD(S)  Topical / Transdermal Drug Delivery (System)
TEA  Triethylamine
TEM  Transmission Electron Microscope
VE  Viable Epidermis
WGA-AF488  Wheat Germ Agglutinin-Alexa Fluor 488 Conjugate
β-gal  β-galactosidase
SUMMARY

The barrier functions of the stratum corneum and the epidermal layers present a tremendous challenge in achieving effective transdermal delivery of drug molecules. Among many nanocarriers that have been tested to improve topical drug delivery, poly(amidoamine) (PAMAM) dendrimers have shown to be a promising skin-penetration enhancer, and yet little is known regarding the fundamental mechanisms behind the dendrimer-skin interactions. In this dissertation research, we have performed a systematic study to better elucidate how dendrimers interact with skin layers using Franz diffusion cells and confocal microscopy. Results indicated that the size, surface charge, and hydrophobicity directly dictate the permeation route and efficiency of dendrimer translocation across the skin layers, providing a design guideline for engineering dendritic nanomaterials as potential transdermal drug delivery vectors. On the knowledge base obtained from using dendrimers, the study was expanded to a dendron micelle (DM) system as a novel topical nanocarrier in order to exploit the advantageous dendritic structure and versatility in drug choice. This study has revealed surface modifications of the DMs affect loading and release profile, without sacrificing efficacy of endoxifen (EDX), a chemo-preventive medicine that is skin impermeable. The DMs, especially those with carboxyl termini (-COOH), induced substantially enhanced permeation of EDX through both full-thickness mouse (up to 20-fold) and split-thickness human (up to 4-fold) skin samples compared to a traditional chemical penetration enhancer (ethanol). These results demonstrate the potential
of dendrimers and DMs as topical drug delivery platforms and provide fundamental understanding on their skin interactions, which will potentially benefit for future development of nanoscale penetration enhancers based on dendritic polymers and other types of materials.
CHAPTER 1.
INTRODUCTION

1.1 Structure and Function of Skin and Topical Drug Delivery

Topical and transdermal drug delivery (TDD) systems have been of great scientific interest, due to their unique advantages. Specifically, topically administered agents can avoid first pass liver metabolism and GI tract incompatibility, minimize side effects by avoiding toxic plasma drug concentration, maintain favorable steady state drug concentrations for prolonged therapeutic effects, and are typically associated with enhanced therapeutic efficacy as well as high patient compliance.\(^1,2\) However, because of the excellent barrier functions, only a limited number of drugs have been found to readily penetrate through intact skin.\(^3\)

To overcome the barrier function of the skin, one have to first understand the anatomical structure and biological function of this organ. The skin is composed of sequential functional layers: the stratum corneum (SC), viable epidermis (VE), dermis (DE), and hypodermis (Figure 1.1). The SC and VE form the epidermal layers. In particular, the 15-30 μm thick SC layer,\(^4\) consisting of a lipid-enriched extracellular matrix filled with ceramides, cholesterol, and free fatty acids, is the major contributor to skin barrier functions.\(^3, 5, 6\) There are also multiple layers of corneocytes, which are keratinized
flattened dead cells, embedded in the SC layer and undergoing constant shedding at the SC surface to the environment. This layer also contains the keratin (a combination of cytokeratin and keratohyaline formed in the VE) that protects the skin tissue from abrasion.

**Figure 1.1 Structure of the skin.** Insert: Toluidine blue staining of the porcine skin cross-section showing stratum corneum (SC), viable epidermis (VE), and dermis (DE). *Polymer Chemistry*, 2013, 4, 2651-2657. Reproduced by permission of The Royal Society of Chemistry.

The VE contains discrete layers of proliferating, differentiating, and differentiated cells called keratinocytes. Keratinocytes are self-generating, stratified epithelial cells responsible for the formation of the SC during their upward migration from the basal layer of the VE to the skin surface. The VE can be divided into three sequential layers according to the different appearances of keratinocytes: the basal layer (stratum basale; the deepest layer), the spinous layer (stratum spinosum), and the granular layer (stratum granulosum). Keratinocytes are formed and aligned in a row on the basal membrane that interfaces the
epidermis and the dermis. These cells undergo mitosis periodically to replace cell loss from the skin surface. After formation at the basal layer, keratinocytes migrate upwards into the second layer, the spinous layer, and develop short projections, known as pricks or spines, that attach to adjacent cells via desmosomes. In this layer, cytokeratin is produced by keratinocytes as an intermediate filament precursor to keratin. Upon migration into the third layer, the granular layer, keratinocytes become squamous cells, and granules of keratohyaline (a precursor to the extracellular keratin) are formed within the cytoplasm.

The VE is considered the most biologically active part of the skin that has multiple cell types. Besides keratinocytes, there are several distinctive cell types in the VE that attach to keratinocytes via desmosome, including melanocytes, Langerhans cells, and Merkel cells. Located at the basal layer of the VE, melanocytes impart colors to the skin and hair by the production of a pigment called melanin, which is especially abundant in sun-exposed skin. Langerhans cells, or epidermal macrophages, are located in the stratum spinosum. Together with the dendritic cells and macrophages in the dermis, Langerhans cells form the immunological cellular system in the skin. Merkel cells located in the basal layer of the VE are responsible for detecting the sense of touch, and are commonly found in highly sensitive areas such as the fingertips. The integrity of the VE is maintained by desmosome attachments between adjacent epidermal cells, which also contribute to the skin barrier functions.
The dermal layer is rich in connective tissues comprised of collagen, elastin, and reticular fibers that network together to provide the physical support for blood vessels and nerve endings. The major cell types in the dermal layer are fibroblasts, mast cells, and macrophages. In addition, appendages such as sweat glands, sebaceous glands, hair follicles, and arrector pili muscles are anchored within this layer. The dermis can be further divided into two separate layers without a distinguishable boundary. A superficial papillary layer comprised of loose connective tissue is subjacent to the epidermal basement membrane and adopts the curve of the basal epithelial ridges and grooves. The lower reticular layer consists of dense irregular collagenous connective tissue with fewer cells and more fibers. A major component of the dermal layer is the extensive network of capillaries that functions to regulate body temperature and make the skin one of the most highly perfused organs in the body.

The subcutaneous tissue, or the hypodermis, is a fatty layer beneath the dermis that anchors the dermis to the underlying muscle or bone. Adipose tissue in this layer serves as a cushion or fat pad. A significant number of fibroblasts are also present in this layer for the synthesis of collagen and elastin, which form a loose connective tissue and allow the skin flexibility and free movement over the underlying structures.

The SC and VE provide an effective protective barrier against the entry of exogenous substances. Without physical intervention, the healthy skin limits permeation of all but only
a small number of molecules with an optimum molecular weight (typically less than 500 Dalton, or Da) and partition coefficient (typically between 1 and 3). A successful TDD system is therefore required to bypass the skin barrier function to enhance transdermal penetration of most drug molecules that have partition coefficients outside of the optimal range. It should be noted that in diseased skin, the barrier function may be compromised to varying degrees, which can result in greater skin permeation of exogenous substances. However, this dissertation will focus on the skin permeation studies using healthy, intact skin from multiple species.

1.2 Skin Penetration Enhancers and Their Mechanisms of Action

The success of TDD depends on the drug’s ability to permeate skin at a rate sufficient to achieve the desirable concentration in the intended area of the body. According to S. Mitragotri et al., transdermal transport of drug molecules (especially hydrophobic molecules) depends largely on the lipid bilayers of the skin. The SC is embedded with several layers of corneocytes with about 10 lipid bilayers filled in between the adjacent corneocyte layers. They pointed out that there are four different pathways for hydrophobic molecule diffusion through the skin layers: free volume diffusion in lipid bilayers (for small molecules with molecular weights (MW) less than 400 Da), lateral diffusion along lipid bilayers (for large molecules with MW greater than 400 Da), transdermal transport through pores, and transport through hair follicles and sweat ducts as a shunt diffusion pathway.
As mentioned above, since many drugs are insufficiently permeable to skin layers, numerous chemicals have been investigated as skin penetration enhancers (PEs). Chemical penetration enhancers (CPEs) are small molecule compounds that can significantly enhance drug penetration through the epidermis. Ideally, the CPEs should be safe and nontoxic to the skin, pharmacologically inert, nonirritating and non-allergenic. Alcohols, fatty acids, phospholipids, essential oils, azones and surfactants have been extensively used as CPEs in topical formulations to enhance skin permeation. In general, CPEs alter skin barrier functions via physicochemical disruption of the SC, promoting drug diffusion into deeper layers of the skin through different pathways. The pathways through which drug molecules can penetrate the skin include polar, nonpolar, or a combination of polar and nonpolar pathways. Changing the protein conformation in the SC can alter the polar pathway, while modifying the rigidity of lipid structure and fluidizing the crystalline matrix of the SC may alter the nonpolar pathway. Some CPEs can act on both polar and nonpolar pathways by altering the multi-laminar pathways for penetrants. CPEs can also alter the partition coefficients of drug molecules, thus increasing their skin permeability. Moreover, CPEs can dissolve skin lipids or denature skin proteins to increase the drug’s diffusivity in the SC.

Although seemingly effective, the application of CPEs usually enhances the permeation not only of the drug molecules, but also of the formulation excipients and the CPEs themselves. One extreme example is dimethyl sulfoxide (DMSO), which has deleterious effects on the
biochemical and structural integrity of the skin. In spite of the excellent penetration enhancement effect of DMSO, its use as a CPE is limited. Consequently, many CPEs possess a strong direct correlation to skin damage, irritation, and allergies. Those side effects have been linked with protein denaturation, lipid extraction, and integrity damage in the SC, especially when the transdermal system is applied repeatedly to a single site before full recovery occurs. The CPE-associated toxicities thus warrant investigation for an alternative means of augmenting topical drug delivery effectiveness.

Advanced colloidal formulations, such as liposomes, micelles, and deformable vesicles, have been developed not only to enhance drug solubility and skin permeation, but also to lessen skin irritation. These formulations have obtained various in vivo and clinical trial successes as topical drug delivery systems, with some of them subsequently being approved by the FDA or similar international regulatory authorities. These lipid- and surfactant-based TDD systems have been extensively reviewed elsewhere.

1.3 Nanoscale Polymeric Penetration Enhancers in Topical Drug Delivery

Driven by the growing interest in polymeric nanomaterials-based formulations for drug delivery, advanced nanomaterials have been investigated for their potential in TDD. Like liposomal formulations, recent nanomaterial designs for TDD aim to overcome the CPE-associated toxicities, without sacrificing delivery efficiency. Of these designs,
polymeric nanomaterials typically offer a unique opportunity as delivery platforms with a high level of modularity, allowing for various functional modifications to enhance TDD.\textsuperscript{24,25} However, polymer designs often require advanced synthetic schemes, intricate characterization, and exhibit inefficient drug loading, requiring further investigation into their handling and multifaceted mechanisms of action. In this section, we highlight polymeric nanomaterials used as polymeric penetration enhancers (PPEs), with an aim of emphasizing their roles in the enhancement of TDD. Polymeric formulations of controlled size and structure offer increased functionality and controlled drug release through various, diverse mechanisms, as discussed below.

1.3.1 Biodegradable Polymers for TDD

Biodegradable, biocompatible polymers have been widely studied for their use in drug delivery, including TDD.\textsuperscript{26-29} Commonly used polymers for drug delivery include polyesters that allow encapsulation of drug molecules and sustained drug release via hydrolysis of ester linkages in their backbones.\textsuperscript{30} This ester cleavage-driven drug release is also useful in TDD given that viable or freshly excised skin is typically abundant in esterase activity, facilitating the hydrolysis process. Moreover, degradation byproducts are generally considered to have minimal SC lipid disturbance, resulting in low skin irritation and limited adverse effects.\textsuperscript{5,31,32} Biodegradable polymer-drug complexes thus provide relatively new routes for controlled drug delivery kinetics to the skin layers, while minimizing skin irritation.
The most commonly studied biodegradable polyesters include polylactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL) (see Figure 1.2A for their chemical structures). For example, PLA nanoparticles (NPs) have been reported to deliver hydrophilic (4-Di-2-Asp) and hydrophobic (Bodipy 630/650) dyes into skin explants, exhibiting minimal PLA penetration and time-dependent dye release. PLGA has been reported to increase stability and facilitate sustained release of encapsulated estradiol and urea transdermally. The PLGA-based estradiol formulations displayed minimal burst release, along with prolonged rates of urea release, compared to those from water-in-oil or oil-in-water emulsions. PCL has been also used to topically deliver minoxidil and octyl methoxycinnamate, although negligible improvement of drug permeation, and a significant burst release was observed. These biodegradable
homopolymers have demonstrated the potential of being PPEs; however, the enhancement in TDD has been marginal. Instead, block copolymers composed of PCL and a hydrophilic block have been shown to significantly improve TDD efficiency, as discussed in the next section.

1.3.2 Amphiphilic Block Copolymers for Effective Skin Delivery

Amphiphilic polymers of tunable hydrophilic-lipophilic balance (HLB) have been used to enhance the skin penetration of drugs and genetic materials. These polymers provide enhanced protection and thermodynamic stability of unstable bioactive agents by encapsulation within self-assembled supramolecular nanostructures. In particular, the advantages of block copolymers as TDD vehicles include their ability to be engineered with an HLB desirable for drug encapsulation and skin interactions.

1.3.2.1 Di-block Copolymers

Several di-block copolymers that self-assemble into micelles, often consisting of a poly(ethylene glycol)-conjugated (PEGylated) surface and a hydrophobic, biodegradable core, have been investigated for TDD applications.

Xue et al. have reported self-assembled methoxy-terminated PEG-PCL (mPEG-PCL) micelles (Figure 1.2B) as potential drug carriers for the transdermal delivery of oridonin.
Micelles with an average diameter of approximately 25 nm were prepared using a thin film hydration method. As oridonin has limited water solubility (0.8 mg/mL at 25°C) and a relatively high Log P value (about 1.7 at 25°C), residence of oridonin in the hydrophobic PCL core is thermodynamically favorable, which further stabilizes the micelle. The drug loading (8.0 ± 0.0%) and encapsulation efficiency (99.5 ± 0.3%) indicated that oridonin is suitable to be encapsulated into mPEG-PCL micelles. It was reported that rapid release of the encapsulated oridonin during the first 11.5 h was observed, followed by a steady-state release over 3 days. Importantly, skin permeation experiments using excised mouse skin demonstrated that micelle formulation is superior to saturated water solution for transdermal oridonin delivery, with approximately a 1.8-fold permeation enhancement.

Self-assembled PEG-PCL micelles have been also shown to efficiently deliver minoxidil through guinea pig skin in vitro. Shim et al investigated the size-dependent permeation of PEG-PCL micelles.\textsuperscript{36} \textsuperscript{1}H-NMR spectroscopy was used to analyze receiver solutions from in vitro Franz diffusion cell experiments. The detected block copolymers from the receiver solutions indicated that the micelles penetrated hairless guinea pig skin concurrently with encapsulated minoxidil. Furthermore, relative to free drug formulation in 30% ethanol, minoxidil encapsulated in the micelles exhibited up to 2.5-fold enhanced skin permeation, altogether supporting increased permeation of minoxidil encapsulated in the nanoparticle formulations.\textsuperscript{36}
1.3.2.2 Poloxamers – Tri-block Copolymers

Poloxamers are nonionic tri-block copolymers with hydrophilic-hydrophobic-hydrophilic units (Figure 1.2C) that undergo self-assembly in aqueous solutions into micelles. The general structure of this type of polymers contains a central hydrophobic part of poly(propylene glycol), flanked by two hydrophilic parts of PEG. Biologically active molecules, such as plasmid DNA, can be encapsulated into the core, with particle stabilization achieved via the surface hydrophilic corona (in aqueous environment).

Using poloxamer-based micelles, Tong et al. have successfully delivered plasmid DNA through mouse skin and observed transgene expression of β-galactosidase (β-gal) in the skin, spleen, brain, and spinal cord after 48 and 72 h of topical administration. The delivery efficiency of β-gal plasmids in the presence of poloxamer micelles was significantly improved compared to free β-gal DNA. The authors also found that transdermal permeation of the plasmid-loaded polymeric micelles (P/PM) was an energy-dependent process. By measuring the apparent permeability coefficient, the authors found that skin permeation was impeded in skin pretreated with an ATP synthesis inhibitor for 15 min (sodium azide, 150 mM). In addition, the skin permeation of P/PM was significantly decreased at 4°C, relative to experiments performed at 37°C, indicating temperature dependence of P/PM skin transport. These results provide insight into the potential mechanism of the observed skin penetration of P/PM into both the epidermal and dermal layers of mouse skin.
1.3.2.3 Star Block Copolymers

The barrier function of the non-polar SC typically prevents permeation of polar molecules. To overcome this, Poree et al have recently developed star block copolymers with two different sizes to deliver polar fluorophores across epidermal layers (Figure 1.3). The construction of the copolymers began with the synthesis of dendritic cores with 6 or 12 tertiary bromide surface functionalities. Polar oligo(ethylene glycol) methacrylate (OEGMA) and non-polar lauryl methacrylate (LMA) were then successively conjugated onto the branching units of the dendritic cores via atom transfer radical polymerization, producing amphiphilic, reversed star block micelles. The hydrophilic interior (OEGMA) facilitated encapsulation of polar fluorophores (green proflavin and red rhodamine B), and the hydrophobic exterior (LMA) stabilized the micelle in the non-polar environment of the extracellular matrix. Notably, topical delivery efficiencies of the encapsulated fluorophore were dependent on the size of the star block copolymers. Fluorescence microscopy showed the 12-arm polymer delivered a larger amount of fluorophores than 6-arm to the viable epidermal and dermal layers of porcine skin. These results indicate that amphiphilicity and size of the delivery systems play a key role in determining the TDD efficiency of small polar molecules.
Figure 1.3 Star-block copolymers with 1, 6, and 12-arm for delivery of hydrophilic dye (proflavin) through the porcine skin to different depths: oligo PEGMA and LMA grafted star-block structures with 1-arm (1a, D-Br₁), 6-arm (1b, D-Br₆), or 12-arm (1c, D-Br₁₂) were prepared and used to encapsulate proflavin for porcine skin delivery. After 2 h of topical treatment, 12-arm star-block copolymer induced the deepest penetration of proflavin throughout the epidermal layers (SC and viable epidermis), whereas the other two copolymers only deposited proflavin in the peripheral layer of the SC. One-arm copolymer formed smaller micelles that deposited more proflavin into the SC than its 6-arm counterpart, due to the enhanced diffusion through the intercellular spaces of the SC (<100 nm). Reprinted with permission from Biomacromolecules, 2011, 12, 898-906. Copyright (2011) American Chemical Society.
1.3.3 Effect of Surface Modifications of Nanoscale PPEs on TDD

1.3.3.1 Chitosan

Chitosan is a cationic polysaccharide composed of polymers of glucosamine and N-acetyl-glucosamine (Figure 1.2D). It has been reported to enhance skin penetration by altering the secondary structure of keratin, promoting hydration of the SC and increasing cell membrane fluidity. Moreover, under mild acidic conditions, chitosan is positively charged, increasing interactions with the negatively charged SC and skin cell membranes, causing depolarization. In this case, cell membrane potential is decreased, leading to reduced skin resistance and enhanced permeation. Chitosan-encapsulated drug molecules have demonstrated sustained release profiles and enhanced safety profiles, while reducing skin irritation.

Surface modification of chitosan-based NPs has been also shown to improve TDD efficiency. Shah et al have prepared oleic acid (OA)-modified, PLGA-chitosan bilayered NPs for the topical co-delivery of anti-inflammatory drugs spantide II (SP) and ketoprofen (KP). Surface-modified NPs had significantly higher skin deposition of drugs, compared to NPs without OA modification. Drug release from the nanoparticles was first-order, with 20% and 59% release at 24 h of SP and KP, respectively. Furthermore, SP and KP co-delivered by the OA-modified NPs have also demonstrated synergistic anti-inflammatory effects, as observed from an in vivo allergic contact dermatitis model (C57BL/6 mice with
edema, or swollen ears). Consistently, the superiority of SP+KP-NP-OA to SP+KP-NP in reducing the ear thickness after 72 h of treatment was observed in an inflamed mouse model (Figure 1.4). This study strongly supports that surface modification of the chitosan NPs using OA enhances transdermal drug delivery of SP and KP.

**Figure 1.4** Chitosan nanoparticles (NPs) improved topical co-delivery of spantide II (SP) and ketoprofen (KP) for the treatment of allergic contact dermatitis (ACD). H&E histological staining of ACD-induced C57/BL mice ears after treatment with a positive control dexamethasone and various formulations. Reduction of mouse ear thickness, as evidence for anti-inflammatory response, observed after 72 h of treatment following the sequence of dexamethasone (positive control) ≈ SP+KP-NPs-OA > SP+KP-NPs+OA ≈ SP+KP-NPs > free SP+KP > no treatment (negative control). The images were taken using an optical microscope with 10 × objective. Reprinted with permission from *Journal of Controlled Release*, 2012, 158, 336-345. Copyright (2012) Elsevier.
1.3.3.2 Dendrimers

Dendrimers are synthetic, spherical macromolecules with tree-like branched structures, characterized by their repeating chemical unit and generational (radial) growth of polymer chains (Figure 1.2E).\textsuperscript{47} Their controlled sizes (3-10 nm), well-defined chemical structure, multifunctionality, and biocompatibility make these nanomaterials attractive for a wide range of promising biomedical applications.\textsuperscript{25} Poly(amidoamine) (PAMAM) dendrimers have been recently studied for their potential application in topical drug delivery. The surface terminal groups of PAMAM dendrimers can be precisely modified, endowing modular controllability over dendrimer surface functionality.

Recent studies have reported that through emulsion or pretreatment, PAMAM dendrimers enhance, by as much as four-fold, the skin permeability of the nonsteroidal anti-inflammatory drugs (NSAIDs), such as ketoprofen and diflunisal,\textsuperscript{48} as well as the hydrophilic drug, such as 5-fluorouracil (5FU).\textsuperscript{49} Notably, permeation of 5FU was enhanced to varying degrees in skin pretreated with generation 4 (G4) or G3.5 PAMAM dendrimers with different surface functional groups. The largest enhancement in drug permeability coefficient ($K_p$) was obtained by amine-terminated G4 (G4-NH$_2$), followed by hydroxylated G4 (G4-OH) and carboxylated G3.5 (G3.5-COOH).\textsuperscript{50}
Venuganti et al. have reported a systematic study, demonstrating that PAMAM dendrimer-skin interactions are highly dependent upon size and surface groups of the materials using porcine ear skin. They employed G2 through G6 PAMAM dendrimers, showing that low generation dendrimers penetrate the skin layers more effectively than the larger generation counterparts. The authors also provided evidence that cationic dendrimers alter skin lipid layers, thereby reducing skin resistance, as measured in terms of transepidermal water loss and skin resistance. Using a series of fluorescein isothiocyanate (FITC)-labeled dendrimers with various surface groups, they also observed better penetration of G4-NH$_2$ into the SC than G3.5-COOH and G4-OH. Interestingly, all dendrimers were constrained in the SC layer after 2 h of treatment. Upon prolonged G4-NH$_2$ treatment, however, partitioning of dendrimers beyond the SC into viable epidermis was observed. The evidence suggests that dendrimer penetration into skin is inversely proportional to size and affected by surface charges of the nanomaterials.

**1.3.4 Prospective**

Overcoming the barrier functions of skin presents a tremendous challenge in developing highly efficient topical delivery systems. Recent advances in nanotechnology offer a series of polymeric nanomaterials that exhibit great potential as effective PPEs. Particularly, nanoscale PPEs allow precise control and modification of their physical parameters, such as size, biodegradability, amphiphilicity/hydrophobicity, and surface charges, which significantly affect their skin interactions. However, the development of these nanoscale
PPEs is still at the preclinical stage, and the majority of the studies reported to date demonstrate experimental conditions that generate varying degrees of discrepancies, necessitating acquisition of a larger body of evidence and standardization of research protocols. Extensive developmental efforts, mechanistic studies of PPE-skin interactions, further toxicity studies, and *in vivo* optimization are all required to ensure improved delivery mediated by these polymeric nanomaterials. Nonetheless, these nanomaterials have great potential to change the paradigm of TDD, playing a key role in the next generation of transdermal applications.

### 1.4 Dissertation Objectives and Outline

The main goal of this dissertation research is to obtain fundamental understanding of the interactions between dendritic nanomaterials and skin layers, thus leading to the development of an effective transdermal drug delivery platform. In particular, skin penetration and retention behaviors of the surface-modified poly(amidoamine) (PAMAM) dendrimers were observed via confocal laser scanning microscope imaging techniques, and surface-modified, PEGylated dendron-based copolymer micelles as derivatives of dendrimers were developed for the hydrophobic drug molecule delivery through skin layers *in vitro* and *in vivo*. This dissertation consists of three major chapters: (1) interactions between dendrimer and skin layers and skin penetration pathway studies, (2) dendrimer
derivation – dendron micelle preparation and \textit{in vitro} transdermal drug delivery studies, and (3) dendritic drug carrier formulation and \textit{in vivo} drug delivery studies.

Chapter 2, the first part of this dissertation, includes the surface modification of PAMAM dendrimers for their skin penetration and retention studies: rhodamine B isothiocyanate was conjugated to the generation 2 and generation 4 PAMAM dendrimers for microscopic and spectrometric detection of dendrimers; surface acetylation and carboxylation of the amine-terminated PAMAM dendrimers were carried out to obtain three types of PAMAM dendrimers with different surface functionalities. Oleic acid conjugation to the dendrimer surface altered the partition coefficient of the dendrimer. These surface modifications have shown effects on skin permeation behaviors of the dendrimers in terms of penetration depth, permeation pathways, and skin retention.

Chapter 3 explores the transdermal delivery of skin-impermeable hydrophobic molecules upon encapsulation using PEGylated dendron-based micelles (DMs), which are derived from the structure of dendrimers and synthesized in house. Surface modification of the dendron micelles affected drug loading efficiency and indirectly resulted in different drug delivery efficiencies. The prepared dendron micelles were fully characterized, and the drug release profiles revealing improved drug stability in aqueous conditions were studied. The efficacy of the hydrophobic drug endoxifen (EDX) after micelle encapsulation was maintained. \textit{In vitro} skin permeation of the DM-loaded drug molecules was investigated in
both mouse and human skin. Significantly enhanced drug permeation was observed using DMs compared to CPEs and liposomes.

Chapter 4 describes the development of the DM system for in vivo drug delivery and proof-of-concept in vivo studies using hairless mice. Different topical gel matrices and occlusive conditions were compared in terms of their compatibility to the DM drug delivery system. In vivo drug delivery efficiency of the DMs was investigated using EDX. Skin deposition and blood concentration of the drug were measured using a LC-MS method. Based on the data obtained from the in vitro skin permeation experiments and the in vivo blood concentration measurements, we have reached the conclusion that the DMs can effectively delivery EDX through the skin layers.

This dissertation will present a systematic understanding of the interactions between the skin layers and the dendritic polymeric nanoparticles, demonstrating the potential of the dendrimers and dendron-based micelles as transdermal drug delivery platforms. Moreover, the in vivo pilot study provided a fundamental understanding for future developments of DM systems as topical EDX delivery platforms to prevent estrogen receptor positive primary breast cancer.
1.5 References


CHAPTER 2.

Effect of Size, Surface Charge and Hydrophobicity of Poly(amidoamine) Dendrimers on Their Skin Penetration

2.1 Introduction

The outermost layer of the skin – the stratum corneum (SC) consisting of multiple lipid layers - functions as a protective barrier against exogenous molecules.\(^1\) In particular, the SC layers are excellent barriers against those molecules with molecular weights over 500 g/mol and those with 1-octanol/PBS partition coefficients (log \(P\)) less than 1 or greater than 3.\(^2\)\(^-\)\(^4\) For this reason, a variety of molecules and materials have been investigated as candidates that enable or facilitate skin permeation of those molecules that are otherwise skin-impermeable. Chemical penetration enhancers (CPE) have been widely used to increase the skin permeability of many therapeutic molecules and anesthetics.\(^2\) However, the penetration-enhancing effect is frequently accompanied by skin irritation and toxicity.\(^2\)\(^-\)\(^5\) By way of contrast, polymer-based permeation enhancers typically do not cause skin irritation, but their large size often prohibits them from penetrating deep into the skin layers, which limits their efficacy.\(^6\)

Dendrimers are synthetic, spherical macromolecules with tree-like branched structures (Figure 2.1). Their well-controlled sizes (3-10 nm), ease of functionalization, high water
solubility, well-defined chemical structure, and biocompatibility make these nanomaterials attractive for a wide spectrum of promising biomedical applications. Poly(amidoamine) (PAMAM) dendrimers have been shown to be advantageous over linear polymers due to their multivalency, which can be precisely controlled by engineering their surface functional groups. Previously, Hong et al. reported a series of studies on the biological interactions between dendrimers and either lipid bilayers or cell membranes. The studies revealed that positively charged PAMAM dendrimers induce nano-scale hole formation (within non-cytotoxic concentrations), whereas neutral or negatively charged PAMAM dendrimers do not. These observations suggested an alternative mechanism of lipid layer permeabilization by positively charged dendrimers, which may be applicable for skin penetration.

A few recent studies have reported that, through emulsion or pretreatment, PAMAM dendrimers enhance, by as much as four-fold, the skin permeability of the nonsteroidal anti-inflammatory drugs (NSAIDs) ketoprofen and diflunisal and the hydrophilic 5-fluorouracil (5-FU). It was also reported that permeation of 5FU was enhanced in skin pretreated with generation 4 (G4) or G3.5 PAMAM dendrimers with different surface functional groups; the order of enhancement in drug permeability coefficient ($K_p$) was G4-NH$_2$ > G4-OH > G3.5-COOH. Meanwhile, the $K_p$ of 5FU was inversely proportional to the molecular weight of the dendrimer, suggesting that amine-terminated, small PAMAM dendrimers are more effective than other types of dendrimers in enhancing skin.
permeability of small drug molecules. However, although quite a few reports have shown enhanced skin permeation of small drug molecules mediated by PAMAM dendrimers, all of those studies used dendrimer-drug complexes to increase drug solubility and loading. Furthermore, the reported skin permeation was frequently assisted by addition of CPEs such as mineral oil and isopropyl myristate or by formulating the complex into emulsions using cetyl alcohol and Brij or polysorbate as emulsifiers. More importantly, most of those reports have focused on skin permeation of small molecules, without providing systematic investigations focusing on the interactions of the dendrimers themselves (low-generations in particular) with the skin layers. Further mechanistic studies of how low-generation dendrimers interact with the skin layers are therefore required for better understanding and potential clinical translation of dendrimer-based transdermal drug delivery.

The primary objective of this study was to investigate the effects of dendrimer size, surface charge, and hydrophobicity as potential key parameters that determine the skin permeation/penetration behavior of dendrimers. The size effect was investigated by comparing G2 and G4 PAMAM dendrimers. The surfaces of G2 PAMAM dendrimers were then modified to be amine-, acetyl-, and carboxyl-terminated to investigate the charge effects (the chemical structures are shown in Figure 2.1). In addition, G2 PAMAM dendrimers were conjugated with oleic acid (OA) to control the hydrophobicities of the nanomaterials. Using those materials, we performed Franz diffusion cell experiments,
confocal microscopy observations, and partition coefficient analysis to assess the dendrimer-skin interactions. This study presents a systematic understanding of the interaction between the skin layers and surface-engineered dendrimers, demonstrating the potential of the dendrimers as a transdermal drug delivery vehicle.

Figure 2.1 Chemical structures of G2 PAMAM dendrimer and ¹H NMR spectra of the starting materials G2-NH₂ and G4-NH₂. The representative structure of the surface modified dendrimers: A) amine- (-NH₂), B) carboxyl- (-COOH), and C) acetyl-terminated (-Ac) dendrimers. D) Starting G2 PAMAM dendrimer with primary amine end groups (G2-NH₂); E) Starting G4 PAMAM dendrimer with primary amine end groups (G4-NH₂).
2.2 Materials and Methods

2.2.1 Materials

PAMAM dendrimers, generations 2 (G2, MW 3,256 g/mol) and 4 (G4, MW 14,215 g/mol), with ethylenediamine cores were purchased from Sigma-Aldrich (St. Louis, MO). Rhodamine B isothiocyanate (RITC), acetic anhydride, triethylamine (TEA), succinic anhydride, oleic acid (OA), \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarboadiimide hydrochloride (EDC), \(N\)-hydroxysuccinimide (NHS), anhydrous methanol, ethanol, dimethyl sulfoxide (DMSO), and 1-octanol were all obtained from Sigma-Aldrich (St. Louis, MO). Calcium- and magnesium-free PBS was purchased from Mediatech, Inc. (Manassas, VA). Poly(ethylene glycol) 400 (PEG400) was obtained from Fisher Scientific, (Fair Lawn, NJ). All other chemicals used in this study were obtained from Sigma-Aldrich and used as received unless otherwise noted.

2.2.2 Synthesis and Characterization of G2- and G4-RITC-NH\(_2\) Conjugates

The reaction scheme for conjugation between G2 / G4 PAMAM dendrimers and RITC is illustrated in Figure 2.2. The reactions were carried out following previous reports\(^{17,20}\). Briefly, G2 and G4 PAMAM dendrimers (10.0 mg, 3.1 \(\mu\)mol and 0.7 \(\mu\)mol, respectively) were dissolved in 1 mL DMSO. RITC (2.5 mg, 4.6 \(\mu\)mol and 0.6 mg, 1.1 \(\mu\)mol, 50% molar excess to G2 and G4, respectively) was first dissolved in 200 \(\mu\)L DMSO and then added to the dendrimer solutions dropwise under vigorous stirring at RT for 24 h, resulting in G2-
RITC-NH$_2$ and G4-RITC-NH$_2$, respectively. Unreacted RITC was removed by membrane
dialysis (Spectra/Por dialysis membrane, MWCO of 500 for G2 and 1,000 for G4,
Spectrum Laboratories Inc., Rancho Dominguez, CA) in 4 L double deionized water
(ddH$_2$O) for 3 days. The purified products were then lyophilized for 2 days and stored at
-20°C. The chemical structure of the conjugates was confirmed by $^1$H NMR in D$_2$O using a
400 MHz Bruker DPX-400 spectrometer (Bruker BioSpin Corp., Billerica, MA). The
numbers of the RITC molecules per dendrimer were calculated from UV/Vis
measurements. Serially diluted RITC (1.3, 2.5, 5.0, 10.0 µg/mL) solutions in 1:1
DMSO/H$_2$O were prepared and used to plot the standard curve for the quantification of the
number of RITC attached to the dendrimer conjugates using a DU800 UV/Vis
spectrophotometer (Beckman Coulter, CA). The surface charges (zeta potential, mV) of
G2-RITC-NH$_2$ and G4-RITC-NH$_2$ were obtained from three repeat measurements of the
aqueous dendrimer solutions at a concentration of 125 µg/mL by quasi-elastic laser light
scattering using a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa
Barbara, CA) as we previously reported.

2.2.3 Preparation and Characterization of Acetylated and Carboxylated G2-RITC
Conjugates

G2-RITC-NH$_2$ was fully acetylated or carboxylated as previously described. Briefly,
G2-RITC-NH$_2$ (10.0 mg, 2.6 µmoL) was dissolved in 1 mL of methanol and acetylated by
adding acetic anhydride (6.1 mg, 5.6 µL, 59.3 µmoL, 50% molar excess of the number of
amines on the surface of G2-RITC-NH₂) and TEA (6.7 mg, 9.2 µL, 65.2 µmol, 10% molar excess of acetic anhydride), under vigorous stirring at RT for 24 h (Figure 2.2). In a separate reaction, G2-RITC-NH₂ (10.0 mg, 2.6 µmol) in 1 mL of DMSO was fully carboxylated by adding succinic anhydride (5.9 mg, 59.3 µmol, 50% molar excess of the number of amines on the surface of G2-RITC-NH₂) in 1 mL of DMSO under vigorous stirring at RT for 24 h (Figure 2.2). The acetylation and carboxylation reactions were confirmed using ¹H NMR measurements (Figure 2.2). The surface charges (zeta potential, mV) of G2-RITC-Ac, G2-RITC-COOH, and G2-RITC-NH₂ were also obtained using the same method as described above.

2.2.4 Preparation and Characterization of G2-RITC-NH₂-OA Conjugates

G2-RITC-NH₂ was reacted with OA at either 5 or 8 molar excess to G2-RITC-NH₂ using EDC/NHS chemistry (Figure 2.3). For the stoichiometry of 1:5 of G2-RITC-NH₂:OA, OA (4.2 µL, 13.2 µmol) was pre-activated by EDC (25.3 mg, 132.0 µmol) and NHS (15.2 mg, 131.9 µmol) in DMSO with vigorous stirring in the dark at RT for 2 h. G2-RITC-NH₂ (10.0 mg, 2.6 µmol) in DMSO was then added and vigorously stirred for 24 h. For the 1:8 ratio, proportionally higher amounts of OA, EDC, and NHS were used under the identical conditions. Unreacted OA was removed by membrane dialysis against ddH₂O using a 500 MWCO membrane (Spectrum Laboratories) for 2 days, followed by lyophilization for 2 days and storage at -20°C. ¹H NMR and mass spectroscopy (MS, Applied Biosystems Voyager-DE Pro matrix assisted laser desorption ionization-time of flight (MALDI-TOF)
mass spectrometer, Carlsbad, CA) were performed to characterize the molecular weights of the dendrimer-OA conjugates as described previously.\textsuperscript{21,23}

![Diagram](image.png)

**Figure 2.2 Reaction schemes of the surface modifications of G2 dendrimers and \( ^1\text{H} \) NMR spectra of A) G2-RITC-Ac and B) G2-RITC-COOH:**

**Top:** Conjugation of G2-RITC-NH\(_2\) (same conditions for G4-RITC-NH\(_2\) conjugation), and surface charge modification by acetylation and carboxylation of G2-RITC-NH\(_2\); B) conjugation of OA to G2-RITC-NH\(_2\).

**Bottom:** The degree of acetylation was determined by comparing the integration values of the characteristic peaks of G2 dendrimer at 3.31 ppm and acetyl group at 2.06 ppm (see arrows). The measured ratio revealed that 94.3\% of the primary amine groups were acetylated. The shape changes of characteristic peaks of dendrimer after carboxylation demonstrate that the surface modification was successful. (\(^1\text{H} \) NMR spectra obtained by Ryan Pearson)
Figure 2.3 Reaction scheme of G2-RITC-NH2-OA conjugation and $^1$H NMR spectra for A) 1:5 and B) 1:8 (G2-RITC-NH2:OA) reaction products:

Top: Conjugation of OA to G2-RITC-NH2. Bottom: The large peak at 1.22 ppm (d and d’) corresponds to the protons of the oleic acid backbone, and the peak at 0.89 ppm (f and f’) is assigned to the methoxy end group of OA. The protons connected to the ethylene bond on oleic acid are observed at 5.30 ppm (e and e’). The methoxy groups in RITC are observed at 0.97 ppm (a and a’) and 1.45 ppm (c and c’), and the proton peak at 7.92 ppm (b and b’) corresponds to the protons on the benzene ring of RITC. The ratio of OA to RITC was calculated based on the integration values of the characteristic values. The measured ratios were ranging from A) 1:2 (the integration ratio of peak d or e to peak a or c) to 1:2.7 (the integration ratio of peak f to peak c) and B. 1:2.3 (the integration ratio of peak d’ to peak c’) to 1:3 (the integration ratio of peak e’ to peak c’). ($^1$H NMR spectra obtained by Ryan Pearson)
2.2.5 Porcine Skin Preparation

Full-thickness porcine skin was collected from the inner thigh area of a 30 lb female American Yorkshire pig (Halsted Packing House, Chicago, IL). The skin samples were collected from the thigh regions as these areas have generally less hair and fat compared to other regions such as dorsal, flank, and belly, to minimize the hair and fat removal process that may cause skin damage. All hairs were removed using small tweezers, and the skin was carefully examined for any defects. Undamaged skin was cut into $10 \times 10 \text{ cm}^2$ squares with similar numbers of hair follicles. The fat and subcutaneous tissues were gently removed using a surgical blade. Each piece of skin was wrapped with aluminum foil, sealed in a zip bag, stored at -80°C, and used within 90 days.

2.2.6 Skin Permeation Tests using Franz Diffusion Cells

The porcine skin was thawed on ice, further trimmed into $1.2 \times 1.2 \text{ cm}^2$ squares, and sandwiched between the donor and receiver chambers of the Franz diffusion cells ($\Phi 7 \text{ mm}$ with $0.38 \text{ cm}^2$ exposure area, PermeGear Inc., Hellertown, PA) with the SC side facing upward, following a previous report. The receiver chambers were then filled with fresh PBS (pH 7.4). After equilibration of the skin at 37°C for 30 min, 100 µL of each dendrimer conjugate at a concentration of 1 mM or control groups (free rhodamine or vehicle (ddH$_2$O)) were applied to each donor chamber, as described elsewhere. Note that for the dendrimer-OA conjugates, 70% ethanol solution was used as a solvent vehicle due to their poor water solubility. The chambers were first covered by Parafilm™ to prevent
evaporation and then by aluminum foil to minimize fast photobleaching of rhodamine. The first sampling (t=0) was done by withdrawing 250 µL of receiver solution from each sampling portal, followed by addition of 250 µL of fresh PBS to maintain a constant total volume in the receiver chamber. Samplings were performed as frequently as every two hours up to 24 h. All sample solutions were kept at 4°C in dark before subsequent analysis.

2.2.7 Measurements of Skin Permeation and Retention

The fluorescence intensity from each receiver solution was detected using a SpectraMAX GeminiXS microplate spectrofluorometer (Molecular Devices Inc., Sunnyvale, CA). The dendrimer-RITC conjugates were detected at 555 nm excitation and 590 nm emission wavelengths. The amount of dendrimer in the receiver solutions was quantified based on standard curves of fluorescence intensities versus concentrations of serially diluted solutions from the 1 mM stock solutions of the various dendrimer conjugates (Table 2.1).

The percent permeation (Permeation%) was calculated by dividing the amount of the conjugates in the receiver solution by the original amount applied. The materials absorbed to the epidermis and dermal layers were directly measured by extracting the conjugates from each skin layer using a cocktail of 1:1:1 ddH2O:ethanol:PEG400 for 6 h. In some experiments, skin absorption was also measured by subtracting the amount of the materials in the donor and receiver solutions from the total amount of the materials applied.
Table 2.1 Characterization of dendrimer conjugates.

<table>
<thead>
<tr>
<th></th>
<th>Surface NH₂ groups</th>
<th>RITC Attached&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RITC Attached&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Measured MW (Da)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Theoretical MW (Da)</th>
<th>ζ-Potential (mV)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2-NH₂</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>3,160-3,253</td>
<td>3,256</td>
<td>+16.6</td>
</tr>
<tr>
<td>G4-NH₂</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>10,709-15,287</td>
<td>14,215</td>
<td>+38.5</td>
</tr>
<tr>
<td>G4-RITC-NH₂</td>
<td>63</td>
<td>1.0</td>
<td>-</td>
<td>9,101-19,000</td>
<td>15,753</td>
<td>+41.2</td>
</tr>
<tr>
<td>G2-RITC-NH₂</td>
<td>15</td>
<td>1.0</td>
<td>1.3</td>
<td>4,155</td>
<td>3,792</td>
<td>+18.8</td>
</tr>
<tr>
<td>G2-RITC-Ac</td>
<td>0</td>
<td>1.0</td>
<td>1.3</td>
<td>4,399</td>
<td>4,398</td>
<td>+2.7</td>
</tr>
<tr>
<td>G2-RITC-COOH</td>
<td>0</td>
<td>1.0</td>
<td>1.3</td>
<td>5,655</td>
<td>5,346</td>
<td>-14.5</td>
</tr>
<tr>
<td>G2-RITC-NH₂-OA₂₃</td>
<td>13</td>
<td>1.0</td>
<td>1.3</td>
<td>4,389</td>
<td>4,638</td>
<td>N/A&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>G2-RITC-NH₂-OA₂₇</td>
<td>10</td>
<td>1.0</td>
<td>1.3</td>
<td>3,708-5,643</td>
<td>5,202</td>
<td>N/A&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured using UV/Vis spectroscopy.
<sup>b</sup> Measured using <sup>1</sup>H NMR.
<sup>c</sup> Measured using MALDI-TOF.
<sup>d</sup> Measured using Zeta Potential/Particle Sizer.
<sup>e</sup> Zeta potential of OA conjugates were not measured due to the limited solubility in aqueous solutions.

To confirm that the skin used was intact, we performed two observations of each square of skin before accepting the data it generated. Firstly, as the dendrimer-RITC conjugates were red in color, we observed immediate color changes (within 1 h) in receiver solutions when damaged skin was used, and such results were excluded. Secondly, we measured the fluorescence from the receiver solutions using a fluorimeter at early time points (0, 2 and 4 h) to ensure that there was the induction period that is typical for skin permeation. Early permeation was considered an indicator for skin damage.
2.2.8 Confocal Laser Scanning Microscopy (CLSM) Observations

For the size comparison study, porcine skin was exposed to either G2-RITC-NH$_2$ or G4-RITC-NH$_2$ for 24 h in the Franz cell setup. The skin area that was exposed to the treatment was carefully collected, rinsed twice with ddH$_2$O for 10 min, and immersed in 10 mL of 10% neutral buffered formalin for 24 h of fixation. The fixed skin pieces were transferred into 70% (v/v) ethanol for 24 h of dehydration, followed by an overnight treatment of 30% (w/v) sucrose solution, before being embedded into cryomolds (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA). Skin was cryosectioned into 10 µm-thick slices and placed on anti-frost glass slides. After drying at RT, the slides were first stained with Wheat Germ Agglutinin-Alexa Fluor 488 conjugate (WGA-AF488, 5 µg/mL in PBS, Invitrogen Corporation, Carlsbad, CA) for 10 min at RT. After washing off the excess WGA-AF488 with ddH$_2$O, skin slides were mounted with antiphotobleaching mounting media with DAPI (Vector Laboratory Inc., Burlingame, CA) and covered with glass cover slips.

For the permeation pathway study, porcine skin was cut into 5×10 mm$^2$ strips and embedded into cryomolds. Skin was cryosectioned into 10 µm-thick slices and placed on anti-frost glass slides. After drying at RT, the skin strips were treated with 200 nM of G2-RITC-NH$_2$, G2-RITC-COOH, or G2-RITC-Ac in PBS at RT for 1 h, and the excess materials were gently washed away using ddH$_2$O. The slides were then fixed by 10% neutral buffered formalin for 10 min at RT and washed again with ddH$_2$O. The slides were
stained with WGA-AF488 for 10 min at RT. After washing off the excess WGA-AF488 with ddH₂O, the skin slides were mounted with antiphotoablation mounting media with DAPI and covered with glass cover slips.

The cross-sections of the skin layers were then visualized using a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM, Carl Zeiss, Germany). The 488 nm line of a 30 mW tunable argon laser was used for the excitation of AF488, a 1 mW HeNe at 543 nm for RITC, and a 25 mW diode UV 405 nm laser for DAPI. Emission was filtered at 505-530 nm, 565-595 nm, and 420 nm for AF488, RITC, and DAPI, respectively.

### 2.2.9 Partition Coefficient Measurements

The partition coefficients of the surface-modified G2-RITC and the dendrimer-OA conjugates were determined using the shake-flask method.²⁶ Equal volumes of spectroscopic grade 1-octanol and calcium- and magnesium-free PBS were stirred together vigorously for 24 h to mutually saturate the two phases. The phases were allowed to separate overnight before aliquots were collected. Each of the water-soluble dendrimer conjugates (except for G2-RITC-NH₂-OA) was dissolved at a concentration of 10 µM in PBS. The G2-RITC-NH₂-OA conjugates were dissolved at 10 µM in 1-octanol. The pH of each PBS solution was adjusted to 7.4 with NaOH and/or HCl. Two milliliters of the PBS phase and 2 mL of the octanol phase were gently added to a 7 mL centrifuge tube. The
tube was placed on a rocker (Fisher Scientific, Pittsburgh, PA) rotating once every 3 s for 5 min and centrifuged at 20-60 ×g for 3 min. The fluorescence of the PBS phase was read using a SpectraMAX GeminiXS microplate spectrofluorometer (Molecular Devices, LLC., Sunnyvale, CA) at 555 nm excitation and 590 nm emission wavelengths.

The partition coefficient, or log $P$, was calculated as

$$\log P = \log \frac{[\text{Dendrimer in Octanol}]}{[\text{Dendrimer in PBS}]}$$

The method was validated by comparing the tested value with the literature reported values of rhodamine$^{26}$ and dendrimers.$^{27}$

### 2.2.10 Statistical Analysis

Data processing was performed using Origin 8.0. Statistical analysis was performed using SPSS 11.5 based on a one-way ANOVA followed by Tukey’s post hoc analysis. Data are considered significant at $p<0.05$.

### 2.3 Results

#### 2.3.1 Characterization of the Various Dendrimer Conjugates

The molecular weights and numbers of the terminal groups of each dendrimer as well as the numbers of fluorophores and OA attached to each dendrimer are summarized in Table 2.1.
The UV/Vis measurements (Figure 2.4A) revealed that, on average, 1.0 RITC was conjugated to each G2 and G4 dendrimer molecule, which were referred to as G2-RITC-NH₂ and G4-RITC-NH₂, respectively. The RITC conjugation was confirmed using ¹H NMR by observing the peak(s) from the newly formed thiourea bond at 6.96 ppm as a result of conjugation between the dendrimers and the isothiocyanates (Figure 2.4B). The G2-RITC-NH₂ conjugates were then surface modified by acetylation or carboxylation.¹³ The degree of acetylation was measured using ¹H NMR shown in Figure 2.2A, which revealed that 94% of the primary amine groups on the G2 surfaces were converted to acetamide. The shape changes of the characteristic peaks of the dendrimers after carboxylation demonstrated that the surface modification was successful (Figure 2.2B).¹³ The zeta potential values of the various dendrimers are also listed in Table 2.1. G4-RITC-NH₂ exhibited the highest positively charged moiety (+41.2 mV), and G2-RITC-NH₂, G2-RITC-Ac, and G2-RITC-COOH showed highly positive (+18.8 mV), nearly neutral (+2.7 mV), and negative (-14.5 mV) surface charges, respectively, confirming the success of the surface modifications. The reaction stoichiometry (1:5 and 1:8 of G2-RITC-NH₂:OA) resulted in two different numbers of OA molecules per dendrimer, as shown in Table 2.1 and Figure 2.3. The ¹H NMR and MALDI-TOF data indicated that approximately 2.3 and 2.7 OA molecules were conjugated per G2-RITC-NH₂ molecule, resulting in G2-RITC-NH₂-OA₂.3 and G2-RITC-NH₂-OA₂.7, respectively.
Figure 2.4 Characterization of G2-RITC-NH$_2$ and G4-RITC-NH$_2$ using A) UV/Vis and B) $^1$H NMR spectroscopy.

The ratio of dendrimer to RITC was calculated based on UV spectra of RITC standards. RITC exhibits a maximum absorption peak at 554 nm, which was shifted after conjugation to dendrimer (see arrows, peak shifted to 555 nm for G2-RITC-NH$_2$, and 548 nm for G4-RITC-NH$_2$). Serially diluted RITC (2.5, 5, and 10 µg/mL) solutions in 1:1 DMSO/H$_2$O were prepared and used to plot the standard curve for the quantification of the number of RITC on the dendrimer conjugates. The ratio was 1:1 for both G2-RITC-NH$_2$ and G4-RITC-NH$_2$. The integration values of the characteristic peaks from $^1$H NMR measurements (see arrows, 3.442 ppm for G2 dendrimer, 6.963 ppm for RITC) also revealed the ratio of approximately 1:1.3. ($^1$H NMR spectra obtained by Ryan Pearson)
2.3.2 Effect of Dendrimer Sizes on Skin Permeation

The effect of dendrimer size was investigated by comparing G2 and G4 PAMAM dendrimers conjugated with RITC. Figure 2.5A-C shows the confocal images of the cross-sections of the porcine skin treated with G2-RITC-NH$_2$ and G4-RITC-NH$_2$. The images demonstrated that G4-RITC-NH$_2$ did not penetrate across the SC, as only a small amount of the dendrimers was retained at the outermost layer of SC (Figure 2.5B). On the other hand, a significantly larger amount of G2-RITC-NH$_2$ was absorbed into the SC layers as well as the underlying viable epidermis (Figure 2.5C). G2-RITC-NH$_2$ also exhibited a significantly stronger fluorescence signal than that of G4-RITC-NH$_2$, indicating that G2 dendrimers were much more strongly absorbed into the skin layers than G4. The Permeation% of dendrimers showed better permeation of G2-RITC-NH$_2$ than G4-RITC-NH$_2$ in general (data not shown). However, the small amount of the permeated dendrimers and the large batch-by-batch variations made accurate quantification of the amount of dendrimers in the receiver solution difficult. We therefore measured the amounts of dendrimers in the skin layers instead of measuring Permeation%. To quantify the dendrimer conjugates in the skin, the skin samples were collected after the Franz cell diffusion experiments, and the conjugates were extracted using a cocktail of 1:1:1 ddH$_2$O:ethanol:PEG400 for 6 h. Figure 2.5D demonstrates the fold increases of the skin-absorbed materials after 24 h. The epidermal absorption of G2-RITC-NH$_2$ was 5.8-fold higher than that of G4-RITC-NH$_2$ after 24 h, indicating that the G2 dendrimer penetrates more efficiently than G4. Due to the better permeation of the smaller-sized G2 dendrimers,
we chose them for subsequent transdermal permeation experiments that investigate the effect of charges (surface groups) and hydrophobicity on dendrimer-skin interactions.

Figure 2.5 Skin permeation efficiencies of various PAMAM dendrimers.  
A-C) CLSM images of the skin cross-sections of microtomed porcine skin layers after treatment with dendrimers.  
A) Vehicle (ddH₂O) control, B) G4-RITC-NH₂, and C) G2-RITC-NH₂.  
(I, overlay of red channel (dendrimer conjugates) and bright field images; II, merged images of dendrimer conjugates (red), cell membrane stained by WGA-AF488 (green), and nuclei stained by DAPI (blue).  
(CLSM images taken by Suhair Sunoqrot)  
D) Fold increase in epidermal accumulations of G2 and G4 PAMAM dendrimers over 24 h (error bars: standard deviation (SD), n=3).  
Note that the smaller molecular size leads to better skin absorption; in this case G4-RITC < G2-RITC.  
E) Fold enhancement in % permeation of the surface modified G2 PAMAM dendrimers after 24 h (error bars: SD, n=3-6).  
Note that the both neutral and negatively charged dendrimers permeate skin more efficiently than the positively charged dendrimers.  
Scale bar: 10 µm. SC, stratum corneum; VE, viable epidermis; DE, dermal layer.  *p<0.05.
2.3.3 Effect of the Surface Charge of Dendrimers on Skin Permeation and Retention

To assess the permeation efficiencies of the G2-RITC conjugates with different surface functional groups, Franz diffusion cell experiments were performed using a 1 mM concentration of the materials in ddH$_2$O without adding any commonly used permeation enhancers. The difference in permeation was observed by analyzing RITC fluorescence in the receiver solutions (Figure 2.5E). Although Permeation% of the dendrimer conjugates was low (less than 3%), there was an up to four-fold increase in permeation for both G2-RITC-COOH and G2-RITC-Ac compared to G2-RITC-NH$_2$. These results show that G2 dendrimers with different surface functional groups behave differently in terms of skin permeation. Confocal images shown in Figure 2.6 visualize the interactions of the various dendrimers with the skin cells, assessed by incubation of the pre-cryosectioned skin slides with the dendrimer conjugates. For clear visualization of the dendrimers in the skin layers, we added the dendrimer conjugates after cryosectioning the skin samples. As seen from the red signals from the cytoplasm (Figure 2.6A), G2-RITC-NH$_2$ was internalized into the individual cells in both the epidermal and dermal layers. By way of contrast, neither G2-RITC-COOH nor G2-RITC-Ac interacted with the cells (Figure 2.6B and 2.6C).
Figure 2.6 Confocal images of cross-sections of microtomed porcine skin layers after 1 h treatment with: A) G2-RITC-NH\(_2\); B) G2-RITC-COOH; or C) G2-RITC-Ac. The dendrimer conjugates (I, red); cell membranes stained by WGA-AF488 (II, green); nuclei stained by DAPI (III, blue); and merged images of all three channels (IV) are shown in each quadrant. Note that G2-RITC-NH\(_2\) strongly interacts with the epidermal/dermal cells whereas G2-RITC-COOH and G2-RITC-Ac do not. Scale bar: 10 µm. SC, stratum corneum; VE, viable epidermis; DE, dermal layer. (CLSM images taken by Suhair Sunoqrot)

### 2.3.4 Effects of Dendrimer Hydrophobicity on Skin Permeation

The hydrophobicity of the materials used is quantified by log \( P \). Note that the log \( P \) values increase with an increase of hydrophobicity of the materials. Figure 2.7A shows that the log \( P \) values of the first three types of surface modified G2 dendrimers are all negative (-0.9 ± 0.2 for G2-RITC-NH\(_2\), -1.0 ± 0.0 for G2-RITC-Ac, and -1.3 ± 0.4 for G2-RITC-COOH). After conjugation with OA, the partition coefficients of the dendrimer-OA conjugates changed from negative to positive (1.2 ± 0.0 for G2-RITC-NH\(_2\)-OA\(_{2.3}\) and 1.4 ± 0.1 for G2-RITC-NH\(_2\)-OA\(_{2.7}\)). To investigate the relationship between the partition coefficient and skin permeation efficiency, the skin permeation efficiencies of the various G2 PAMAM
dendrimers with differing log $P$ values were compared using the Franz diffusion cells. As the amounts of dendrimers that permeated across the full skin layers were relatively negligible (less than 3%), the amount of materials remaining in the donor chambers after 24 h were recorded to estimate the amounts in the skin layers (Figure 2.7B). While a large portion (88.6%) of the hydrophilic G2-RITC-COOH was still dispersed in the donor solution, the OA-dendrimer conjugates tended to partition more into the skin. As more OA was conjugated, less material was detected in the donor solution (44.1% and 36.8% of the original amounts of G2-RITC-NH$_2$-OA$_{2.3}$ and G2-RITC-NH$_2$-OA$_{2.7}$, respectively), which correlates well with the partition coefficient results and confirms that the partition coefficient is a good indicator for the skin partitioning behavior of materials. The skin absorption of the rest of the conjugates was also calculated by measuring the amounts in the donor chambers: 62.7% for G2-RITC-NH$_2$ and 48.0% for G2-RITC-Ac.
Figure 2.7 Relationships between hydrophobicity and skin retention of the surface modified G2 dendrimers: A) Partition coefficients of various G2 PAMAM dendrimers measured using the shake-flask method. The experimental groups include G2-RITC-COOH, G2-RITC-Ac, G2-RITC-NH₂, and two types of OA-conjugated dendrimers: G2-RITC-NH₂-OA₂.₃ and G2-RITC-NH₂-OA₂.₇. The observed higher skin deposition of the G2-RITC-NH₂-OA conjugates is likely a result of their higher partition coefficients than other types of dendrimers.

B) Skin deposition and retention of various G2 PAMAM dendrimers and their conjugates after 24 h of the Franz cell experiment (error bars: standard error from the donor solution, n=3).

2.4 Discussion

Although several reports have studied PAMAM dendrimers as a potential skin penetration enhancer,¹⁵⁻¹⁹ the skin permeation and retention behaviors of PAMAM dendrimers themselves are largely unknown. Therefore, in this study, we wanted to reveal the role of size, surface charge, and hydrophobicity of dendrimers in the skin permeation/deposition of the materials in a systematic manner. To achieve this objective, our study progressed with validating three hypotheses: (i) smaller dendrimers penetrate better than larger ones; (ii)
surface modification of G2 PAMAM dendrimers enhances or alters skin permeability; and (iii) the partition coefficient (hydrophobicity) determines the permeation efficiency of the dendrimer conjugates.

The first hypothesis was assessed by comparing the size effect of dendrimers in terms of permeation efficiency and penetration depth into the skin layers (Figure 2.5). It is generally known that the smaller molecules penetrate through the skin layers more efficiently than their larger counterparts. However, it is difficult to compare the size effect in polymeric materials while maintaining other parameters constant due to their intrinsic heterogeneity in structure and chain length. Dendrimers offer precise control over their size, providing an excellent platform for systematic studies to investigate the effects of not only size, but other parameters as well. We therefore compared the skin permeation G2 and G4 PAMAM dendrimers conjugated with RITC to investigate the size effect. The Franz diffusion cell experiments revealed that the G2 conjugates displayed better skin permeation properties (up to 3.5% and up to 0.6% for G2 and G4 after 24 hrs, respectively, data not shown), which is in a good agreement with literature.

Furthermore, the confocal images of skin cross-section shown in Figure 2.5 visualize that G2 conjugates penetrate deeper into the skin layers compared to G4 PAMAM dendrimers, validating the first hypothesis. Although the dendrimer conjugates used in this study underwent an extensive purification process, it needs to be noted that the conjugates may
contain a degree of larger impurities, such as dimers, which may further prevent the skin penetration of particularly larger (G4) dendrimers.

Any molecule larger than 500 g/mol is generally considered impermeable through the skin.\(^6\) However, G2-RITC-NH\(_2\), which has a molecular weight of as high as 5,000 g/mol, still exhibits a degree of permeability and deep penetration through the porcine skin, which implies that it utilizes an alternative mechanism of penetration. Venuganti and Perumal also found, through transepidermal water loss, skin resistance measurements, and ATR-FTIR studies, that cationic dendrimers alter the skin lipid layers. G2 PAMAM dendrimers reduced skin resistance to a greater extent than higher generations of dendrimers.\(^{16}\) Moreover, a series of papers published by Hong et al. demonstrated that cationic PAMAM dendrimers, such as primary amine terminated ones, induced nano-scale holes on supported lipid bilayers.\(^{8,11,12}\) This membrane permeabilization mechanism plays a key role in the cellular internalization of PAMAM dendrimers and other positively charged polymers that have been commonly used for non-viral cell transfection or gene delivery.\(^{12}\) The reduced skin resistance and membrane permeabilization by positively charged dendrimers may explain the observed skin permeation/penetration of the materials.

Our finding from the size comparison study, i.e., G2>G4, led us to further investigate the other two hypotheses using G2 PAMAM dendrimers as basal materials. Hypothesis 2 was assessed by a series of assays including permeation tests and confocal microscopy
observations on cross-sections of the porcine skin. We have found that G2-RITC-NH₂ permeated the skin less effectively than G2-RITC-COOH and G2-RITC-Ac (Figure 2.5E). Interestingly, when delivering 5FU through the skin pretreated with PAMAM dendrimers with different surface functional groups, it was reported that the order of enhancement in $K_p$ of 5FU was G4-NH₂ > G4-OH > G3.5-COOH. However, the permeation behaviors of surface-modified dendrimers themselves do not necessarily parallel the permeation enhancement effects for a small molecule. As previously reported, amine-terminated PAMAM dendrimers internalized into the cells non-selectively. The negatively charged dendrimers, on the other hand, did not internalize or bind to the cells. Due to the concentration gradient across the skin and possibly charge repulsions between the dendrimers and the negatively charged cell membrane, it is hypothesized that they go through the skin layers through an extracellular pathway that could be faster than the transcellular pathway taken by G2-RITC-NH₂. In contrast, the surface of G2-RITC-Ac is nearly neutral. It may simply follow the concentration gradient to go through the skin layers extracellularly, which also may result in a faster penetration compared to G2-RITC-NH₂. Since the theoretical molecular weight of G2-RITC-Ac (4,398 g/mol) is smaller than G2-RITC-COOH (5,346 g/mol), and based on the conclusion from the first hypothesis, it may go through the skin layers faster than G2-RITC-COOH. Due to the small size and flexible/deformable nature of PAMAM dendrimers even after surface modification, they may go through the skin layers more easily by taking the extracellular route, which results
in the higher permeation efficiencies observed with G2-RITC-COOH and G2-RITC-Ac compared to G2-RITC-NH₂.

This hypothesis was further tested by confocal microscopy observations. As shown in Figure 2.6A, G2-RITC-NH₂ internalized into the skin cells within 1 h. This could explain the lower skin permeation efficiency of G2-RITC-NH₂, compared to G2-RITC-COOH and G2-RITC-Ac, which did not internalize into the cells (Figure 2.6B and 2.6C). Amine-terminated dendrimers internalized into individual cells both in the epidermal and dermal layers by interactions between their positively charged termini and the negatively charged cell membranes.¹¹ This increased uptake leads to higher accumulation of the materials in the skin layers, which makes them potential candidates for localized treatment of skin diseases. Meanwhile, carboxylated and acetylated dendrimers appeared to penetrate the skin layers better than their amine-terminated counterparts (Figure 2.5E). Charge repulsions, particularly between the carboxylated dendrimers and the cell membranes, may have forced them to take an extracellular route, allowing this rapid diffusion. This result highlights the potential advantage of using the carboxylated (or acetylated) dendrimers for systemic administration of active compounds through the skin, which requires fast and deep penetration through the skin layers and access to the circulation.

The third hypothesis regarding the effect of hydrophobicity on skin permeation was tested by altering the 1-octanol-to-PBS partition coefficient (log P) of G2-RITC-NH₂ by
conjugation with different amounts of OA. The reported partition coefficient for G2-NH₂ is -2.0,²⁷ and that of OA is 7.3 at pH 7.4,²⁹ which indicates that the G2 PAMAM dendrimer is hydrophilic whereas OA is highly hydrophobic. Since the optimum range of log P for SC partitioning is 1-3,³ neither of them is easily SC-permeable when used separately. We tested if the covalent conjugation of these molecules could significantly change their partitioning behavior, and potentially make their joint log P fall into the optimum range for best skin permeability. The results showed that by attaching OA, the log P values for the conjugate was reversed from negative to positive (Figure 2.7A). The transition from negative to positive was dependent on the number of the hydrophobic molecules attached. Increasing the number of OA on the surface of G2 dendrimers increased the hydrophobicity of the final conjugates.

Hydrophobic modification of the dendrimers resulted in log P values (1.2 and 1.4 for G2-RITC-NH₂-OA₂,³ and G2-RITC-NH₂-OA₂,₇, respectively) that theoretically permit the dendrimer conjugates to readily partition into the SC. In fact, the results from the Franz cell experiments showed significantly enhanced skin partitioning of the dendrimer-OA conjugates (Figure 2.7B). The increase in %permeation into the receiver solutions was marginal; however, this can be attributed to the use of pure PBS in the receiver chambers as opposed to adding ethanol as reported by others.¹⁷, ¹⁹ Further studies need to done to confirm the upper limit of the partition coefficient that dendrimer-OA conjugates can or should not exceed. Whether the value should be less than 3 for optimal skin permeation is
not yet tested in our case. A linear relationship between the partition coefficient and skin permeability might exist, which could be the subject of our future investigations.

In this study, we have demonstrated that physicochemical properties of PAMAM dendrimers directly affect the skin interactions of the macromolecules. As noted, our results provide a guideline for the future development of transdermal drug delivery systems. To summarize, amine-terminated dendrimers would be beneficial for the localized transdermal delivery given their enhanced skin deposition and retention. Acetyl- or carboxyl-terminated ones would be more effective for systemic delivery through topical administration, given their enhanced permeation. Furthermore, smaller dendrimers would exhibit enhanced skin permeation and strong dendrimer-skin interactions, particularly when their hydrophobicity is optimized through conjugation with hydrophobic molecules such as drug molecules. In addition to the potential application of the surface modified dendrimers for transdermal delivery, the modularity in surface engineering enables them to be applied for controlled intestinal absorption after oral administration. The paracellular and transcellular permeation pathways, observed using Caco-2 cells, well correlate to our results presented in this paper, indicating the potential of the surface-engineered dendrimers to overcome the challenge of low permeability through the intestinal barriers.
2.5 Conclusion

Collectively, the results highlighted in this study confirmed the three hypotheses and allowed us to reach three main conclusions: i) smaller dendrimers penetrate the skin better than larger ones, i.e., the skin permeation and penetration depth of G2 are superior to those of G4; ii) surface modifications of PAMAM dendrimers increase skin permeation efficiencies and dictate penetration pathways; and iii) the G2-RITC-NH₂-OA conjugates with log $P$ values between 1-3, the reportedly optimal range for skin partitioning, result in enhanced skin deposition, compared to not only unmodified dendrimers but also all other G2-RITC conjugates used in this study. Our results indicate that by adjusting the stoichiometry of the dendrimer-model drug conjugation, the partition coefficient can be manipulated, which serves well as a predictor of skin permeation of the material. After surface modification with charged moieties and adjustment of log $P$ values, G2 PAMAM dendrimers could be further modified through conjugation with drug molecules, targeting moieties, and imaging probes to become multifunctional, programmed nanocarriers to achieve controlled therapeutic administration through the transdermal route.
2.6 References


CHAPTER 3.

Dendron-Based Micelles for Topical Delivery of Endoxifen: A Potential Chemo-
Preventive Medicine for Breast Cancer

3.1 Introduction

Endoxifen (4-hydroxy-N-desmethyl tamoxifen or EDX) is the primary metabolite of tamoxifen and is known to be responsible for the overall effectiveness of tamoxifen against estrogen receptor-positive (ER+) breast cancer.\textsuperscript{1} The conversion of tamoxifen to its active form EDX is dependent on the activities of cytochrome P450 isoforms (e.g. CYP2D6, and CYP3A4/5).\textsuperscript{1, 2} Although controversial,\textsuperscript{3} women with genetically impaired CYP2D6 activity (homozygous for two null CYP2D6) have been reported to have a higher propensity for breast cancer recurrence with tamoxifen treatment,\textsuperscript{1} a concern which also applies to the prevention setting. Thus, direct administration of EDX to the breast through its skin envelope could potentially avert the problem of inefficient metabolism of tamoxifen. Furthermore, local transdermal delivery to the breast should decrease side effects such as hot flashes, vaginal atrophy, higher risk of endometrial cancer and thromboembolic events, which are the collateral damage of systemic exposure to tamoxifen.\textsuperscript{4-6} Instead, a localized, topical EDX delivery system to the breast would offer a promising alternative way to minimize its systemic exposure,\textsuperscript{7} which could potentially reduce the side effects observed from conventional oral tamoxifen/EDX treatment and help women with impaired CYP2D6 activities.
For EDX to be effectively delivered through the skin layers, it must traverse through multiple barriers. In particular, the stratum corneum (SC), the topmost skin layer, is the most significant barrier that needs to be overcome to effectively deliver drugs across the skin layers.\(^8\) EDX is a relatively hydrophobic small molecule with an octanol-water partition coefficient (logP) of 4.09.\(^9\) As this logP value is beyond the optimum range (between 1 and 3) for efficient skin permeation,\(^10\)-\(^14\) EDX alone cannot effectively pass through the skin layers without the use of penetration enhancers.\(^15\)

Chemical penetration enhancers (CPEs), such as ethanol and sodium dodecyl sulfate (SDS), have been commonly used to enhance the skin permeability of therapeutic molecules that are otherwise skin impermeable.\(^12\) However, significant irritation and adverse effects have been often observed because of skin dehydration and/or SC lipid disruption, which is proportional to the penetration enhancement abilities of the individual CPE.\(^12,\)\(^16\) To overcome the problem of skin irritation, nanocarriers such as polymeric micelles have been introduced as potential platforms for transdermal drug delivery due to their nanoscale size, biocompatibility, high drug and gene adaptability, tunable surface functionality, and controlled release profiles.\(^17\)-\(^20\) A few types of amphiphilic block copolymer-based micelles prepared from methoxy-poly(ethylene glycol)-poly(e-caprolactone) (mPEG-PCL),\(^21\) poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) (PEG-PPG-PEG, or pluronics),\(^17\) and methoxy-poly(ethylene glycol)-hexyl substituted polylactide (mPEG-hsPLA) block copolymers\(^19\) have been reported to successfully enhance transdermal drug
delivery of oridonin (anticancer), econazole nitrate (antifungal), and plasmids (carries β-galactosidase gene), respectively.

In the previous study, we investigated the permeation of polyamidoamine (PAMAM) dendrimers across skin layers.¹⁰ We reported that generation (size) and surface charge of the dendrimers played significant roles in their penetration behaviors. Lower generation G2 dendrimers penetrated the skin better than larger G4 dendrimers and surface-modification by acetylation or carboxylation produced an enhancement of dendrimer permeation. However, drug delivery using dendrimers is limited due to the number of available surface functional groups, where the only method to stably carry drug molecules is through chemical conjugation that could also result in decreased drug efficacy.²² The lack of a highly reactive functional group on EDX further limits the use of dendrimers for this particular drug molecule.

To maintain drug efficacy with maximized loading, we have recently developed biocompatible, amphiphilic PEGylated dendron-based copolymers (PDCs), which are a hybridization of dendritic structure and linear-block copolymers.²³ At concentrations higher than their critical micelle concentration (CMC), they can self-assemble into dendron micelles (DMs) (Figure 3.1). The DMs displayed CMCs on the order of $10^{-8}$ M, demonstrating their remarkable thermodynamic stability for hydrophobic drug encapsulation and stability in aqueous phase. Additionally, the DMs are almost completely
covered by a dense layer of PEG, which confers similar properties of dendrimers to the DMs such as high-density peripheral functional groups and provides ease of surface functionalization.\textsuperscript{22}

Here, we hypothesized that the EDX-loaded DMs would achieve enhanced skin permeation, water solubility/stability, and controlled release profile of EDX without a cost of its efficacy compared to free EDX. To test our hypotheses, we compared the skin permeation efficiency of EDX delivered by the DMs to that by cationic liposomes and a traditional CPE (ethanol) using both hairless mouse and human skin samples. We have also investigated the surface charge effect of the DMs on drug loading, \textit{in vitro} drug release, and skin permeation by modulating the end-group functionalities of the DMs. Our data indicate that the DMs have great potential as a novel platform for controllable topical delivery of EDX.

\textbf{3.2 Materials and Methods}

\textbf{3.2.1 Materials}

EDX (N-Desmethyl-4-hydroxy Tamoxifen, $M_w$ 373.49, E/Z 1:1 mixture) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, MW 698.54), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, MW 677.93), and Cholesterol (MW 386.65) were obtained from
Avanti® Polar Lipids, Inc. (Alabaster, AL). Surface modified PEGylated dendron-based copolymers (PDC) and their respective dendron micelles (DM-(end group)) were prepared in house as described previously with the following end-groups (-NH₂, -COOH, and -Ac).²⁴ Coumarin-6 (Cou6), 17β-estradiol (E2), all solvents for chemical reactions, including dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dichloromethane (DCM) and methanol, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise specified.

3.2.2 Preparation of EDX-loaded DMs with Different Surface Functional Groups

EDX-loaded DMs were prepared using a dialysis method as described in our earlier publications.²⁴ Briefly, 10 milligrams of the surface modified PDC were dissolved in 1 mL (10 mg/mL) of DMF along with 1.5 mg of EDX. The polymer-EDX mixture was then transferred to a dialysis membrane (MWCO 3,500) and dialyzed for 24 h against 500 mL of double distilled water (ddH₂O). After dialysis, the micelle solution was collected into 1.5 mL centrifuge tubes and centrifuged at 10,000 rpm for 5 min at 25°C. The supernatant was carefully collected and freeze-dried for 2 days to obtain the EDX-loaded DM. Characterizations including particle size, surface charge, morphology observations, and drug loading measurements were described in 3.2.5.
3.2.3 Preparation of EDX-loaded Cationic Liposomes

EDX-loaded liposomes were prepared by a thin film hydration method. DOTAP (4.5 mg), DMPC (4.3 mg), Cholesterol (1.2 mg) (2:2:1 molar ratio), and EDX (1.5 mg) were dissolved in 1 mL of chloroform. The solvent was evaporated using a rotary evaporator until the materials formed a thin, dried film at the bottom of the round-bottom flask. Two milliliters of ddH$_2$O were added to rehydrate (completely re-dissolve) the lipid film by vortexing at high intensity. The rehydrated materials were then sonicated for 30-60 min in a water bath sonicator. The size of the liposome was further refined by extrusion 40 times through a polycarbonate membrane (pore size 100 nm) at 40 psi. The liposomes prepared were then collected into 1.5 mL centrifuge tubes and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was carefully collected and stored in a separate tube for characterization. The liposome pellet was re-suspended in 1 mL of 5% sucrose/water solution for lyophilization (1 day).

3.2.4 Preparation of Two-dye DMs with Different Surface Functional Groups

To prepare two-dye dendron micelles, 2 mg of acetylated, RHO-labeled PDC$^{24}$ was mixed with 10 mg of unlabeled, PDC-NH$_2$ in 1.2 mL DMF, and 2% (wt%) of Cou6 was added to the mixture. The materials were fully dissolved and mixed by brief vortexing followed by gentle stirring for 2 h. The Cou6-encapsulated, RHO-labeled two-dye dendron micelles were prepared using the dialysis method, and the final products were obtained as powder after lyophilization as described above.
3.2.5 Characterization of Drug-loaded DMs and Liposomes

Particle size (diameter, nm) and surface charge (zeta potential, mV) of the EDX-loaded micelles and liposomes were measured from three repeat measurements by quasi-elastic laser dynamic light scattering (DLS) using a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA).\textsuperscript{24,26} The materials were dispersed in ddH\textsubscript{2}O to a concentration of 500 µg/mL and briefly vortexed prior to each measurement.

The micelle morphology was also observed using transmission electron microscopy (TEM, JEM-1220, JEOL Ltd., Japan).\textsuperscript{24,26} A drop (5 µL) of micelle suspension (0.2 mg/ml) after filtration (pore size 0.45 µm) was placed on a 300 mesh copper grid coated with carbon. The sample was stained with a drop of 2% phosphotungstic acid and dried at room temperature in a desiccator for 1 day. The diameter of each type of micelle was measured using 10 randomly selected particles from each TEM image. The average and standard deviation were calculated.

Drug loading was determined using a reversed-phase (RP) HPLC\textsuperscript{27} and the conditions were used for all other EDX detection experiments. One hundred micrograms of the lyophilized micelles were dissolved in 1 mL of DMSO, followed by vortexing and 6 h of incubation at room temperature with gentle shaking before measurement. A series of EDX (2.0, 7.8, 31, 125 and 500 ng/mL) solutions in DMSO were prepared and used to plot the standard curve.
for the quantification of drug loading using RP-HPLC. All samples were filtered through 0.2 µm Teflon syringe filters. A CHIPhearsor (Jelight Co., Inc., CA) was used as a pre-column photochemical reactor to convert endoxifen into its fluorescent derivatives at 254 nm. Ten microliters of each sample were injected into a Symmetry C18 column (3.5/5 µm, 4.6 × 150 mm, Waters, Milford, MA) connected to a Waters HPLC system composed of a 717 Autosampler and a 600E series pump under isocratic mode. The mobile phase was composed of 46% (v/v) acetonitrile and 54% (v/v) KH$_2$PO$_4$ buffer (20 mM, pH 3.0). The flow rate was maintained at 1 mL/min. The column temperature was set at 25°C. EDX was detected at 254 nm excitation and 390 nm emission wavelengths using a Waters 474 Fluorescence detector. Loading was expressed as micrograms of EDX per milligram of micelle or liposome (µg EDX/mg micelle or liposome). Loading efficiency was calculated from the ratio of the actual measured loading to the theoretical loading (For micelles, the theoretical loading was the amount of EDX added divided by the mass of each PDC used in the formulation.$^{26}$ For liposomes, the theoretical loading was the amount of EDX added divided by the mass of lipids plus sucrose used in the formulation).$^{28}$

### 3.2.6 In Vitro Drug Release Test

The drug release test was performed using a vertical Franz diffusion cell device (Φ7 mm with 0.38 cm$^2$ exposure area, PermeGear Inc., Hellertown, PA). A cellulose membrane (MWCO 3,500) was sandwiched between the donor and receiver chamber. The micelles and liposomes loaded with the same amount of EDX were suspended in ddH$_2$O and 100 µL
of each solution were applied to each donor chamber. The receiver solution (release media), composed of 30% ethanol and 70% normal saline, was stirred constantly at 600 rpm and the temperature was maintained at 37 °C. At predetermined time intervals, 250 µL of release medium were removed and replaced with an equal amount of fresh receiver solution and analyzed using RP-HPLC (see 3.2.5 for detailed method descriptions). The release profile was obtained by plotting the cumulative EDX release% into the receiver solution against time.

3.2.7 In Vitro Anti-proliferation Assay of Breast Cancer Cells

The ER+ MCF-7 and the ER− MDA-MB-231 breast cancer cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle's medium (DMEM, Mediatech, Inc., VA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen™) and 1% (v/v) penicillin/streptomycin in a humidified incubator at 37°C and 5% CO₂. When the cell monolayers were approximately 70% confluent, the culture medium was replaced with an estrogen-depleted medium (stripped medium) consisting of phenol-red free DMEM (Mediatech, Inc., VA) supplemented with 10% (v/v) charcoal-stripped fetal bovine serum (Invitrogen™), 1% (v/v) 200 mM L-Glutamine, and 1% (v/v) penicillin/streptomycin under the same incubation conditions mentioned above. To remove any residual estrogen, the monolayer was washed and replenished with fresh stripped medium every day for 3 days; when cells were >90% confluent, the monolayer was detached from the cell culture flask with phenol red-free trypsin and counted using a
hematometer. Cells were seeded onto a 96-well plate at a concentration of 2,000 cells per well in 100 µL of the stripped medium. The cells were allowed to attach and stabilize overnight, after which 100 µL of fresh medium with or without 0.1 nM of estradiol (E2) were added to each well, followed by EDX-loaded or empty DM treatment. An ER-negative cell line, MDA-MB-231, was also employed as a control. The cells were maintained in L-15 cell culture media supplemented with 10% (v/v) charcoal-stripped fetal bovine serum (InvitrogenTM), and 1% (v/v) penicillin/streptomycin under the same incubation and treatment conditions mentioned above. After the stripped cells were incubated in the phenol red-free medium with or without 0.1 nM of E2 overnight, 100 µL of stripped medium containing 0.1 nM of E2 with various concentrations of EDX (0, 10, 50, 100, and 500 nM) and 0.01% DMSO (vehicle), EDX-loaded DMs with equivalent drug concentrations, or empty DMs with equivalent micelle concentrations were added to each well in 96-well plates (n=3). The treatment was repeated every other day up to the twelfth day. The cell proliferation was assessed using a CellTiter 96 AQueous One Solution (MTS) Reagent (Promega Corp., Madison, WI) according to the manufacturer’s protocols. Briefly, the culture media were removed and the cells were washed with pre-warmed PBS and fresh basal medium; then, 100 µL of fresh complete medium, along with 20 µL of the MTS reagent, were added to each well. The plate was incubated at 37°C in a humidified, 5% CO2 atmosphere for 2 h. The UV absorbance was measured at 490 nm using a Labsystems Multiskan Plus microplate reader (Labsystems, Finland).
3.2.8 Preparation of Mouse and Human Skin Samples and Franz Diffusion Cell Experiments

The research use of hairless mice was approved by the Office of Animal Care and Institutional Biosafety Committee at the University of Illinois at Chicago. Full thickness mouse skin (average thickness 360 ± 20 µm, mean ± SD) was collected from the dorsal side of the 6-8 weeks old SKH1 hairless mice (Charles River Laboratory, Boston, MA). Subcutaneous fat and blood vessels were carefully removed using cotton swabs. The signed informed consents were obtained from the patients before the acquisition of human skin samples from the operating room. The research use of human skin samples was approved by the Institutional Review Board at the Northwestern University, under the approved protocol number CR4_STU00023488. The split-thickness skin (STS) samples from two subjects were prepared and used as described in a previous study. The thickness of the STS samples was 390 ± 30 µm (mean ± SD).

Undamaged skin was cut into 1.2 × 1.2 cm² squares, rinsed with normal saline, and sandwiched between the donor and receiver chambers of the Franz diffusion cells with the SC side facing upward. The receiver chambers were filled with fresh normal saline containing 30% ethanol (v/v). After equilibration of the skin at 37°C for 30 min, 100 µL of each formulation were applied to the donor chambers. For mouse skin permeation experiments, four EDX formulations in ddH₂O (DM-NH₂, DM-Ac, DM-COOH and liposomes containing same amount of drug) were compared with free EDX in ddH₂O.
containing 60% ethanol. For human skin permeation experiments, three formulations were compared: 1) EDX-loaded DM-COOH in ddH$_2$O (4 mg/mL); 2) EDX in phosphate buffer (PB, 2 mM KH$_2$PO$_4$, 4 mM Na$_2$HPO$_4$, pH 7.0) containing 60% of ethanol (v/v); and 3) EDX in the 60% ethanol in the presence of 0.5% (v/v) of oleic acid (OA) as used in previous study. The donor chambers were covered with Parafilm™ to avoid evaporation. The first sample collection was performed at 0 h by withdrawing 250 µL of the receiver solution from each sampling port and replacing with 250 µL of fresh receiver solution. Sample collection was performed at predetermined time points up to 24 h. All solutions were kept at 4°C in dark before analysis.

3.2.8 Preparation of Mouse and Human Skin Samples and Franz Diffusion Cell Experiments

Full thickness mouse skin (average thickness 360 ± 19 µm, mean ± SD) was collected from the dorsal side of the 6-8 weeks old SKH1 hairless mice (Charles River Laboratory, Boston, MA). Subcutaneous fat and blood vessels were carefully removed using cotton swabs. The signed informed consents were obtained from the patients before the acquisition of human skin samples from the operating room. The split-thickness skin (STS) samples from two subjects were prepared and used as described in a previous study. The thickness of the STS samples was 389 ± 29 µm (mean ± SD).
Undamaged skin was cut into $1.2 \times 1.2\ cm^2$ squares, rinsed with normal saline, and sandwiched between the donor and receiver chambers of the Franz diffusion cells with the SC side facing upward. The receiver chambers were filled with fresh normal saline containing 30% ethanol (v/v). After equilibration of the skin at 37°C for 30 min, 100 µL of each formulation were applied to the donor chambers. For mouse skin permeation experiments, four EDX formulations in ddH₂O (DM-NH₂, DM-Ac, DM-COOH and liposomes) containing the same amount of drug were compared with free EDX in 60% ethanol. For human skin permeation experiments, three formulations were compared: 1) EDX-loaded DM-COOH in phosphate buffer (PB, 2 mM of KH₂PO₄ and 4 mM of Na₂HPO₄, pH 7.0) (4 mg/mL); 2) EDX in PB containing 60% of ethanol (v/v); and 3) EDX in the same solution as in 2) plus 0.5% of oleic acid (OA). The donor chambers were covered with Parafilm™ to avoid evaporation. The first sample collection was performed at 0 h by withdrawing 250 µL of the receiver solution from each sampling port and replacing with 250 µL of fresh receiver solution. Sample collection was performed at designated time points up to 24 h. All solutions were kept at 4°C in dark before analysis.

### 3.2.9 CLSM Imaging of the Mouse Skin Treated with Two-dye Dendron Micelles

SKH-1 hairless mouse skin was exposed to the two-dye dendron micelles with different surfaces for 24 h in the Franz cell setup. The skin area that was exposed to the treatment was carefully collected, rinsed twice with ddH₂O for 10 min and embedded into cryomolds (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA). Skin was cryosectioned into 80
µm-thick slices and placed on anti-frost glass slides. The slides were then treated with 10% neutral buffered formalin for 10 min at RT and rinsed with ddH₂O. The fixed skin samples were then mounted with antiphoto-bleaching mounting media with DAPI (Vector Laboratory Inc., Burlingame, CA) and covered with glass cover slips. The slides were visualized using a Zeiss LSM 510 Meta CLSM (Carl Zeiss, Germany). The 488 nm line of a 30 mW tunable argon laser was used for the excitation of Cou6, a 1 mW HeNe at 543 nm for RHO, and a 25 mW diode UV 405 nm laser for DAPI. Emissions were filtered at 505-530 nm, 565-595 nm and 420 nm for Cou6, RHO, and DAPI, respectively.

3.2.10 Measurements of Skin Permeation and Retention

After the Franz cell experiment, donor solutions were collected and kept at 4°C in dark before analysis. Skin was also collected and thoroughly cleaned. The effective exposure area on the skin was cut and homogenized in a 1.5 mL centrifuge tube. DMSO was used as an EDX extracting reagent. After 12 h of extraction, the samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for analysis. RP-HPLC was used to detect EDX from the receiver solutions, skin extracts, and donor solutions using the conditions described above. For two-dye micelles treated skin, the fluorescence intensity in the donor and receiver solutions, as well as in the skin extracts were detected using a SoftMax Pro spectrofluorometer (Spectra MAX, Molecular Devices Inc., Sunnyvale, CA). For the detection of Cou6, the excitation wavelength was 444 nm and the emission
wavelength was 510 nm. For the detection of RHO from the dendron micelle, the excitation wavelength was set at 555 nm, and the emission wavelength was 590 nm.

3.2.11 Statistical Analysis

Data processing and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) based on a one-way ANOVA followed by Tukey’s post hoc test. Data were considered significant at $p < 0.05$.

3.3 Results and Discussion

A DM platform was developed to achieve effective EDX encapsulation and delivery via topical administration. PDCs were synthesized from the combination of a single hydrophobic core-forming block PCL with multiple hydrophilic PEG chains mediated by a G3 polyester dendron. Encapsulation of EDX into DMs through self-assembly provided a water-soluble drug formulation, while maintaining the surface functionality of the DMs and offered the potential to deliver EDX without the use of CPEs.

The particle size, zeta potential, and drug loading values are listed in Table 3.1. The encapsulation of EDX into DMs yielded number-weighted average diameters between 40 and 50 nm with a narrow size distribution, which were in agreement with images obtained
using transmission electron microscopy (TEM) (Figure 3.1A-C). In contrast, EDX-loaded liposomes employed as a control group had an average size of 100 nm in diameter. The smaller size of DM resulted in a larger surface-area-to-volume ratio, which would facilitate better interaction with the skin surface.\textsuperscript{12} The zeta potential measurements revealed that amine-terminated dendron micelles (denoted as DM-NH\textsubscript{2}) and liposomes were positively charged (42.9 ± 1.6 mV and 28.6 ± 0.3 mV, respectively) when measured in ddH\textsubscript{2}O (pH 5.6). Following surface modification, the surface charge of acetamide-terminated micelles (denoted as DM-Ac) was decreased to nearly neutral (-2.7 ± 1.4 mV), and that of carboxyl-terminated micelles (denoted as DM-COOH) was reversed to a negative value (-23.2 ± 3.5 mV). Drug loading measurements revealed that liposomes encapsulated the least amount of EDX (0.4 ± 0.0 µg/mg liposome), likely due to their aqueous core and smaller hydrophobic regions than micelles, which hindered effective encapsulation of hydrophobic molecules.\textsuperscript{34} Interestingly, DM-COOH encapsulated the highest amount of EDX (29.7 ± 2.0 µg/mg DM-COOH, almost 70-fold higher than in liposome), while DM-NH\textsubscript{2} and DM-Ac demonstrated modest drug loading (3.0 ± 1.3 µg/mg DM-NH\textsubscript{2} and 5.7 ± 0.2 µg/mg DM-Ac).
Table 3.1 Characterization of EDX-loaded dendron micelles (DMs) and liposomes

<table>
<thead>
<tr>
<th>Materials</th>
<th>Size (nm)a</th>
<th>ζ-Potential (mV)</th>
<th>Drug Loadingb</th>
<th>Loading%c</th>
<th>EE%d</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDX/DM-NH₂</td>
<td>48.7 ± 7.1</td>
<td>42.9 ± 1.6</td>
<td>3.0 ± 1.3</td>
<td>0.3 ± 0.1%</td>
<td>2.0 ± 0.9%</td>
</tr>
<tr>
<td>EDX/DM-Ac</td>
<td>37.4 ± 6.2</td>
<td>-2.7 ± 1.4</td>
<td>5.7 ± 0.2</td>
<td>0.6 ± 0.0%</td>
<td>3.8 ± 0.1%</td>
</tr>
<tr>
<td>EDX/DM-COOH</td>
<td>48.4 ± 6.1</td>
<td>-23.2 ± 3.5</td>
<td>29.7 ± 2.0</td>
<td>3.0 ± 0.2%</td>
<td>19.8 ± 1.4%</td>
</tr>
<tr>
<td>EDX/Liposome</td>
<td>100.5 ± 20.9</td>
<td>28.6 ± 0.3</td>
<td>0.4 ± 0.0</td>
<td>0.1 ± 0.1%</td>
<td>0.0 ± 0.0%</td>
</tr>
<tr>
<td>Cou-6/DM-RHO-NH₂</td>
<td>75.2 ± 8.3</td>
<td>-</td>
<td>1.2 ± 0.1</td>
<td>0.1 ± 0.0%</td>
<td>6.1 ± 0.7%</td>
</tr>
<tr>
<td>Cou-6/DM-RHO-Ac</td>
<td>24.0 ± 4.2</td>
<td>-</td>
<td>1.6 ± 0.2</td>
<td>0.1 ± 0.0%</td>
<td>8.2 ± 1.1%</td>
</tr>
<tr>
<td>Cou-6/DM-RHO-COOH</td>
<td>24.5 ± 3.3</td>
<td>-</td>
<td>1.2 ± 0.1</td>
<td>0.1 ± 0.0%</td>
<td>6.2 ± 0.7%</td>
</tr>
</tbody>
</table>

aMeasured using dynamic light scattering.
bMeasured using RP-HPLC, expressed as “µg of drug / mg of DMs”
cEquation 1. Calculation of Drug Loading%:
\[
\text{Loading\%} = \frac{\text{Total amount of EDX encapsulated (µg)}}{\text{Total weight of nanoparticles(µg)}} \times 100\%
\]
dEquation 2. Calculation of Encapsulation Efficiency% (EE\%):
\[
\text{Encapsulation Efficiency\%} = \frac{\text{Amount of EDX encapsulated(µg)}}{\text{Amount of EDX added for encapsulation(µg)}} \times 100\%
\]

All data presented are Mean ± SD.
The substantially higher drug loading of DM-COOH may be explained by the ion-pairing phenomenon. According to Turco Liveri et al.,\textsuperscript{35} hydrophobic drug molecules encapsulate into the core of micelles through three steps of aqueous, hydrophilic corona, and hydrophobic core pseudophases. In particular, the aqueous pseudophase is important during the encapsulation process using dialysis where initial interactions between drug molecules and the surface groups of the micelles occur. As a result, differently charged surface groups may be responsible for the observation of distinctly different drug loading of the DMs. Depending on the pKa of the drug encapsulated and pH of the aqueous solvent,
ion pairing between the surface groups and the drug molecules may occur, inducing drug loading thermodynamically favorable. In the case of DM-COOH, EDX with an estimated pKa above 9.0 (estimated using Physico-chemical property predictors, ChemAxon, Cambridge, MA) will be positively charged at neutral pH, whereas the carboxyl group of DM-COOH will be negatively charged based on the zeta potential measurement, facilitating the formation of ion pairs.

Next, the release profiles of EDX from the DMs and liposomes were monitored. As shown in Figure 3.2, cationic liposomes completely released the encapsulated EDX within 8 h, indicating that the liposomes were unable to stably encapsulate EDX. On the other hand, release of EDX from the surface-modified DMs occurred in a controlled manner following a biphasic release profile. The initial fast release of EDX within the first 8 h could be attributed to the possibility that a portion of EDX molecules were adsorbed on the surface of the DMs during the micelle preparation process. The remaining encapsulated EDX was then released slowly and steadily from the DMs over 144 h (6 days). The release of EDX appeared to be surface charge-dependent. Although applied with the same initial dose based on EDX, among three types of DMs, DM-COOH with the highest drug loading exhibited fastest drug release, whereas DM-NH$_2$ had the lowest drug loading and slowest release rate.
**Figure 3.2** *In vitro* EDX release from liposomes and surface modified DMs. The release of EDX from DMs followed the general trend of Liposome faster than the DM-COOH, followed by the DM-Ac and the DM-NH$_2$ over 6 days. Note that the liposomes reached 100% release of EDX only after 8 h. **A**) Release profiles of EDX from various formulations over the first 8 h. **B**) Release profiles of EDX over 6 days. Error bars: Standard error (SE, n=3).

To ensure that EDX maintains its ER-dependent anti-proliferative effect after encapsulation into the DMs, MTS assays were performed on ER$^+$ MCF-7 and ER-negative (ER$^-$) MDA-MB-231 breast cancer cells. Free EDX exhibited a concentration-dependent growth inhibition against MCF-7 cells (Figure 3.3) but not against MDA-MB-231 cells (Figure 3.4). DM-loaded EDX treated at the same concentration as free EDX demonstrated comparable efficacy. The empty micelles, on the other hand, did not cause significant cytotoxicity towards MCF-7 cells at a concentration up to 1 µM (Figure 3.5), which is in agreement with our previous report where concentrations of surface modified empty DMs were nontoxic to KB cells at concentrations up to 100 µM.$^{24}$ These results indicate that the observed growth inhibition of MCF-7 cells is solely due to the fact that EDX maintains its ER-
specific anti-proliferative efficiency after encapsulation, and that the EDX-loaded DMs are non-toxic, suitable for delivery of chemo-preventive medicine.
**Figure 3.3** *In vitro* anti-proliferative effect of EDX in its free form and in surface-modified DMs evaluated using MTS assay. The anti-proliferative effect of EDX encapsulated into DMs is comparable to that of free EDX. All groups were treated with 0.1 nM of E2 (β-estradiol) except for the vehicle group (0.01% DMSO). Error bars: standard deviation, SD, n=3. *p*<0.05; *** *p*<0.001; **** *p*<0.0001.

![Relative Viability % of MDA-MB-231 Cells (Mean ± SD)](image)

**Figure 3.4** *In vitro* cell viability after EDX or EDX-loaded DM Treatments. Estrogen receptor-negative (ER-) MDA-MB-231 breast cancer cells were treated with EDX or EDX-loaded DMs with different concentrations. Neither free EDX nor EDX-loaded DMs exhibited significant anti-proliferative effect against MDA-MB-231 cells, indicating the EDX’s effect against cell proliferation is ER-dependent. EDX/DM-COOH, EDX/DM-Ac, and DM-NH₂ are EDX-loaded DMs with different surface functional groups. All groups were treated with 0.1 nM of E2, and the cell viability was relative to the cells that were not treated with EDX formulations. Error bars: standard deviation, SD, n=3.
Figure 3.5 In vitro cell viability after empty DM Treatments. MCF-7 cells were treated with empty DMs with different concentrations for 3 days. The empty DMs did not exhibit significant anti-proliferative effect against MCF-7 cells, indicating the biocompatibility of the PEGylated dendron-based copolymers. Empty DM-COOH, DM-Ac, and DM-NH$_2$ are empty DMs with different surface functional groups. All groups were treated with 0.1 nM of E2, and the cell viability was relative to the cells that were not treated with DMs. Error bars: standard deviation, SD, n=3.

We then compared our DM systems with CPEs and cationic liposomes, which have been commonly used for transdermal drug delivery,\textsuperscript{37} in terms of skin permeation and retention. The skin permeation parameters of EDX in various formulations are summarized in Table 3.2. Using hairless mouse skin, we observed that the rate of EDX permeation (flux) was proportional to drug loading, i.e., DM-COOH $>>$ DM-Ac $>$ DM-NH$_2$ with the flux of 155, 67, and 45 ng/cm$^2$/h, respectively (Figure 3.6A). This finding is consistent with our previous report where the penetration behaviors of PAMAM dendrimers were surface group dependent.\textsuperscript{10} Similar to the drug loading, the substantially enhanced EDX permeation with DM-COOH can be also explained by ion pairing. The charge
sequestration of EDX by ion pairing may potentially facilitate skin permeation of EDX because charged drug molecules typically cannot partition into or permeate across the skin.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Skin Type</th>
<th>Vehicles</th>
<th>Flux\textsuperscript{a} (ng/cm\textsuperscript{2}/h)</th>
<th>Lag time\textsuperscript{b} (h)</th>
<th>Permeability Coefficient (Kp, cm/h)</th>
<th>Diffusion Coefficient (D, cm\textsuperscript{2}/h)</th>
<th>Enhancement Ratio (ER)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>EtOH</td>
<td>17.1</td>
<td>11.0</td>
<td>4.6 \times 10^{-4}</td>
<td>6.1 \times 10^{-6}</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Liposome</td>
<td>44.1</td>
<td>8.3</td>
<td>46.7 \times 10^{-4}</td>
<td>8.0 \times 10^{-6}</td>
<td>102.4</td>
</tr>
<tr>
<td></td>
<td>DM-NH\textsubscript{2}</td>
<td>45.0</td>
<td>9.0</td>
<td>79.5 \times 10^{-4}</td>
<td>7.4 \times 10^{-6}</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>DM-Ac</td>
<td>66.5</td>
<td>9.8</td>
<td>91.7 \times 10^{-4}</td>
<td>6.8 \times 10^{-6}</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>DM-COOH</td>
<td>154.7</td>
<td>9.9</td>
<td>37.9 \times 10^{-4}</td>
<td>6.8 \times 10^{-6}</td>
<td>8.3</td>
</tr>
<tr>
<td>Human</td>
<td>EtOH</td>
<td>7.5</td>
<td>12.3</td>
<td>6.7 \times 10^{-4}</td>
<td>2.0 \times 10^{-3}</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>EtOH w/ OA</td>
<td>14.7</td>
<td>11.7</td>
<td>13.3 \times 10^{-4}</td>
<td>2.2 \times 10^{-3}</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>DM-COOH</td>
<td>30.2</td>
<td>11.4</td>
<td>27.3 \times 10^{-4}</td>
<td>2.2 \times 10^{-3}</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}J = -D \frac{\partial C}{\partial x} Flux of EDX is obtained from the slope of the steady state skin permeation curve.

\textsuperscript{b}Lag time is the time between EDX application (t=0) and the beginning of the steady state permeation.

It is noteworthy to compare the topical drug delivery potential of the DMs to that of liposomes, given that cationic liposomes have been used in various transdermal delivery applications.\textsuperscript{38} The highest permeation coefficient was obtained by using liposomes (4.7 \times 10^{-2} cm/h) in this study. However, the poor EDX loading (0.4 µg EDX/mg liposome) necessitates a larger amount of liposomes to deliver the same amount of EDX compared to DMs. It is likely that liposomes would be a good delivery vehicle for hydrophilic or amphiphilic molecules that can be incorporated into the aqueous core of the liposomes at high efficiency.\textsuperscript{39} For EDX that is highly hydrophobic, however, DM-COOH that achieves
the highest drug loading of EDX is the superior drug carrier among the group tested in this study.

To finally validate the efficiency of DM-COOH as a transdermal delivery system for EDX, we used split-thickness human skin samples. Compared to CPEs (ethanol and ethanol-oleic acid (OA) combination), DM-COOH showed significantly more efficient skin permeation of EDX across the human skin, as measured by a higher EDX flux, shorter lag time, and greater permeability coefficient (Figure 3.6). As shown in Figure 3.6B, DM-COOH achieved a substantially increased flux of EDX (30.2 ng/cm²/h) that is 4-fold and 2-fold higher than 60% ethanol (7.5 ng/cm²/h) and the ethanol-OA system (14.7 ng/cm²/h) through the human skin, respectively. The permeation% and skin retention of EDX also followed the order of DM-COOH > ethanol-OA > ethanol. The obtained skin permeation parameters were lower than those obtained from a previous study using ethanol-OA combination,33 which is likely due to the lower amount of EDX applied to the human skin in this experiment. Although the human skin permeation of EDX was lower than mouse skin (likely due to the thickness difference in SC; ~26 µm for human40 vs. ~15 µm for mouse skin41), the results assured that the trend observed in the mouse skin is translatable to human skin, demonstrating that DMs, DM-COOH in particular, is a promising topical delivery system for EDX.
Figure 3.6 Skin permeation of EDX delivered by various vehicles across mouse and human skin. 
A) Mouse skin permeation of EDX over 24 h. B) Human skin permeation of EDX over 24 h. Note that DM-COOH induces permeation of the highest amount of EDX across the skin layers, while liposomes allow a significantly lower amount of EDX to permeate. Ethanol (60% in ddH$_2$O) in PB (2 mM KH$_2$PO$_4$, 4 mM Na$_2$HPO$_4$, pH 7.0) allows the least amount of EDX to permeate as compared to other vehicles that are dispersed in pure ddH$_2$O. OA, oleic acid. Error bars: standard error, SE, n=3-6**p<0.005; ***p<0.001.

To better understand how the DMs facilitate the delivery of small hydrophobic molecules through skin layers, two-dye fluorescent-labeled DMs were prepared. Coumarin-6 (Cou6) was encapsulated into the core of the micelles and the surface of the micelles was conjugated with rhodamine (RHO). The CLSM images shown in Figure 3.7A demonstrate the localization of Cou6 in mouse skin after 24 h treatment using the Franz cells. As seen from the green signal of Cou6 in the images, DMs induced high levels of the encapsulated Cou6 to reach both the epidermal and dermal layers. By way of contrast, free Cou6 could not diffuse effectively into the skin layers even in the presence of ethanol. DMs that were labeled with RHO, on the other hand, did not show any presence in the skin layers other than the stratum corneum, indicating that the micelles do not penetrate into the skin. The
skin retention of Cou6 was also analyzed using a spectrofluorometer. Figure 3.7B shows that all three types of micelles delivered significantly higher amounts of Cou6 through the mouse skin (DM-COOH: 67 ± 6%, DM-Ac: 68 ± 2%, and DM-NH₂: 66 ± 4%) than free Cou6 delivered by ethanol (27 ± 3%). However, given the similarity of the drug loading, there was no significant difference among surface modified DMs in depositing the encapsulated Cou6 into the skin layers. This observation indicated that permeation of EDX using the DMs, is directly related to the drug loading. Therefore, it will be important to investigate case-by-case for different drugs in order to have the proper surface groups on the DMs for optimum encapsulation to obtain optimal skin permeation.
**Figure 3.7** Skin deposition of Cou6-encapsulated, RHO-labeled two-dye DMs. 

A) CLSM images of the SKH1 hairless mouse skin cross-sections after treatment for 12 h with Free Cou6 in ethanol (Free Cou6), Cou6-encapsulated DM-NH₂ (Cou6/DM-NH₂), DM-Ac (Cou6/DM-Ac), and DM-COOH (Cou6/DM-COOH). Images are merged to show localization of Cou6 (green) and nuclei stained by DAPI (blue). Note that the RHO-labeled DMs are not detectable in the skin layers (no red signal). Scale bar: 20 µm. SC, stratum corneum; VE, viable epidermis; DE, dermal layer. 

B) Skin deposition of Cou6 delivered by DMs measured using a spectrophotometer after skin extraction with DMSO. Note that ethanol allows a significantly lower amount of Cou6 deposited into the skin layers. All DMs induce similar amounts of Cou6 deposited into the skin regardless of their surface functionalities. Error bars: standard error, SE, n=3. **p<0.005.

As demonstrated above, DMs could efficiently encapsulate and deliver hydrophobic small molecules into the skin layers. According to studies using monomeric micelles for topical drug delivery, the mechanism behind this phenomenon was explained as skin barrier perturbation, but not skin irritation. As reported by Ghosh et al., the size of the micelle could directly dictate how easily it permeates the skin, thus determining whether it will cause skin irritations. They claimed that the skin penetration of micelles was mostly
through aqueous porous pathway, and the average size of skin aqueous pores is $2.9 \pm 0.5$ nm. The measured radius of SDS micelles was $2.0 \pm 0.1$ nm, thus they could cause skin irritation; but the larger micelles (sodium cocoyl isethionate (SCI) micelle with $3.4 \pm 0.1$ nm radius) did not. In our study, all of the prepared micelles were much larger than the SCI micelle (Table 3.1); thus, they were not permeable to the skin, as confirmed by CLSM observations of the two-dye micelle system (Figure 3.7A). However, according to our permeation results, these micelles effectively facilitated skin translocation of Cou6 and EDX despite the fact that the micelles were almost skin impermeable. From the CLSM images (Figure 3.7A), the permeated dye was not confined to hair follicle regions, which indicated that micelles delivered the encapsulated hydrophobic molecules mainly through free-volume diffusion (through lipid bilayers) and lateral diffusion (along lipid bilayers). However, the process of how the micelles interact with the skin to facilitate higher skin permeation of the small molecules requires further study. Moreover, the CLSM images also demonstrated that the DMs caused greater skin permeation of Cou6 than ethanol did (Figure 3.7), which confirmed the superiority of DMs over ethanol for the delivery of hydrophobic molecules through the skin.

3.4 Conclusion

In this study, we have demonstrated that the DMs can successfully encapsulate EDX and enhance its skin permeation into mouse and human skin. Through our investigation, the
higher skin permeation was attributed to the higher drug loading in, and the faster drug release from the DMs, especially those with carboxyl surface groups. Skin localization studies using the two-dye DMs demonstrated that DMs deliver the encapsulated hydrophobic molecules through the skin without entering the skin layers as a whole, thus skin irritation from the polymers can be potentially avoided. This study presents the potential of DMs as topical drug delivery platform and also indicates that the DM structures may need to be individually designed depending upon the properties of drug molecules.
3.5 References


CHAPTER 4.

*In Vivo* Transdermal Delivery of Endoxifen using Dendron Micelles as Drug Carriers and Polymeric Penetration Enhancers

### 4.1 Introduction

As described in Chapter 3, EDX is an active metabolite of tamoxifen (TAM) that has been proven effective for the prevention and treatment of ER+ breast cancer.¹ The bioconversion of TAM to its active form EDX is dependent on the cytochrome P450 2D6 (CYP2D6) isoform.¹, ² Depending on CYP2D6 activities of individual patients, oral administration of TAM can vary in terms of concentrations of EDX in the breast tissues, resulting in different therapeutic outcomes. Although controversial, it has been reported that women diagnosed with genetically impaired CYP2D6 activity have a higher propensity for breast cancer recurrence upon TAM treatment.¹ (JM Rae, 2013 Clinical Pharmacology and Therapeutics) Thus, prescribing the active form EDX to those patients may be an alternative way to overcome the individual variations of CYP2D6 activities.

In the treatment of ductal carcinoma *in situ* (DCIS, the earliest form of breast cancer),³, ⁴ EDX is required only at the localized region in the breast (especially in the milk duct networks). Since systemic exposure after oral TAM intake often causes severe side effects in patients, including hot flashes, vaginal atrophy, endometrial cancer, and thromboembolic
events, topical delivery of EDX to the breast appears to be an attractive route for the treatment of DCIS as well as prevention of other ER-positive breast cancers. However, EDX is a highly hydrophobic small molecule with a partition coefficient (log$P$) of 4.09. As this log$P$ value is far beyond the optimum range (between 1 and 3) for efficient skin permeation, EDX alone cannot effectively penetrate through the skin layers without the use of drug carriers or penetration enhancers.

The preceding chapter showed promising topical EDX delivery efficiency in vitro of the dendron micelle (DM) system. This DM system increased the solubility of highly hydrophobic drug molecules in aqueous solutions and demonstrated efficient loading, prolonged release, maintained efficacy, and enhanced skin permeation of the model drug. In this chapter, we have further investigated the DM’s EDX delivery potential in vivo using hairless mice. The in vivo experimental setup was first optimized by testing various occlusive conditions and formulations. Different formulations based on the DMs containing EDX were applied to the mouse skin and their interactions were monitored. Cross-sections of the mouse skin layers were observed using the fluorescently labeled DMs after topical application to reveal the potential mechanism of the enhanced drug permeation. The skin deposition and plasma concentrations of EDX delivered by the DM systems were evaluated using a validated liquid chromatography-mass spectrometry (LC-MS) method. This study provides a preliminary in vivo result that is necessary for
future development of the DM systems as a topical EDX delivery platform that is engineered to prevent ER+ breast cancer.

**4.2 Materials and Methods**

**4.2.1 Preparation of EDX-loaded and Empty DMs using a Dialysis Method**

Ten milligrams of the carboxylated dendrons were dissolved in 2 mL of DMF in the presence or absence of 1 mg of EDX (Z-isomer, TRC, Toronto, CA). The polymer-EDX solution was then transferred to a dialysis membrane (MWCO 3,500) and dialyzed for 24 h against 500 mL of double distilled water (ddH₂O). After dialysis, the micelle solution was collected into 1.5 mL centrifuge tubes and centrifuged at 10,000 rpm for 5 min at 25°C. The supernatant was carefully collected and freeze-dried for 2 days to obtain the EDX-loaded and empty DMs.

**4.2.2 Preparation of EDX-loaded and Empty DMs using a Solvent Evaporation Method**

Ten milligrams of the carboxylated dendrons were dissolved in 1 mL of THF along with or without 1 mg of EDX. Using a glass syringe equipped with a blunt needle, the THF solution was added drop-wise to 6 mL of sterile ddH₂O in a 20 mL amber vial with vigorous stirring (1,100 rpm). The solution was stirred overnight at RT with the lid open to evaporate the organic solvent. After solvent evaporation, the micelle solution was first
purified as described below in detail, followed by freeze-drying for 2 days to yield the EDX-loaded and empty DMs.

4.2.3 Purifications of EDX-loaded DMs after Solvent Evaporation

The aqueous micellar solution was collected into 1.5 mL eppendorf tubes and centrifuged at 10,000 rpm for 5 min at 25°C. The supernatant was carefully collected in aliquots, and was either freeze-dried directly or dialyzed (MWCO 3,500) against ddH2O for 24 h before freeze-drying. The drug loading was measured in comparison with micelles prepared in the same batch but without purification, and those prepared in parallel using the dialysis method described above.

4.2.4 Drug Loading Measurements using the Reversed-phase (RP) HPLC

Drug loading was determined using a protocol that was described previously.13,20 Briefly, one milligram of the lyophilized DM was dissolved in 1 mL of DMSO, followed by vortexing and 6 h of incubation at room temperature with gentle shaking before measurement. Serially diluted EDX (15.6, 31.3, 62.5, 125.0, 250.0, 500.0 and 1000.0 ng/mL) solutions in DMSO were prepared and used to plot the standard curve for the quantification of drug loading using RP-HPLC. All samples were filtered through 0.2 µm Teflon syringe filters. A CHIPhEarser (Jelight Co., Inc., CA) was used as a pre-column photochemical reactor to convert endoxifen into its fluorescent derivatives at 254 nm. Ten
microliters of each sample were injected into a Symmetry C18 column (3.5/5 µm, 4.6 × 150 mm, Waters, Milford, MA) connected to a Waters HPLC system composed of a 717 Autosampler and a 600E series pump under isocratic mode. The mobile phase was composed of 46% (v/v) acetonitrile and 54% (v/v) KH$_2$PO$_4$ buffer (20 mM, pH 3.0). The flow rate was maintained at 1 mL/min. The column temperature was set at 25°C. EDX was detected at 254 nm excitation and 390 nm emission wavelengths using a Waters 474 Fluorescence detector. Loading was expressed as micrograms of EDX per milligram of micelle or liposome (µg EDX/mg micelle or liposome). Loading efficiency was calculated from the ratio of the measured loading to the theoretical loading (For micelles, the theoretical loading was the amount of EDX added divided by the mass of the dendron copolymer added into the formulation).$^{21}$

4.2.5 Characterization of DMs using DLS and TEM

Particle sizes (diameter, nm) of the DMs were measured from three repeat measurements by quasi-elastic laser dynamic light scattering (DLS) using a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA).$^{14,21}$ The materials were dispersed in ddH$_2$O to a concentration of 500 µg/mL and briefly vortexed prior to each measurement. The average and standard deviation of the DM diameters were calculated.
The micelle morphology was observed using transmission electron microscopy (TEM, JEM-1220, JEOL Ltd., Japan). After filtration (pore size 0.45 µm), a drop of micelle suspension (0.2 mg/ml) was placed on a 300 mesh copper grid coated with carbon. The sample was stained with a drop of 2% phosphotungstic acid and dried at room temperature in a desiccator for 1 day. The diameter of each type of micelle was measured using 10 randomly selected particles from each TEM image. The average and standard deviation of the DM diameters were calculated.

### 4.2.6 In Vitro Drug Release Test

The drug release test was performed using a vertical Franz diffusion cell device (Φ7 mm with 0.38 cm² exposure area, PermeGear Inc., Hellertown, PA). A cellulose membrane (MWCO 3,500) was sandwiched between the donor and receiver chambers. The DMs were suspended in ddH₂O, and 100 µL of each solution were applied to each donor chamber. The amount of DMs added to each donor chamber was determined based on the concentration of EDX, so that each chamber received the same amount of EDX. The receiver solution (release media), composed of Normal Saline, was stirred constantly at 600 rpm and the temperature was maintained at 37 °C. At predetermined time intervals, 250 µL of release medium were removed and replaced with an equal amount of fresh receiver solution and analyzed using RP-HPLC as described above. The release profile was obtained by plotting the cumulative EDX release% against time.
4.2.7 Preparation of Topical Gel Formulations

The topical gel formulations were prepared freshly before applying to the skin. To prepare the DM-containing gel matrix, 100 mg of the freeze-dried DM powder were dissolved in 10 mL of ddH₂O. To prepare a gel containing free EDX, the drug was first dissolved in absolute ethanol, and then diluted with ddH₂O to a final ethanol content of 60% (v/v). To these solutions, 150 mg of the Klucel hydroxypropylcellulose (1.5%, w/v) was added to form a gel matrix (Klucel gel, Hercules Incorporated, Wilmington, DE). Blank gel was prepared by directly dissolving the powder of Klucel hydroxypropylcellulose in ddH₂O to a final concentration of 1.5% (w/v). The prepared gel was homogenized using a magnetic stirrer (300 rpm) at 4°C before use. The final concentration of EDX in both EDX-containing formulations was 1 mg/mL.

Poloxamer gel formulation was prepared by dissolving 100 mg of DMs in 10 mL of Poloxamer 407 (20%, v/v) solution and mixing at 4°C. The final concentration of EDX in the Poloxamer gel was 1.0 mg/mL. Polyethylene glycol (PEG) gel was prepared by mixing 500 µL of PEG 400 solution with 500 mg of PEG 1455 powder (1:1, v/w). The mixture was incubated with periodic stirring on a hot plate until the PEG 1455 melted into the solution. A PEG gel was formed after cooling down the mixture at RT. Finally, 10 mg of DMs were triturated into 290 mg of the PEG gel for application. The final concentration of EDX in the PEG gel was 1:300 (w/w, 1 mg EDX per 300 mg gel).
4.2.8 Mouse Skin Preparations and Diffusion Cell Setups

Freshly excised SKH-1 hairless mouse (Charles River Laboratory, Boston, MA) skin from the dorsal side with an average thickness of 433.3 ± 27.6 μm (mean ± SD) was used in this study at 6 wk.\textsuperscript{16} Subcutaneous fat and blood vessels were carefully removed using cotton swabs. The full thickness skin was cut into 1.2 × 1.2 cm\textsuperscript{2} squares, rinsed with normal saline, and sandwiched between the donor and receiver chambers of the Franz diffusion cell with the stratum corneum side facing upward.\textsuperscript{8} The receiver chambers were filled with freshly prepared normal saline containing 30% ethanol (v/v) as a drug-solubilizing reagent. Receiver solutions were stirred constantly at 600 rpm and the temperature was maintained at 37°C. After equilibration of the skin for 30 min, topical formulations were applied to the donor chambers. The first sample collection was performed at 0 h by withdrawing 250 μL of the receiver solution from each sampling port and replacing with 250 μL of fresh receiver solution. Sample collection was performed at designated time points. All samples were kept at 4°C in dark before analysis using RP-HPLC.

4.2.9 In Vitro Comparison of Topical Gel Formulations

Three types of topical gels containing DMs were compared with respect to their EDX delivery efficiencies \textit{in vitro} using the Franz diffusion device. To each donor chamber, one of the three topical gel formulations containing DMs loaded with 50 μg of EDX was applied to the skin (n=3 for each formulation). The donor chambers were covered with
Saran wrap to avoid evaporation. Each formulation was tested in triplicates. Receiver solution samples were collected at 0, 24, 48, and 72 h.

4.2.10 In Vitro Comparison of Occlusive Conditions

In this study, “occlusive condition or occlusion” means the *in vitro* skin permeation experiment was performed when the donor chamber or skin surface was covered with occlusive materials to prevent evaporation of moisture.\textsuperscript{22, 23} By way of contrast, the “non-occlusive condition” means no occlusive material was used during the experiment. In this section, three progressive experiments were performed to optimize the occlusive conditions.

To investigate whether occlusive conditions could prevent moisture-loss from micelle formulations and thus facilitate better skin permeation, Saran wrap was used as an occlusive material to cover the top of the donor chambers to create an occlusion after topical gel applications. In parallel, non-occlusive conditions were created by not covering the donor chambers during the gel treatment. Equal amounts of the Klucel gel formulation containing EDX-loaded DMs were applied to the donor chambers (each received 100 \( \mu \)g of EDX in 100 \( \mu \)L of topical gel; to avoid drying of the gel on the surface of the skin under non-occlusive conditions, more gel was applied). After the Saran wrap was applied to the
donor chambers, receiver solution samples were collected at 0, 24, 48, and 72 h. Each condition was tested using the skin from 3 mice in triplicates.

Another experiment comparing the locations of occlusion was carried out by placing the occlusive material (Saran wrap) either on top of the donor chambers (“non-contact mode” - there was still a space between the occlusion and the skin), or below the donor chambers (“contact mode” - occlusive material was first applied to the gel treated skin and then sandwiched in between the donor and the receiver chambers). Equal amounts of the Klucel gel formulation containing EDX-loaded DMs were applied to the donor chambers (each received 50 µg of EDX). After the Saran wrap was applied to the donor chambers, receiver solution samples were collected at 0, 24, 48, and 72 h. Each mode was tested three times in triplicates.

The third experiment was carried out to investigate whether the biocompatible dermatological tapes are equally effective to provide occlusion as compared to the Saran wrap. Tegaderm (3M Health Care, St. Paul, MN) and Bioclusive (Systagenix, Yorkshire, UK) were used in place of Saran wrap to create contact mode occlusions. Equal amounts of the Klucel gel formulation containing EDX-loaded DMs were applied to the donor chambers (each received 50 µg of EDX). Receiver solution samples were collected at 0, 24, 48, and 72 h. Each material was tested using 3 mice in duplicates.
4.2.11 In Vivo Skin Permeation Experiments

4.2.11.1 In Vivo Experiment Under Non-occlusive Conditions: Each SKH-1 hairless mouse in the DM treatment group (24 mice in total) was treated with 100 µL of the Klucel gel containing 1 mg of EDX-loaded DMs (average drug loading 100 µg). The treatment was repeatedly applied every 24 h for up to 72 h, and 4 mice were sacrificed for blood collection and necropsy on the first day at 6 h after one time treatment. On the following days at 24 h, 48 h, 72 h, and 96 h, five mice were sacrificed at each time point for the same purpose. In this case, the mice that were sacrificed by 24 h also received one time treatment; the ones that were sacrificed by 48 h received two treatments; and the rest had received three repeated treatments. A positive control group with 24 mice was treated with free EDX dispersed in ethanol gel containing oleic acid (OA, 0.5%, v/v) and EDX (100 µg of EDX per 100 µL gel), and was processed in the same manner as described above. Rhodamine-labeled empty DMs (1 mg/mouse/day) were used as control materials, and 9 mice in the control group were treated the same way for 3 days. Another control group with 3 mice was treated with OA-containing ethanol gel as a negative control, and were sacrificed at the 96 h for the collection of blank blood and skin samples.

4.2.11.2 In Vivo Experiment Under Occlusive Conditions: Each SKH-1 hairless mouse in the DM treatment group (9 mice in total) was treated with 100 µL of the Klucel gel containing 1 mg of EDX-loaded DMs (average drug loading 100 µg). The treatment was applied for 72 h under occlusive conditions provided by TegaDerm. These mice were
sacrificed for blood collection and necropsy at the end of the treatment. A positive control group with 9 mice was treated with free EDX dispersed in ethanol gel containing oleic acid (OA, 0.5%, v/v) and EDX (100 µg of EDX per 100 µL gel), and was processed in the same manner as described in the DM group. Another control group with 9 mice was treated with Klucel gel containing 35% ethanol and same concentration of EDX, and the animals were sacrificed at 72 h for the collection of blank blood and skin samples. Rhodamine-labeled empty DMs (1 mg/mouse) were used as control materials, and 7 mice in the this group were treated the same way for 3 days and the plasma sample were collected for the use of blank plasma.

4.2.12 Blood Collection and Necropsy

After anesthesia with a mixture of Ketamine (100 mg/kg) and Xylazine (8 mg/kg, i.p.), blood samples were collected using a cardiac puncture method. The blood samples were centrifuged at 15,000 rpm for 20 min at 4°C, and the plasma was carefully collected and stored at -20°C before EDX extraction. After euthanasia, the topical gel treated mouse skin (on the dorsal side) was gently cleaned using alcohol pads and excised for EDX deposition analysis. All skin samples were rinsed in normal saline and dried on filter paper before snap-frozen in liquid nitrogen and stored at -80°C.
4.2.13 Preparation of Calibration Standards, Quality Controls, and Internal Standard Solutions

The EDX (analyte) and 4-hydroxy Tamoxifen (4-OHT, internal standard, I.S.) stock solutions with concentrations of 1 mg/mL were prepared in acetonitrile. Two sets of the calibration samples were prepared freshly for each analysis. Ten microliters of the analyte (0, 13.3, 40.0, 200.0, 400.0, 800.0, and 1600.0 ng/mL) and 10 µL of the I.S. (400 ng/mL) were spiked into 100 µL of the mouse serum (Sigma Aldrich, St. Louis, MO). After vortex mixing for 1 min, 280 µL of acetonitrile were added to yield the calibration standards with the theoretical analyte concentrations of 0, 0.03, 0.1, 5.0, 10.0, 20.0, and 40.0 ng/mL. These standard solutions were further vortex-mixed for 1 min before being centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was carefully collected and the concentration of EDX was analyzed using LC-MS. Two sets of the quality controls (QCs) were prepared in the same manner to obtain the final concentrations for the low QC at 0.1 ng/mL, the middle QC at 10.0 ng/mL, and the high QC at 40.0 ng/mL. The final concentration of the I. S. was 10 ng/mL in all samples.

4.2.14 LC-MS Analytical Methods

An AB Sciex 5500 QTRAP Tandem Mass Spectrometer was used for analyte detection. The parameters and settings used in this study were summarized in Table 4.1.18, 19
Table 4.1 Summary of LC-MS analytical method used for \textit{in vivo} study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Settings</th>
<th>Parameters</th>
<th>Settings</th>
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</thead>
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<td>Cell exit potential</td>
<td>9.0 V</td>
</tr>
</tbody>
</table>

\textbf{4.2.15 Drug Extraction from the Plasma Samples}

The drug extraction procedure was obtained from published reports.\textsuperscript{18,19} Briefly, to 100 \(\mu\)L of plasma, 10 \(\mu\)L of the I.S. in acetonitrile (4-OHT, 400 ng/mL) were added and vortex mixed for 1 min. Then, 290 \(\mu\)L of acetonitrile were added to the mixture and another round of vortex mixing was performed for 1 min. This solution was centrifuged at 12,000 rpm for 10 min at 4\(^\circ\)C. The supernatant was carefully collected and the concentration of EDX was analyzed using LC-MS (QTrap 5500, AB Sciex, Framingham, MA).

\textbf{4.2.16 Drug Extraction from the Skin Samples}

EDX was extracted from the skin samples according to previously reported procedures.\textsuperscript{8,13} Briefly, skin samples were collected and thoroughly cleaned. The effective exposure area on the skin was excised and homogenized in a 1.5 mL centrifuge tube. DMSO was used as an EDX-extracting reagent. After 12 h of extraction at RT, the samples were centrifuged at
10,000 rpm for 10 min at RT, and the supernatant was collected for analysis using the RP-HPLC.

4.2.17 Fluorescence Microscope Imaging of the Mouse Skin after DM Treatments

SKH-1 hairless mouse skin was exposed to the rhodamine-labeled (RHO) empty DMs for 3 days. The skin area that was exposed to the treatment was carefully collected, rinsed twice with ddH₂O for 10 min and embedded into cryomolds (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA). Skin was cryo-sectioned into 80 µm-thick slices and placed on antifrost glass slides. The slides were then treated with 10% neutral buffered formalin for 10 min at RT and rinsed with ddH₂O. The fixed skin samples were then mounted with anti-photobleaching mounting media with DAPI (Vector Laboratory Inc., Burlingame, CA) and covered with glass cover slips. The slides were visualized using an inverted microscope equipped with a fluorescence illuminator (IX 70-S1F2, Olympus America, Inc., Center Valley, PA, USA). Images were recorded using a 10× objective and a CCD camera (QImaging Retiga 1300B, Olympus America, Inc., USA).

4.2.18 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) based on a one-way ANOVA followed by Tukey’s post hoc test. Data were considered significant at $p < 0.05$. The number of animals required for the in vivo study
was determined by performing a priori power analysis using G*Power 3.1 software. With at least 7 animals per treatment group, there was 80% power to detect an effect size of 1.7 using a two-tailed t-test with a Type I error rate of 10%. Data was analyzed using one-way ANOVA followed by Tukey’s post hoc test.

4.3 Results and Discussion

4.3.1 Characterization of DMs Prepared using Different Methods

The drug loading, particle size, and zeta potential values are listed in Figure 4.1. The loading of EDX into DMs prepared using a SE method followed by centrifuge (SE/centrifuge) yielded number-weighted average diameters of 60.4 nm with a narrow size distribution, which were in agreement with TEM images (Figure 4.1). Zeta-potential measurements revealed that all the DMs were negatively charged, and particularly those prepared using the SE/centrifuge method had the zeta-potentials with minimum variations. The DMs prepared using other methods displayed higher zeta-potential values, implying surface adsorption of the positively charged EDX. Drug loading measurements revealed that DMs with less negative zeta-potentials showed EDX loading efficiencies with large batch-to-batch variations (21.7 ± 15.6% and 85.7 ± 22.1% for SE/centrifuge + dialysis and SE/non-processed, respectively), probably due to the varied amounts of EDX absorbed to the DM surfaces. On the other hand, the SE/centrifuge method-prepared DMs exhibited
significantly higher drug loading (6.9-fold higher than the dialysis method-prepared DMs) with a relatively small batch-to-batch loading variation (87.7 ± 8.0%). As a result, the SE/centrifuge method was chosen to prepare EDX-loaded DMs used throughout this in vivo study.

4.3.2 In Vitro Drug Release Studies

The EDX-loaded DMs prepared using different methods were further compared in terms of drug release kinetics. As shown in Figure 4.1A, all DMs released EDX in a similar manner with biphasic release profiles. The initial burst release of EDX in the first 2 h could be attributed to the possibility that a portion of EDX molecules were surface adsorbed to the DMs. EDX encapsulated in the hydrophobic PCL core was released slowly and steadily from the DMs over 11 days (Figure 4.1B). The release profiles of the DMs appeared to be drug loading-dependent. Among DMs with different degrees of drug loading, those with higher drug loading exhibited slower drug release. This phenomenon can be explained by the hydrophobic interactions of EDX. When drug loading is higher, more EDX molecules are encapsulated within the cores, which may augment the hydrophobic interactions among EDX molecules, hindering the drug from being easily released from the micelles.
**Figure 4.1** Characterization of and *in vitro* EDX release from DMs prepared using different methods.

Top: drug loading, particle size, and zeta potential measurements of EDX-loaded DMs prepared by either a dialysis method followed by centrifuge (indicated in black), or by a solvent evaporation (SE) method followed by various processing approaches (indicated in red, blue, and pink). *a* Measured using RP-HPLC; *b* Measured using Zeta Potential/Particle Sizer. All data are presented as mean ± standard deviation (SD, n=3). **Middle:** Representative TEM images of DMs prepared using different methods. Scale bars: 200 nm. (*TEM images were taken by Ryan Pearson.*) **Bottom:** *in vitro* EDX release from DMs prepared using different methods. All DMs released EDX in the similar manner with biphasic release profiles. **A)** Cumulative EDX release in the first 24 h. **B)** EDX loaded in the hydrophobic PCL core was released slowly and steadily from the DMs over 11 days. Error bars: Standard error (SE, n=3).

<table>
<thead>
<tr>
<th>Preparation Methods</th>
<th>EDX Loading* (µg/mg)</th>
<th>Loading%</th>
<th>Loading Efficiency%</th>
<th>Size* (nm)</th>
<th>Zeta Potential* (mV)</th>
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<td>Dialysis + Centrifuge</td>
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<td>12.7 ± 3.7%</td>
<td>43.6 ± 6.4</td>
<td>-25.5 ± 4.7</td>
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<td>SE/ Centrifuge</td>
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<td>8.8 ± 0.8%</td>
<td>87.7 ± 8.0%</td>
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<td>-16.4 ± 7.6</td>
</tr>
</tbody>
</table>

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**Figure 4.1** Characterization of and *in vitro* EDX release from DMs prepared using different methods.

Top: drug loading, particle size, and zeta potential measurements of EDX-loaded DMs prepared by either a dialysis method followed by centrifuge (indicated in black), or by a solvent evaporation (SE) method followed by various processing approaches (indicated in red, blue, and pink). *a* Measured using RP-HPLC; *b* Measured using Zeta Potential/Particle Sizer. All data are presented as mean ± standard deviation (SD, n=3). **Middle:** Representative TEM images of DMs prepared using different methods. Scale bars: 200 nm. (*TEM images were taken by Ryan Pearson.*) **Bottom:** *in vitro* EDX release from DMs prepared using different methods. All DMs released EDX in the similar manner with biphasic release profiles. **A)** Cumulative EDX release in the first 24 h. **B)** EDX loaded in the hydrophobic PCL core was released slowly and steadily from the DMs over 11 days. Error bars: Standard error (SE, n=3).
4.3.3 In Vitro Comparison of Topical Gel Formulations

In search of a suitable topical gel matrix for the in vivo applications, three candidate gel matrices were prepared. Skin permeation of EDX was tested in vitro to select the best gel matrix. The ideal gel matrix should work as a reservoir for the EDX-loaded DMs that provides optimal fluidity (without overflow beyond the predetermined exposure area, yet with required drug diffusion) and the required steady state drug permeation.\textsuperscript{27} As Figure 4.2, compared to the hydroxypropyl cellulose gel (Klucel gel, 1.5%, w/v), the PEG and Poloxamer gel matrices inhibited the permeation of EDX by 4.8-fold and 7.4-fold, respectively ($p < 0.001$). Furthermore, the Klucel gel did not alter the permeation flux of EDX significantly compared to that obtained from using water solution of DMs, indicating that the Klucel gel was a compatible matrix for the DM delivery system (data not shown).

Using the same Klucel gel in a study of 55 postmenopausal women with invasive ER-positive breast cancer, Rouanet et al. reported promising results.\textsuperscript{28} They found that topical application of 4-hydroxy tamoxifen (4-OHT, another active metabolite of tamoxifen) to the breast region showed localized effect on inhibiting tumor proliferation. The anti-proliferative effect of the topically applied 4-OHT at 1-2 mg/day was comparable to that of the orally administered tamoxifen (TAM) at the standard dose (20 mg/day). Moreover, plasma level of 4-OHT was much lower than TAM, and the topical 4-OHT gel appeared to be well tolerated. This report, combined with our observations, indicates that the Klucel
gel system is safe and effective for topical application of TAM metabolites. Hence, we selected it for the following studies.

**Figure 4.2** *In vitro* comparisons of topical gel formulations for *in vivo* applications. EDX-loaded DMs were incorporated into Poloxamer, PEG, or Klucel topical gel formulations, and the skin permeation of EDX was investigated using Franz diffusion cells over 72 h. Error bars: Standard error (SE, n=3). ***p < 0.001.

4.3.4 *In Vitro Comparison and Optimization of Occlusive Conditions*

4.3.4.1 *Comparison of Occlusive vs. Non-occlusive Conditions*

Using the Klucel gel system, we first investigated skin permeation of EDX under both occlusive and non-occlusive conditions. When occlusive conditions were applied, the Permeation% of EDX through the mouse skin was significantly higher than observed in
non-occlusive conditions ($p < 0.001$). Occlusive conditions allowed 4.5-fold, 8.2-fold, and 9.3-fold higher Permeation% than the non-occlusive conditions at 24, 48, and 72 h, respectively (Figure 4.3). In non-occlusive conditions, extended treatment did not demonstrate greater skin permeation of EDX, indicating that the DMs dried on the gel-treated skin surfaces when exposed to air. On the other hand, a steady state EDX flux (721.1 ng/cm$^2$ h) was observed in the occlusive condition up to 72h, demonstrating the effectiveness and necessity of the occlusive condition for the DM delivery system.

![Figure 4.3](image)

**Figure 4.3** In vitro comparison of skin surface treatments after Klucel gel application. EDX-loaded DMs were incorporated into Klucel topical gel formulations and was applied to the skin surface. The skin permeation of EDX was investigated using Franz diffusion cells over 72 h. Occluded conditions were created by covering the donor chambers of the Franz cell device with Saran Wrap. Non-occluded conditions were created by not using the Saran Wrap to cover the donor chambers. Error bars: Standard error (SE, n=3). ***$p < 0.001$. 
Previous reports have suggested that under occlusive conditions, water molecules can incorporate themselves into the polar head groups on the intercellular lipids in the SC. This causes the SC to become less hydrophobic and behave more similarly to the viable epidermis, resulting in higher permeability.\textsuperscript{29,30} Water molecules can also bind to keratin in the corneocytes embedded in the SC, causing swelling of cells and intercellular lipid structure disruption. It is the loosening of the SC structure that results in the increased permeability of drug molecules.\textsuperscript{31,32} We have conducted a pilot \textit{in vivo} study using hairless mice treated with EDX-loaded micelles in Klucel gel. In a test \textit{in vivo} study, under non-occlusive conditions, different degrees of drying and chipping of the DMs on the skin surface were observed in all animals after 24 h (data not shown). This caused dosing differences in the animals. Thus, non-occlusive conditions should not be used for the topical delivery of DM formulations. In contrast, moisture was well retained on the mouse skin in occlusive conditions, and no drying of the DMs was observed over the 72 h treatment period (data not shown). This observation encouraged us to further investigate EDX delivery in occlusive conditions.

\textit{4.3.4.2 Comparison of Contact Mode vs. Non-contact Mode Occlusions}

For the \textit{in vivo} application of occlusive conditions, the occlusive materials had direct contact with the topical gel on the skin surface (henceforth “contact mode”). However, the \textit{in vitro} Franz cell experiment typically had the occlusive materials on top of the donor chamber, leaving a space between the gel treated skin and the occlusive materials.
(henceforth “non-contact mode”). To investigate whether the location differences led to different skin permeation outcomes, we have compared the permeation of EDX in vitro under these two modes using a Franz diffusion device. After treating the mouse skin with the same amount of EDX gel, we have observed a 3.3-fold and a 2.8-fold higher Permeation% from the contact mode groups than from the non-contact mode groups at 24 and 48 h, respectively (Figure 4.4, \( p < 0.05 \)). These results indicate that contact mode occlusion can further prevent moisture loss from the gel and the SC, which leads to better skin permeation.

![Figure 4.4 In vitro comparison of contact mode vs. non-contact mode of occlusions after Klucel gel applications. EDX-loaded DMs were incorporated into Klucel topical gel formulation and was applied to the skin surface. The skin permeation of EDX was investigated using Franz diffusion cells for 48 h. Error bars: Standard error (SE, n=3). *\( p < 0.05 \).](image)
4.3.4.3 Comparison of Different Occlusive Materials

For better skin compatibility, we have tested three occlusive materials under contact mode occlusions. Besides Saran Wrap, which was previously used, two types of dermatological tapes, TegaDerm (3M, St. Paul, MN) and Bioclusive (Systagenix, Yorkshire, UK), were introduced to the Franz diffusion cell experiments. Although a trend of increasing Permeation% of EDX was observed following the order of TegaDerm < Saran Wrap < Bioclusive, there was no significant difference among the three occlusive materials (Figure 4.5). Previous reports have demonstrated the biocompatibility of and animal compliance with the TegaDerm treatments.\textsuperscript{33} Considering the breathability and the established biocompatibility study reports, we have chosen the 3M TegaDerm as our occlusive material for future \textit{in vivo} studies.

![Figure 4.5 In vitro comparison of different occlusive material after Klucel gel applications. EDX-loaded DMs were incorporated into Klucel topical gel formulation and was applied to the skin surface. The skin permeation of EDX was investigated using Franz diffusion cells for 48 h. Error bars: Standard error (SE, n=3).](image-url)
4.3.5 LC-MS Method Developments and Validations

To evaluate the in vivo drug delivery efficiency, we have implemented a protein precipitation method to extract EDX from plasma samples and a LC-MS method for the determination of plasma EDX concentrations. According to the “FDA Bioanalytical Method Validation Guidances”, the acceptable range of Recovery% should be within 80-120% of the theoretical value. Using the QC samples prepared at 3 different concentrations (low, middle, and high QCs), we have obtained the absolute and relative recovery% at 89.3 ± 4.0% and 103.4 ± 4.6% from the low QC, 87.3 ± 3.7% and 96.6 ± 4.1% from the middle QC, and 98.8 ± 0.2 and 108.1 ± 0.2% from the high QC, respectively (Table 4.2). The data indicate that the current protein precipitation method can effectively extract EDX from the plasma samples and can be used for future in vivo studies.

Table 4.2 Recovery of EDX from quality control plasma samples (QC) measured using LC-MS.

<table>
<thead>
<tr>
<th></th>
<th>Absolute Recovery%</th>
<th>Relative Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low QC</td>
<td>89.26 ± 3.99</td>
<td>103.37 ± 4.62</td>
</tr>
<tr>
<td>Mid QC</td>
<td>87.30 ± 3.70</td>
<td>96.64 ± 4.14</td>
</tr>
<tr>
<td>High QC</td>
<td>98.79 ± 0.16</td>
<td>108.11 ± 0.17</td>
</tr>
</tbody>
</table>

The calibration curve over the entire ranges of concentrations was satisfactorily described by linear regression of the peak-area ratio of EDX to its I.S. (4-OHT), versus the
concentrations of EDX in standard samples. The determination coefficients \((R^2)\) of all calibration curves were higher than 0.99 with back-calculated concentrations of the calibration samples within ± 15% of nominal values (± 20% at the lower limit of quantification, LLOQ).

The LLOQ was also determined in this study and was designated as the lowest standard on the calibration curve. According to the same FDA Guidance, the analyte response at the LLOQ should be at least 5 times higher than the blank response. We have obtained an 11.0 ± 0.6 times higher response at LLOQ than the blank response, demonstrating that the current analytical method is robust and can provide enough response to the analyte at all tested concentrations.

**4.3.6 In Vivo EDX Delivery Studies**

**4.3.6.1 Localization Study using Rhodamine-labeled Empty DMs (RHO-DMs)**

In the previous Chapter, we showed that the DMs facilitated the skin permeation of free EDX and dye molecules *in vitro*, but without penetrating through the SC by itself. In this Chapter, we employed the rhodamine-labeled empty DMs (RHO-DMs) and confirmed this phenomenon *in vivo*. Fluorescence microscopy observations of the cross-sectioned mouse skin after 72 h of RHO-DMs treatment revealed that the DMs were mainly located in the first few layers of the epidermis (Figure 4.6). Ghosh et al. have reported that the skin
penetration of micelles was mostly through an aqueous porous pathway and that the average size of skin aqueous pores is 2.9 ± 0.5 nm. The size of SDS micelles was 2.0 ± 0.1 nm; thus, they can cause skin irritation upon skin penetration and lipid disruption, whereas larger micelles (sodium cocoyl isethionate micelle with 3.4 ± 0.1 nm radius) did not.\textsuperscript{34,35} With respect to this theory, skin irritation is less likely to happen in the case of DMs treatment, since the DMs have an average size around 50.0 nm and their skin penetration is observed to be minimal.

**Figure 4.6 In vivo skin localization study of rhodamine (RHO)-labeled empty DMs.** RHO-DMs were incorporated into the Klucel gel formulation and the skin localization of the DMs after 72 h of topical treatment to hairless mice was investigated using fluorescence microscopy (FLU, n=3). \textbf{A)} RHO-labeled empty DMs; \textbf{B)} Skin cell nuclei stained by DAPI (blue); \textbf{C)} Bright field image of the skin cross-section; \textbf{D)} Merged image of all three channels. Scale bar: 200 µm. SC, stratum corneum; VE, viable epidermis; DE, dermal layer. Note that the DMs are mainly located at the epidermal layers without further skin penetration. (\textit{In vivo studies were performed by help of Oukseub Lee})
4.3.6.2 Skin Deposition and Blood Concentration of EDX

In this study, both the DM aqueous gel and the OA-containing ethanol gel systems have facilitated skin deposition of EDX over 96 h (Figure 4.7A and 4.7B). However, the progression of EDX deposition over the course of 96 h was different between the two systems. The OA gel system enabled an initial EDX deposition of 2.6 ± 0.4 µg/cm² in the first 6 h and maintained a stable skin deposition of EDX at 5.0 ± 0.4 µg/cm² from 24 to 96 h. On the other hand, the DM gel system had initially deposited 2.9 ± 0.1 µg/cm² of EDX into the mouse skin, followed by a gradual increase to 5.4 ± 2.3 µg/cm² at 24 h and 7.0 ± 2.3 µg/cm² at 48 h, possibly due to the sustained drug release properties of the DMs. However, a sudden decrease of skin deposition was observed at 72 h (to 1.2 ± 0.2 µg/cm²). This decrease can be attributed to the drying of the DM topical gel system on the skin after 72 h, indicating new treatment should be applied every 2-3 days, or the design of this drug delivery system should be further optimized.

The commercially available Estraderm® 100 patch is a 3.5-day use topical delivery system for estradiol (E2, Novartis Pharmaceuticals Corporation, East Hanover, NJ). It has an initial loading dose of 8 mg of estradiol (E2) that can deliver 100 µg of the drug each day through the skin with 20 cm² exposure area (loading dose 400 µg/cm²), resulting in an average plasma concentration of 32-67 pg/mL•day. The cumulative Permeation% of E2 is 1.25% with a steady state permeation flux of 5 µg/cm²•day (or 208 ng/cm²•h).
Figure 4.7 *In vivo* skin deposition and plasma concentration of EDX after topical gel treatments under non-occlusive conditions. EDX-loaded DMs in Klucel gel (indicated as DMs) or free EDX dispersed in Klucel gel containing 0.5% OA and 60% ethanol (indicated as OA) were applied to hairless mouse skin (each animal received 100 µg of EDX) and the *in vivo* EDX delivery efficiencies were compared. Skin deposition profiles of EDX over 96 h are depicted in A) for DMs and B) for OA formulations. Plasma concentrations of EDX over 96 h treatment of topical gel formulations containing C) DMs and D) OA are also obtained. Error bars: Standard deviation (SD). (*In vivo studies were performed by help of Oukseub Lee; LC-MS analysis were performed by help of Di Hu*)

In our previous *in vitro* studies investigating the EDX delivery efficiency through an OA/ethanol co-solvent system (0.5% OA and 60% ethanol as CPEs in phosphate buffer, pH 7.0), Dr. Khan’s group obtained 4.4% permeation of EDX with a flux of 100 ng/cm²•h and 3.2% permeation of E2 with a flux of 200 ng/cm²•h (as a positive control). The
experiment was performed using split thickness human skin for 24 h with an initial loading
dose of 78.9 µg/cm² for both drugs. In the in vitro study described in Chapter 3, we used a
lower initial loading dose of EDX (30.3 µg/cm²), and observed a flux of 14.7 ng/cm²•h
with 0.6% permeation from the OA/ethanol co-solvent system. In this condition, the DMs
achieved an enhanced flux of 30.2 ng/cm²•h with 1.3% permeation after 24 h. This
Permeation% of EDX obtained from the DM system was comparable to that obtained from
the Estraderm patch, even though the initial loading dose in DM system was only about
one-thirteenth (7.6%) of that was in the Estraderm patch. This indicates that DMs have a
potential to be used as an efficient delivery platform for hydrophobic drug molecules.

Regarding the current in vivo study on the DM delivery system, with the initial loading
dose of 0.1 mg/cm², we obtained the median of EDX plasma concentrations of 60.5, 65.1,
and 60.4 pg/mL at 24, 48, and 72 h, respectively, which are significantly lower than the
positive control (free EDX in Klucel gel containing 0.5% OA and 60% ethanol) (Figure
4.7C and 4.7D). The lower plasma concentration and similar skin deposition profiles of the
DM systems to those of the OA system can be possibly attributed to the slow, controlled
release capacity of the DM, which may prohibit rapid diffusion of EDX into the blood
circulation. Although the plasma concentration obtained from this study was relatively
lower than the positive control, it was still comparable to the reported plasma level obtained
from the 3.5-day use (twice weekly) Estraderm® patch (32-67 pg/mL on average). Although
this cross-species comparison cannot provide conclusions regarding which
system is better than the other, from the preliminary in vivo data obtained in this study, we can at least confirm that the DM system can deliver EDX through the mouse skin. Future studies should be done using a better animal model (such as hairless rats) for comprehensive evaluations of the in vivo pharmacokinetic profiles and tumor prevention efficiencies.38,39

In the second in vivo EDX permeation study (under occlusive conditions), Klucel gel containing 100 µL of EDX either loaded in DMs or dispersed in the OA/ethanol co-solvent system was applied to the hairless mice. The treatments were maintained for 72 h under occlusive conditions provided by the TegaDerm tapes. The skin deposition of EDX after 72 h of treatments was measured using HPLC. Compared to 1.6% obtained from the OA-ethanol group (Figure 4.8A), a lower Permeation% of EDX was obtained from the DM group (0.8%, Figure 4.8B), which is similar to the ethanol group. The plasma level of EDX at 72 h was quantified using LC-MS. Results demonstrated that, comparable amount of EDX had entered into the systemic circulation by help of either DMs or OA-ethanol system (Figure 4.8C-D), demonstrating the DM system can deliver EDX without the use of CPEs, and its potential of being an effective polymeric penetration enhancer (PPE). Interestingly, as compared to the non-occlusive conditions at 24, 48, and 72 h, the application of occlusive dressing for 72 h did not significantly increase the EDX level in the plasma from the OA-ethanol co-solvent group. On the other hand, occlusive conditions significantly enhanced the plasma level of EDX in the DM group (****p < 0.0001), indicating the drug
delivery efficiency is more application condition-dependent for the DM system. Although the results obtained from this *in vivo* study did not reveal significant difference in skin permeation of EDX between the CPE and PPE groups, future improvements on the DM system design as well as *in vivo* toxicity and skin irritation studies are necessary to fully evaluate the potential of DM system as a topical drug delivery platform.
Figure 4.8 *In vivo* skin deposition and plasma concentration of EDX after topical gel treatments under occlusive conditions. EDX-loaded DMs in Klucel gel (indicated as DMs) or free EDX dispersed in Klucel gel containing 0.5% OA and 60% ethanol (indicated as OA) were applied to hairless mouse skin (each animal received 100 µg of EDX) and the *in vivo* EDX delivery efficiencies were compared. Skin deposition profiles of EDX at 72 h are depicted in A). EtOH, ethanol (60%, v/v); DM, dendron micelle; EtOH/OA, ethanol (60%v/v) with OA (0.5%, v/v). Plasma concentrations of EDX at 72 h treatment of topical gel formulations containing B) OA and C) DMs are also obtained. Error bars: Standard deviation (SD, n=4-7). (*In vivo* studies were performed by help of Oukseub Lee; LC-MS analysis were performed by help of Di Hu)
4.4 Conclusion

We have found that the DMs prepared using a solvent evaporation method can successfully load EDX and enhance its skin permeation into hairless mouse skin both *in vitro* and *in vivo*. Through our investigation, the Klucel gel system exhibited the highest skin permeation in occlusive conditions. *In vivo* skin localization studies using the rhodamine-labeled empty DMs demonstrated that DMs would not enter the skin together with the hydrophobic molecules, allowing the possibility to avoid skin irritation. *In vivo* skin deposition and plasma concentration measurements revealed the effectiveness of delivering EDX through the mouse skin using DM system in aqueous condition. This study presents the potential of DMs as a topical drug delivery platform and also indicates that the DM topical gel system may need to be designed into a patch system for future *in vivo* pharmacokinetics and efficacy studies.
4.5 References


27. Krishnaiah, Y. S.; Al-Saidan, S. M. Limonene Enhances the in Vitro and in Vivo Permeation of Trimetazidine across a Membrane-Controlled Transdermal Therapeutic System. *Current Drug Delivery* **2008**, *5*, 70-76.


5.1 Conclusions

Through this dissertation, fundamental understanding of the interactions between dendritic nanomaterials and skin layers was obtained. Based on the rationale design, an effective topical drug delivery platform was developed. In particular, skin permeation, penetration pathways, and skin retention behaviors of the surface-modified poly(aminodiamine) (PAMAM) dendrimers were first investigated using fluorescence spectrometry and confocal laser scanning microscopy. Derived from the structure of dendrimers, surface-modified dendron-based copolymer micelles were then developed for efficient hydrophobic drug delivery through skin layers \textit{in vitro} and \textit{in vivo}. This dissertation consists of three major chapters: (1) interactions between dendrimer and skin layers and skin penetration pathway studies, (2) dendrimer derivation – dendron micelle preparation and \textit{in vitro} transdermal drug delivery studies, and (3) dendron micelle drug carrier formulations and \textit{in vivo} drug delivery studies.

Chapter 2 includes the surface modification of PAMAM dendrimers for their skin penetration and retention studies: rhodamine B isothiocyanate was conjugated to the generation 2 (G2) and G4 PAMAM dendrimers for microscopic and spectrometric
detection of dendrimers; surface acetylation and carboxylation of the amine-terminated PAMAM dendrimers were carried out to obtain three types of PAMAM dendrimers with different surface functionalities. Oleic acid conjugation to the dendrimer surface altered the partition coefficient of the dendrimer. These surface modifications have shown effects on skin permeation behaviors of the dendrimers in terms of penetration depth, permeation pathways, and skin retention. Major findings from this study are listed below:

- Smaller PAMAM dendrimers (G2) penetrate the skin layers more efficiently than the larger ones (G4).
- G2 PAMAM dendrimers that are surface modified by either acetylation or carboxylation exhibit increased skin permeation and likely diffuse through an extracellular pathway.
- Unmodified amine-terminated G2 PAMAM dendrimers show enhanced cell internalization and skin retention but reduced skin permeation.
- Conjugation of oleic acid (OA) to G2 dendrimers increases their 1-octanol/PBS partition coefficient, resulting in increased skin absorption and retention.

Chapter 3 explores the transdermal delivery of skin-impermeable hydrophobic molecules upon encapsulation into PEGylated dendron-based copolymer micelles (DMs), which are derived from the structure of dendrimers and synthesized in house. Surface modifications of the DMs have shown to affect drug loading and indirectly resulted in different drug delivery efficiencies through the mouse and human skin. The physicochemical properties
(size, surface charge, and drug loading) of DMs were characterized, and the sustained drug release profiles revealing improved drug stability in aqueous conditions were obtained. The efficacy of the hydrophobic model drug endoxifen (EDX) after DM encapsulation was well maintained. *In vitro* skin permeation of the DM-loaded drug molecules was investigated in both mouse and human skin. Significantly enhanced drug permeation was observed using the DMs compared to chemical penetration enhancers (CPEs) and liposomes. Major findings from this study are listed below:

- EDX can be successfully loaded within our recently developed biocompatible and biodegradable DMs with various surface functional groups (amine, or -NH2; carboxyl, or -COOH; and acetyl, or -Ac) and distinctive surface charges moieties.
- All EDX-loaded DMs displayed spherical morphologies with similar average diameters of approximately 50 nm using transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively.
- End-group modification was shown to significantly affect the drug loading where the DM-COOH micelles displayed a 10-fold increase in loading compared to DM-NH2, and a more than 70-fold increase compared to cationic liposomes.
- DMs displayed a biphasic release profile of EDX over 6 days, whereas cationic liposomes displayed burst release properties and the encapsulated drug was lost within 8 h.
• DMs were found to substantially enhance the permeation of EDX through both full-thickness mouse (up to 20-fold) and split-thickness human (up to 4-fold) skin samples relative to ethanol, a traditional CPE.

• The fold-enhancement of skin permeation was also greater than that of the cationic liposomes used as a positive control (up to 3.5-fold).

• The flux of EDX through the skin layers obtained from Franz diffusion cell experiments followed the order of DM-COOH greater than DM-Ac, and followed by DM-NH$_2$, which correlated with the drug loading results.

Chapter 4 describes the development of the dendron micelle formulations for in vivo drug delivery, and the results from in vivo proof-of-concept studies using hairless mice were presented. Several DM preparation and drug loading methods were compared to promote drug loading and cost-effectiveness. Different topical gel matrices and occlusive conditions were compared to evaluate their compatibility with and potential for the DM drug delivery system. In vivo drug delivery efficiency of the DMs was also investigated using the model drug endoxifen (EDX). Skin deposition and plasma concentration of the drug were measured using a liquid chromatography-mass spectrometry (LC-MS) method. Based on the data obtained from the in vitro skin permeation experiments (described in Chapter 3) and the in vivo plasma concentration measurements, the in vitro-in vivo correlation (IVIVC) using the DM drug delivery system was also discussed. Major findings from this study are listed below:
• DMs can be prepared using a solvent evaporation (SE) method to improve loading efficiency of EDX.

• The drug permeation flux of EDX through hairless mouse skin provided by the Klucel gel formulation was comparable to that obtained from an aqueous delivery system (water).

• Applying the Klucel gel in occlusive conditions can improve the drug permeation into the mouse skin in vitro.

• The DM aqueous gel formulation has facilitated comparable in vivo skin deposition of EDX to the positive control (a CPE gel – Klucel gel containing 60% ethanol and 0.5% oleic acid, v/v) over a period of 96 h.

• In non-occlusive conditions, the DM aqueous gel formulation resulted in much lower plasma concentration of EDX compared to the positive control (the CPE gel). However, in occlusive conditions, comparable plasma concentrations of EDX were observed from both groups, indicating that the application method-dependent drug delivery of the DM platforms.

• Compared to the report data obtained from the use of the Estraderm® patch, the in vitro and in vivo data obtained from this study indicated that the DM systems have comparable or even greater transdermal drug delivery potential than CPE formulations.
Taken together, this dissertation presented a systematic study of the interactions between the skin layers and the dendritic polymeric nanomaterials, as well as the development of an effective topical drug delivery system for EDX based on the dendritic nanostructures. The in vitro permeation data obtained from both hairless mouse and human skin indicate that the DM system exceeds the performance of CPEs and liposomal formulations in delivering EDX. However, our in vivo study revealed that the permeation effect of the DM system is similar to that of the CPE formulation (Klucel gel containing 60% ethanol and 0.5% oleic acid). This discrepancy is possibly due to the following reasons:

1) Skin conditions are different: the mouse skin used for in vitro experiments is in contact with donor and receiver solutions, thus more hydrated than the mouse skin in the in vivo experiments. Since hydration can improve the skin permeability, higher skin permeation was observed from the in vitro experiments. Moreover, the results obtained from the in vivo study also clearly demonstrated that the skin permeation of EDX through DM system depends more on the occlusive conditions as compared to the CPE formulations, indicating that a hydrated environment is important for the DM systems to be effective. Nonetheless, the barrier function of the live animal skin can be stronger than the excised skin used for in vitro experiments. This is probably due to the capability of self-regeneration of the barrier function is active in the in vivo situation where the excised skin does not have such ability anymore.

2) The experimental settings are different: the optimal sink conditions were applied to the in vitro Franz diffusion cell settings, with 30% ethanol in the receiver solutions to extract
and solubilize the skin-permeated EDX. This setting is more relevant to the human skin, which contains subcutaneous fat tissue in the hypodermal layers that serve as a reservoir for the skin-permeated EDX and potentially enhance the skin permeation of EDX, while maintaining localized effect. However, the in vivo experiments were performed using hairless mice, which do not have subcutaneous fat beneath the mouse skin. This anatomical difference may account for the differences in skin permeation efficiencies between in vitro and in vivo systems.

3) The application methods also affect the skin permeation outcomes: as observed from the in vivo experiments, occlusive conditions can significantly increase the plasma concentration of EDX, as compared to non-occlusive conditions. Although improvement was made, the current occlusive dressing is still far from optimal due to technical limitations. Potential improvements will be discussed in the next section.

Interestingly, the DM delivery system dose not require any organic solvent (solubilizer) to stabilize the water insoluble EDX, but can still achieve effective drug delivery. This is attributed to the remarkable thermodynamic stability (CMC as low as $10^{-8}$ M) of the amiphilic dendron copolymers to form micelles encapsulating the hydrophobic drug. Furthermore, the DMs are almost completely covered by a dense layer of PEG that confers biocompatibility (potentially less skin irritant than using CPEs) and water solubility to the drug-loaded DMs. The PEG layer also provides similar properties of dendrimers to the DMs such as high-density peripheral functional groups and confers modularity to those
surface groups. Nonetheless, surface modifications of the DMs in this study demonstrated various drug loading and skin permeation efficiencies. Different drug molecules can be encapsulated to specific DMs with proper surface functionalities depending on the drug molecules’ physicochemical properties and compatibility to the DMs, providing versatility to the DM platform.

5.2 Future Direction

This dissertation research reveals that dendritic nanomaterials have the potential of being used as drug carriers and penetration enhancers for topical drug delivery. However, the systems presented here are still far from an ideal topical drug delivery system. A series of evaluations and assessments, followed by optimization, would be necessary to develop a highly effective system. For example, a more clinically relevant animal model should be selected for comprehensive evaluations of the in vivo pharmacokinetics profiles and tumor prevention efficiencies, which may potentially lead to a better understanding on in vitro-in vivo correlation (IVIVC). A commercially available topical delivery system could also be included in this study to serve as a positive control in the future.

To improve application accuracy and prevent drying of the DM gel, a reservoir or matrix style patch can be developed to better maintain the stability of the gel matrix and refine the exposure area to the skin. We can potentially start the development process by learning from the design of an Estraderm patch, which contains five proceeding layers from the
visible surface towards the surface attached to the skin: 1) a backing layer composed of polyester (PE)-ethylene vinyl acetate (EVA) copolymers that provides occlusive conditions; 2) a drug reservoir of estradiol (E2) in a hydroxylpropyl cellulose (Klucel) gel matrix containing ethanol as an E2 solubilizer and penetration enhancer; 3) a release rate controlling membrane made of EVA; 4) an adhesive formulation of light mineral oil and polyisobutylene to secure the patch in place onto the skin; and 5) a protective layer made of siliconized PE film attached to the adhesive layer before the patch is applied to the patient.\(^4\)

As compared to the Estraderm\(^\text{®}\) patch, in our current delivery system, DMs are also dispersed in the Klucel gel matrix, but instead of using ethanol and a rate controlling membrane as seen in an Estraderm patch, the PEGylated dendron-based copolymers have functioned as both solubilizers for EDX and rate controlling drug release materials. Although seemingly advantageous in terms of the system simplicity and potential avoidance of ethanol-induced skin irritation, the current design has drawbacks of providing a refined exposure area and a larger drug reservoir that can hold more DMs in a bigger volume of gel matrix. Moreover, to avoid disruption of the treatment by the hairless mouse, a much larger piece of TegaDerm was applied to cover the smaller treated area. This occupied at least half of the trunk region on the treated animal and can potentially provide more stress to the individual. It is critical to design a better system for DMs before one can move forward to \textit{in vivo} efficacy studies. This also brings us the notion that using a hairless rat model might be more suitable for future PK studies, in that rats have larger
skin area that one can manipulate and greater volume of blood that one can collect at multiple time points without sacrificing the animals, all of which can potentially introduce less individual-to-individual variations.\textsuperscript{5,6}

Furthermore, the current drug delivery systems that are intended for localized drug delivery turned out to have a degree of systemic exposure of the absorbed drug in mice (about 10\% by 72 h).\textsuperscript{7} However, this might not necessarily be the case once the system is applied to human skin, due to the differences in skin thickness between the two species, as well as the presence of more fat tissues in the human breast that could serve as a reservoir for EDX to exert local effect without entering into the systemic circulation.\textsuperscript{6,8}

Although the current DM system can efficiently deliver EDX through the skin, their potential in breast cancer prevention has not yet been fully evaluated. Moving forward from the current research findings, the breast cancer prevention studies can be conducted using an \textit{in vivo} xenograft model. Ductal carcinoma in situ (DCIS) is characterized by the presence of hyperplastic epithelial cells within the milk ducts of the breast, contained by an unperturbed myoepithelial cell layer and intact basement membrane.\textsuperscript{9} DCIS, although non-lethal, is an immediate precursor of invasive breast cancers, making the prevention and diagnosis of DCIS of critical importance.\textsuperscript{10} To investigate the anti-proliferative effect of the EDX-loaded DM system, NOD-SCID IL2Rgamma\textsuperscript{null} (NSG) mouse can be used to establish a mouse DCIS model by intra-ductal injection of DCIS specimens (xenograft)
obtained from patient surgical or biopsy samples. After formation of the epithelium inside the ducts, EDX-loaded DMs will be applied topically to the whole breast region or intra-ductal area to evaluate the tumor inhibitory effect of the topically administered EDX. The treatment will be applied every 3 days for up to 3 weeks, and oral tamoxifen will be given to the mice in the control group. The tumor volumes will be measured twice weekly. Blood, mammary glands, ovaries, and skin samples will be collected at the end of the treatments for drug concentration and histological analyses (Ki67 and ERα). If significant tumor inhibition was obtained and minimal side effect was observed using the DM system, in vivo toxicology studies followed by clinical trials will be also conducted in the future to fulfill the development process.

The DMs offer a promising drug delivery platform not only for the delivery of EDX, but they can also be tailored for the delivery of other drug molecules, imaging probes, and genes. For example, indomethacin was successfully encapsulated into the DMs and a sustained drug release for up to 6 days was observed. Moreover, the DM system is also suitable for systemic drug delivery due to its modularity for efficient surface modification and targeting ligand conjugation, as well as its thermodynamic stability after injecting into the blood circulation (demonstrated by their low CMC). Future efforts combined with work presented in this dissertation will play a critical role to advance DM system developments for prevention and treatment of breast cancer and other diseases.
5.3 References


BIBLIOGRAPHY


86. Krishnaiah, Y. S.; Al-Saidan, S. M. Limonene Enhances the in Vitro and in Vivo Permeation of Trimetazidine across a Membrane-Controlled Transdermal Therapeutic System. *Current Drug Delivery* 2008, 5, 70-76.


APPENDIX A Approved ACC Animal Protocol

August 12, 2013

Seunghyo Hong
Biopharmaceutical Sciences
M/C 863

Dear Dr. Hong:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on 8/12/13.

Title of Application: Dendrimer-based Transdermal Drug Delivery

ACC Number: 12-166

Modification Number: 2

Nature of Modification: Addition of start-up funds as funding source.

Protocol Approved: 8/24/2012

Current Approval Period: 8/24/2012 to 8/21/2013. Protocol is eligible for 1 additional year of renewal prior to expiration and resubmittion.

Current Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

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Phone (312) 996-1972 • Fax (312) 996-9088
APPENDIX.B Approved IRB Protocol

3/6/2012

Dr. Boro Kulesza
Pathology
p-kulesza@northwestern.edu

IRB Project Number: CR4_STU00023488
Project Title: NCI 01X1I: Breast Cancer Program: Tissue and Specimen Collection Facility
Project Sites:
Northwestern Medical Faculty Foundation (NMFF)
Northwestern University (NU)
Northwestern Memorial Hospital (NMH)

Sponsor Information (Grant #, if applicable): Avon Products Foundation, Inc.

Submission Considered: Continuing Review Submission Number: CR4_STU00023488
Submission Review Type: Expedited as per 45 CFR 46.110(b).
Review Date: 2/16/2012
Status: APPROVED Approval Period: (3/10/2012 - 3/9/2013)

Dear Dr. Kulesza,

The IRB considered and approved your submission referenced above through 3/9/2013. As Principal Investigator (PI), you have ultimate responsibility for the conduct of this study, the ethical performance of the project, and the protection of the rights and welfare of human subjects. You are required to comply with all NU policies and procedures, as well as with all applicable Federal, State and local laws regarding the protection of human subjects in research including, but not limited to the following:

- Not changing the approved protocol or consent form without prior IRB approval (except in an emergency, if necessary, to safeguard the well-being of human subjects).
- Obtaining proper informed consent from human subjects or their legally responsible representative, using only the currently approved, stamped consent form.
- Promptly reporting unanticipated problems involving risks to subjects or others, or promptly reportable non-compliance in accordance with IRB guidelines.
- Submit a continuing review application 45 days prior to the expiration of IRB approval. If IRB re-approval is not obtained by the end of the approval period indicated above, all research related activities must stop and no new subjects may be enrolled.

IRB approval includes the following:

Written Consent Form/Consent Form and Authorization for Research: NCI 01X1I ICF dated 01.10.12 final clean copy.doc
Protocol: NCI 01X1 Protocol dated 01.18.12.pdf

For more information regarding IRB Office submissions and guidelines, please consult
http://www.northwestern.edu/irb/auth/OPRS/irb.
This Institution has an approved Federalwide Assurance with the Department of Health and Human Services: FWA00001549.
APPENDIX.C Permission of Reprint/Reproduce

Title: Synthesis of Amphiphilic Star Block Copolymers and Their Evaluation as Transdermal Carriers
Author: Dawanne E. Poree, Marco D. Giles, Louise B. Lawson, Jibao He, and Scott M. Grayson
Publication: Biomacromolecules
Publisher: American Chemical Society
Date: Apr 1, 2011
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Effect of Size, Surface Charge, and Hydrophobicity of Poly(amidoamine) Dendrimers on Their Skin Penetration

Yang Yang, Suhair Sunqrot, Chelsea Stowell, Jingli Ji, Chan-Woo Lee, Jin Woong Kim, Seema A. Khan, and Seungpyo Hong

Publication: Biomacromolecules
Publisher: American Chemical Society
Date: Jul 1, 2012
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- W. E. van Doren Scholar for Graduate Research

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