School of Pharmacy

Skin Penetration Enhancement Techniques

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Doctor of Philosophy
of
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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:  .................................................................

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ABSTRACT

Transdermal drug delivery is an effective alternative to conventional oral and injectable drug delivery routes. It offers painless and convenient once daily or even once weekly dosing for a variety of clinical indications. The major limitation to successful transdermal drug delivery is the efficient barrier properties of the skin. Significant research efforts have been focused on developing strategies to overcome these barrier properties. These strategies include the use of physical and chemical penetration enhancers. Physical skin penetration enhancers use an external energy source to alter the barrier properties of the skin. The current research focuses on some of these physical skin penetration enhancers on a range of drug molecules and peptides.

The first technology investigated was Dermaporation that utilised pulsed electromagnetic energy. This technology enhanced the epidermal permeation of naltrexone *in vitro* as compared to passive diffusion. A 5-fold increase in naltrexone permeation was observed during Dermaporation application when compared to passive administration. Multiphoton tomography-fluorescence lifetime imaging microscopy (MPT-FLIM) analysis of the permeation of gold nanoparticles in the *ex vivo* human skin revealed increased penetration across the skin. These results demonstrated that the channels created by dermaporation must be larger than the 10 nm diameter of the applied nanoparticles.

The second technology investigated was an unpowdered magnetic film array technology (ETP), which utilised unpowdered magnetic energy. Chapter 3 presents enhanced epidermal permeation of urea with ETP. A 4-fold increase in urea penetration was observed across human epidermis in the *in vitro* permeation study. Optical resonance tomography was used to visualise the changes in epidermal thickness due to urea permeation as an indication of increased hydration. The results revealed an increase in epidermal thickness at 30 min, to 16% for ETP induced urea permeation as compared to 3% with urea from occlusion. These results further substantiated our previous findings that magnetic energy creates hydrophilic diffusion channels or pores in the skin.

The third technology investigated was low-frequency sonophoresis that utilises cavitation bubbles as a force to create channels for drug delivery in the skin. Chapter
4 presents enhanced human skin permeation of 5-aminolevulinic acid \textit{in vitro} and curcumin dye \textit{in vivo} with low-frequency sonophoresis. Two different sources of ultrasound devices that generated low-frequency sonophoresis were investigated. MPT-FLIM analysis was utilised to investigate the effects of sonophoresis on human skin \textit{in vivo}. This revealed that there was substantial disturbance in the epidermal cells due to cavitation by sonophoresis. Permeation of curcumin was found in the deeper layers of the epidermal membrane with 55 kHz sonophoresis and was confined to the more superficial layers of skin with 21 kHz sonophoresis. Permeation of 5-aminolevulinic acid across human skin increased significantly when compared to passive permeation.

The fourth technology investigated in this research was iontophoresis which utilises a small electric current to drive charged and uncharged molecules across the skin. Chapter 5 presents enhanced epidermal permeation of a range of model therapeutic and cosmetic peptides. Various key parameters such as pH, concentration and presence of counterions and co-ions that are essential for effective iontophoretic delivery of these peptides were investigated. The iontophoretic delivery of 5-aminolevulinic acid revealed a 15-fold enhancement when compared to passive diffusion. For dipeptide (Ala-Trp) the mean cumulative amount increased iontophoretic delivery from 0.4±0.4, 0.1 µg/cm² to 16.0±8.8, 3.6 µg/cm² (Mean±SD, SEM) when the donor pH was reduced from 7.4 to 5.5. The corresponding current intensity (0.38 mA/cm²) normalised flux was 36.1±19.5, 11.2 µg/(mA.h) for iontophoretic Ala-Trp. For the tetrapeptide (Ala-Ala-Pro-Val) the mean cumulative amount that permeation with 2h iontophoresis was 350.4±45.9, 15.3 µg/cm² (Mean±SD, SEM) compared to zero passive permeation. A 4-fold increase in acetyl hexapeptide-3 delivery occurred with iontophoresis compared with passive application. In addition it was observed that lowering of donor solution pH and the presence of counterions and co-ions reduced the iontophoretic delivery of acetylhexapeptide-3. Iontophoresis provided a significant enhancement factor for the decapeptide, triptorelin acetate with a 16-fold increase in epidermal permeation compared with passive permeation. The iontophoretic permeation was concentration dependent with mean cumulative amounts of 48±28, 14 µg/cm² (Mean±SD, SEM) achieved with 9 mM concentration of triptorelin acetate.
Overall the technologies investigated in this research work presented enhanced permeation of drug molecules and peptides. In addition MPT-FLIM was demonstrated to be an efficient visualisation tool for permeation within the skin. This research demonstrates the effectiveness of physical skin permeation enhancement techniques and extends our understanding of these technologies.
1 CHAPTER 1

INTRODUCTION
1.1 TRASDERMAL DRUG DELIVERY SYSTEMS

In the past the transdermal route of delivery was confined to topical creams and ointments for dermatological disorders. However in recent years there has been a great deal of interest in the transdermal delivery of drugs which has lead to the successful commercialization of transdermal patches such as scopolamine, nitroglycerin, clonidine, oestradiol, testosterone and nicotine. The transdermal route of delivery offers the advantage of avoiding local gastrointestinal irritation and hepatic first-pass metabolism thus facilitating effective use of drugs with short biological half-lives, allows administration of drugs with a narrow therapeutic index, providing controlled plasma levels of potent drugs and improving patient compliance. However the primary disadvantage of this delivery route is the inability to administer high doses of drugs, thus restricting its application to potent drugs. This chapter presents a brief introduction to transdermal drug delivery including discussion of various factors and parameters that play an important role in this route of drug delivery.

1.2 STRUCTURE AND FUNCTION OF HUMAN SKIN

Skin is the largest complex organ in the human body. Apart from being a barrier and protecting against the hostile environment, its functions include maintaining the internal aqueous environment, homeostasis, essentially in terms of heat regulation, blood pressure control and excretory roles. It is also the major sensory organ in the human body sensing the various environmental influences such as heat, touch, pressure and pain. The skin undergoes continuous regeneration, produces immunological and histological responses to both physical and chemical assaults including drug molecules applied deliberately or accidentally. Human skin is structurally divided into three layers: epidermis (stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale), dermis and the hypodermis or subcutaneous fatty layer (Figure 1.1 and 1.2).
Figure 1.1: Structure of human skin (1).

1.2.1 EPIDERMIS:

The epidermis is a dynamic, constantly self-renewing tissue, in which a loss of the cells from the surface of the stratum corneum (desquamation) is balanced by cell growth at the stratum basale. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum undergo a number of changes in both structure and composition. The keratinocytes synthesize and express numerous different structural proteins and lipids during their maturation.
1.2.1.1 STRATUM CORNEUM:

The stratum corneum (SC) is the outermost non-viable layer of the epidermis and the final product of epidermal cell differentiation. In general the SC consists of 15 to 25 flattened, stacked, hexagonal and cornified anucleated cells, each cell approximately 40µm in diameter and 0.5µm thick and is a 10-20µm thick layer when dry. Thus the
cells in this layer are termed corneocytes. During the process of cornification the cellular components and cytoplasm disappear and the remaining protein constituents remodel. Corneocytes are thus composed of insoluble bundled keratins (3-4) surrounded by a cell envelope stabilised by cross linked proteins (5) and covalently bound lipid (6-7). Interconnecting the corneocytes of the stratum corneum are polar structures such as corneodesmosomes which contribute to its cohesion by acting like molecular rivets. The SC has been represented as a brick and mortar model, in that the corneocytes are embedded in a mortar of lipid bilayers (8-9). The SC constitutes the major diffusional barrier of the skin. The barrier properties of SC may be related to its very high density (1.4 g/cm³ in the dry state), its low hydration of 15-20% compared to the usual 70% for the epidermis and the presence of cell envelope protein, which is very resistant to chemical attack. SC also functions as a barrier against entry of harmful materials including microorganisms and the loss of internal body components, particularly water, to the external environment. Typically the lamellar bodies carrying the precursors for SC barrier lipids consist of glycosphingolipids, free sterols and phospholipids. At the stratum granulosum/SC interface these lamellar bodies, are enzymatically converted into nonpolar products which assemble into lamellar structures surrounding the corneocytes. Glycolipids hydrolyse generating ceramides (CER), while phospholipids are converted into free fatty acids (FFA). The change in lipid composition and cell structure results in the formation of a very densely packed structure in the stratum corneum. This mutual arrangement in the lamellar domains is a key process in the formation of the skin barrier (10).

1.2.1.2 STRATUM LUCIDUM:

The Stratum lucidum (Latin for "clear layer") is a thin, clear layer of dead skin cells that are located beneath the stratum corneum and seen particularly on thick skin areas, such as that on the palms of the hands and soles of the feet. Within this layer the cells flatten and are filled with eleidin, an intermediate form of keratin, and the nucleus disintegrates (11).

1.2.1.3 STRATUM GRANULOSUM:

One to three layers thick and so called because its cells acquire granular structures and contain enzymes that begin degradation of the viable cell components such as
nuclei and organelles. The keratohyalin granules mature the keratins within the cell. As the cells approach the upper layers of the stratum granulosum, the cells extrude lamellar granules into the intercellular spaces (11).

1.2.1.4 STRATUM SPINOSUM:

The spinous or prickle cell layer of the skin, found on the top of the stratum basale and together with the stratum basale layer is termed the malpighian layer. Cells of the stratum spinosum initiate the keratinisation process by synthesizing intermediate keratin filaments, called cytokeratins, which are anchored to the desmosomes joining adjacent cells to provide structural support that helps the skin resist abrasion. It stratum spinosum forms a hydrophobic barrier between the stratum granulosum and stratum lucidum that prevents dehydration (11).

1.2.1.5 STRATUM BASALE:

The stratum basale also termed the basal layer or stratum germinativum is a continuous single layer that consists of columnar epithelial cells that look similar to cells found in other tissues within the body. The basal cells are termed the stem cells of the epidermis as they proliferate and create daughter cells that migrate to the superficial layer differentiating. The cells undergo regular mitosis, replicating every 3.5-7h to generate one cell layer per day in normal skin. Other cells in this layer include melanocytes, which are responsible for pigmentation of the skin and hair, Langerhan cells, which are the antigen presenting cells of the skin and play an important role in conditions such as contact dermatitis, and Merkel cells which are cutaneous sensors found predominantly around the touch sensitive areas of the body (lips and fingertips) (11).

1.2.2 DERMAL-EPIDERMAL JUNCTION:

The dermal-epidermal junction plays a major role in the regulation of epidermal proliferation but does not pose a primary barrier to diffusion of any compounds. Since the epidermis does not contain any blood vessels, nutrients, xenobiotics, waste products and growth factors must diffuse through his junction to access the cutaneous circulation in the dermis. Similarly, drug molecules administered to the skin surface that are aimed at the systemic circulation must clear this junction and all the epidermal layers above it (12).
1.2.3 **DERMIS:**

The dermis provides nutritive, immune and structural support for the epidermal layers in addition to important role in regulating temperature, pressure and pain. It is a course reticular layer, 0.1-0.5cm thick, consisting of collagenous fibres (70%) in a semigel matrix of mucopolysaccharides. The vast network of fibrous, filamentous and amorphous connective tissue determines the tensile strength and elasticity of the skin and provides the physical support for extensive nerve and vascular networks. Appendages such as the pilosebaceous units (hair follicles and sebaceous glands) and sweat glands (eccrine and apocrine) are derived from this region. The main cells present are the fibroblasts which produce the connective tissue components (collagen, laminin, fibronectin, vitronectin), mast cells that provide immune and inflammatory response and melanocytes (12).

1.2.4 **SUBCUTANEOUS FATTY LAYER:**

The hypodermis is predominantly made up of adipocytes (fat cells) arranged in lobules. This is the layer which anchors the skin to the underlying body constituents. The main roles of the hypodermis are storage of energy, metabolism, providing insulation to the body and mechanical protection against injury. All the principal blood vessels and nerves are carried to the skin in this layer. The hypodermis can be relatively thick in the order of several millimetres or completely absent depending on the region of the body (11).
Figure 1.3: Routes of Penetration through human skin 1. Intercellular, 2. Transcellular and 3. Appendageal (13).
1.3 ROUTES OF TRANSDERMAL DRUG DELIVERY

The skin is an effective barrier to percutaneous absorption of compounds as well as to water loss from the body. The SC provides the primary barrier and the lipids play an important role in the barrier properties of skin. Since the rate-limiting step for skin absorption of most molecules is considered to be this non-viable layer, transdermal permeation is governed by the laws of diffusion.

From the time a molecule first comes in contact with the skin to the time it reaches the systemic circulation, there are multiple, complex permeation processes. Figure 1.3 and 1.4 represents the various partition-diffusion routes that a molecule could go through before reaching the systemic circulation. In addition to these multiple partitioning and diffusion processes, the molecules entering human skin could bind to SC components like keratins or bind to certain receptors in the skin or potentially be rendered inactive or activated (prodrugs) due to metabolic activity in the skin.
Figure 1.3 illustrates three pathways by which a molecule can cross the SC layer: (a) the appendageal pathway such as hair follicles and sweat ducts (shunt route), (b) transepidermal pathway (intercellular lipid domains in the stratum corneum and/or by trans-cellular polar route through the keratin bundles) (15-16).

The appendages essentially act as a pore that bypasses the stratum corneum barrier. The available diffusional area of the shunt route is approximately 0.1% of the total skin area (17-18). Eccrine sweat glands may be numerous in several areas of the body (e.g. palms and soles), but their opening into the skin is still very small. These ducts are either empty or actively secreting sweat that would be expected to diminish inward diffusion of topically applied agents. The duct of the sebaceous gland are also filled but with lipoidal sebum. Despite their small fractional area the skin appendages may provide the main portal of entry into the subepidermal layers of the skin for ions (19-20) and large polar molecules (21-23). The role of the appendageal pathway in the transport of low-to medium-molecular weight non-electrolytes is still unclear. The shunt routes are considered important for delivering vesicular structures to the skin and for targeting their contents to the pilosebaceous units. Iontophoretic drug delivery that uses an electrical charge to drive molecules into the skin is largely dependent on the shunt route. The charge is carried through the stratum corneum via the path of least resistance and the shunt route provides less resistance than the SC bulk (24-25).

It is well known that lipid solubility is usually a prerequisite for good permeation through the SC layers. The intercellular spaces of the SC comprise a tiny portion of its overall volume; these spaces traditionally have been discounted as a possible pathway. Water-soluble substances traverse the SC via diffusion within the aqueous regions situated near the outer surface of intracellular keratin filaments, and lipid-soluble molecules through diffusion in the lipid matrix region between these filaments (18). The intercellular and transcellular pathway in percutaneous penetration of a drug molecule depends on its diffusivity in lipids and proteins, partitioning of the drug molecule between these domains and the geometry of the SC (8, 26-27).
1.4 PERMEANT PHYSICOCHEMICAL CHARACTERISTICS FOR SKIN DELIVERY

The relative importance of the possible routes of permeation through the skin depends on the chemical characteristics of the permeant (28). In general drug permeation across the stratum corneum obeys Fick’s first law (equation 1.1) where steady-state flux \( J \) is related to the diffusion coefficient \( D \) of the drug in the stratum corneum over a diffusional path length or membrane thickness \( h \), the partition coefficient \( K \) between the stratum corneum and the vehicle, and the applied drug concentration \( C_0 \) and \( C_i \) is the concentration inside the membrane which is assumed to be constant(13):

\[
J = D K \frac{(C_0 - C_i)}{h}
\]

Equation 1.1

From the above equation the ideal properties of a molecule for optimal skin penetration would include molecular weight < 600 Dalton, good/optimal oil and water solubility (>1 mg/mL) (to maintain sufficient membrane concentration gradient \( C_0 \)), optimal lipophilicity log partition coefficient \( K \) \( (\log P \approx 1-3) \) and low melting point \( (< 200^\circ C) \) which correlates with good solubility(14). Apart from these characteristics, the ionisation state or polarity of the molecule is important as nonionized molecules generally permeate more readily than ionized drugs (8, 29) because the free-energy change required for an ionized drug to partition into the oil phase is greater than that for a nonionized drug. However, if the water solubility of the ionized form is greater than that of the neutral form, then the ionized drug may display a greater transport rate (8).

1.5 PENETRATION ENHANCEMENT TECHNIQUES

Over the past 3-4 decades considerable research has been directed towards strategies to circumvent the barrier properties of skin. Barry and co-workers introduced the concept of classifying penetration enhancers according to their primary effect, namely (a) disruption of stratum corneum lipids, (b) interaction with intracellular proteins and (c) improved partitioning of the drug (30-32). Therefore an ideal penetration enhancer should increase skin permeability by reversibly disrupting the SC structure whilst avoiding injury to deeper living tissues and provide an additional driving force for transport into the skin. Penetration enhancers are broadly classified
as physical and chemical techniques based on their mode of action. These techniques are not mutually exclusive and considerable research has been published on combined strategies of these techniques.

1.5.1 CHEMICALS AS PENETRATION ENHANCERS

This approach is a logical extension of the traditional pharmaceutical toolbox as it primarily involves designing new formulations with chemical excipients that will act as penetration enhancers. Many effective chemical enhancers disrupt the highly ordered lipid bilayer structures in the stratum corneum by inserting amphiphilic molecules into these bilayers to disorganize molecular packing or by extracting lipids using solvents and surfactants to create defects or micro-cavities of nanometre scale in the lipid packing. Hundreds of chemicals have been identified as penetration enhancers including off-the-shelf compounds such as sulfoxides (dimethylsulfoxide (DMSO & DCMS), dimethylformamide (DMF), pyrrolidones (N-methyl-2-pyrolidone, 2-pyrolidone, 5-methyl-2-pyrrolidone and 1-ethyl-2-pyrrolidone), fatty acids such as lauric acid (C₁₂) and cis-unsaturated oleic acid (C₁₈), alcohols (ethanol, propylene glycol) and terpenes (menthol, 1,8-Cineole, Limonene, etc), surfactants (sodium lauryl sulphate (anionic), cetyltrimethyl ammonium bromide (cationic) and dodecyl betaine (non-ionic), phospholipids (non-vesicle) and urea. Briefly a few most popular include water (described in the next section) which acts by simple hydration (achieved by occlusion)(33-34). DMSO in sulfoxides acts by changing the intercellular keratin confirmation from α helical to β sheet (35-36). Many agents such as antifungal, antibiotics, barbiturates, steroids, and local anaesthetics have been successfully enhanced with DMSO. But due to toxicity, high irritancy and odour, DMSO has limited use. Ethanol in alcohols is the most preferred solvent of choice in transdermal patch system. It acts by solubilising the lipid component of the stratum corneum (37-38) and has successfully enhanced the flux of levogestrol, oestradiol, hydrocortisone, 5-fluorouracil, etc. Oleic acid is the most popular long chain fatty acid. An extensive experiment of fatty acids and alcohols, sulfoxides, amines and surfactants as penetration enhancers by Aungst revealed that saturated alkyl chain lengths of C₁₀-C₁₂ attached to a polar head group yields a potent enhancer (39). The mechanism of penetration enhancement is by fluidizing the lipids and by phase separation within the membrane (40-42). Other specifically designed and synthesized for this purpose include A zone (1-dodecylazacycloheptan-2-one)
and SEPA (2-n-nonyl-1,3 dioxolane) (43) that exerts its action on the hydrophilic component of the stratum corneum layers by hydrating the skin.

Urea (carbamide) is a hydrotrope that works on the hydrophilic component of the stratum corneum layers by hydrating the skin. Urea has been used as a humectant in moisturizing creams since 1943 (44) and is a natural component of the stratum corneum Natural Moisturizing Factor (NMF). Moisturizers containing urea have been reported to improve stratum corneum barrier function, reduce trans-epidermal water loss (TEWL), increase skin capacitance and reduce irritation (45-46). Due to marginal penetration enhancing properties, modified analogues of urea have been synthesized in attempts to combine the water-holding and keratolytic properties of urea with moieties that have shown enhancement activity such as C12 alkyl chains. Wong and co-workers synthesized series of cyclic urea analogues and found them to be as potent as a zone for enhancing the permeation of indomethacin across shed snake skin and hairless mouse skin (47).

The effects of these penetration enhancers on skin have been demonstrated using various techniques like differential scanning calorimetry (DSC) (48-51), electron spin resonance (ESR) (52-53), Fourier transform infrared spectroscopy (FTIR) (42, 54-55), Raman spectroscopy (56) and X-ray diffraction (57). Generally the chemical structure of some of these compounds consist of a polar head group with a long alkyl chain (58) and are more effective for hydrophilic permeants, although increased delivery of lipophilic permeants has also been reported (13). This approach of penetration enhancement techniques has been successful on a wide range of small molecules but their clinical application is limited due to safety issues and skin irritation (59-61).

1.5.2 HYDRATION (WATER AND OCCLUSION)

One of the safest and most widely used methods to increase drug delivery through human skin is to increase the water content of the tissue. Under normal conditions the water content in SC is about 15-20% of its dry weight (depending on the external environment). Once the skin is saturated with water (high humidity) or during occlusion, transepidermal water loss is reduced or stopped and hence there is an increase in water content of the stratum corneum; as it approaches equilibrium with the water content in the deeper layers, the skin is fully hydrated, leading to
greater drug flux. In general increasing SC hydration increases transdermal delivery of both hydrophilic (62) and lipophilic permeants (63). Though the mechanisms of action are unclear the effects of increasing stratum corneum hydration are evident.

1.5.3 CHEMICAL AND FORMULATION MODIFIERS

The chemical nature of the permeant can be modified to provide a drug form that has better partitioning / permeation characteristics. These modifications include: prodrugs to enhance dermal and transdermal delivery of drugs with unfavourable partition coefficients that cleave within the body to release the active drug molecule; formation of ion pairs in which an opposite charged permeant and excipient forms a complex and allows the permeant to partition into the membrane; using coacervates, a specialized form of ion pairing; forming eutectic systems which are based on the theory that lowering the melting point to increase solubility, including solubility in skin lipids (11).

1.5.4 VESICLE OR PARTICLE DELIVERY

Liposomes consist of lipid molecules arranged in one or more bilayers that fully enclose an aqueous volume. The lipid molecules are usually phospholipids with or without cholesterol which increases the rigidity of the liposome. A variety of possible mechanisms exist to explain the enhanced drug delivery using liposomes including adsorption onto and fusing with the skin surface, permeation into the stratum corneum to some extent before fusing with stratum corneum, thereby providing a reservoir for therapeutic agents (11). Despite extensive research, the effectiveness of liposomal delivery remains controversial. Most applications are for cosmetic agents or treatment of pilosebaceous units in conditions such as alopecia and acne for example tretinoin liposome formulation (64) and clindamycin (65). Alternative vesicle compositions have also been investigated including niosomes (non ionic surfactants) (66-67), ethosomes (traditional liposomes but with high levels of a low ethanol- usually ethanol) (68) and Transfersomes (containing surfactant ‘edge activators’) (69-71). The later vesicle formulations have chemical structures that confer flexibility to the vesicle and are claimed to allow them to squeeze through small spaces in the skin.
1.6 PHYSICAL PENETRATION ENHANCEMENT METHODS AND MECHANISM

1.6.1 STRATUM CORNEUM ABLATION

This involves partial removal of the SC which as expected is an effective enhancement method. A number of techniques have been investigated including laser, radio frequency and direct heat. Laser ablation involves directly applying high energy laser to the skin surface to remove a substantial amount of stratum corneum layers and therefore increase drug permeability. Low-intensity Erbium: YAG (yttrium-aluminium-garnet) at 2940 nm laser light is used for cosmetic procedures (plastic surgery) for treatment of rhytides, photodamage, scars, depigmentation, etc. In 1991 Nelson et al showed enhanced (2.1 fold) permeation of INF-γ and hydrocortisone across porcine skin using a much lower intensity (2790 nm) mid-infrared laser (72). Similarly Lee et al reported use of low intensity (0.35-0.45 J and 0.91-1.17 J/cm²) YAG laser (light emission at 2940 nm) enhanced skin (nude mouse) permeability of both lipophilic and hydrophilic drugs invivo (73). Since then the technique has been evaluated on small drugs and macromolecules (74-75).

1.6.2 RADIOFREQUENCY (RF) ABLATION

RF cell ablation technology uses radiofrequency waves to create micro-channels in the epidermis by cell ablation. The science behind the technology has been used earlier to remove living cells to cut tissue and/or destroy small tumours in the kidney and liver. RF ablation is performed by placing a conducting wire on a particular area and passing an alternating electric current at a frequency below 100 KHz through that area. These vibrations create heat or thermal energy that causes water evaporation and cell ablation. The commercial technology (Viaderm, TransPharma Medical Ltd, Lod, Israel) consists of a microelectronic system of RF electrodes that cause use of cell ablation thus creating micro-channels and is used as a pre-treatment with a patch containing the drug then placed on top of the pre-treated skin. The technology has been evaluated for transdermal delivery of drug molecules, hydrophilic polypeptides and other large molecules, genes and vaccines (Figure 1.5) (76-78).
Figure 1.5: Viaderm radiofrequency generating device by TransPharma Medical Ltd, Lod, Israel (78)

1.6.3 JET-PROPELLED INJECTORS

Figure 1.6: The basic design of jet-propelled particle delivery device (79).
The system basically consists of compressed gas (usually helium) leading into a small chamber that contains the drug particles bound between two thin plastic membranes. Beyond the second membrane the nozzle narrows and then widens to the point of delivery. Upon activating the gas bursts the plastic membranes holding the drug powder, which is fired down the nozzle at around 500 m/s into the stratum corneum. Sarphie et al demonstrated inulin delivery through hairless guinea pig from a prototype PowderJect device and showed large particles (39-52 µm) had better delivery than small particles (21-24 µm) (80). The depth of penetration is achieved by optimizing the physical properties of the device, such as velocity of the carrier gas, discharge pressure and particle size (81). Degano et al showed intradermal DNA immunization against influenza A virus in mice using PowderJet system (82). The application of a prototype jet propulsion system (PowderMed) developed in the UK has been focused on vaccine delivery to induce immunization through intradermal and intramuscular injections (83-84). Recently Liu et al group have developed a prototype device (Figure 1.6) that is a countoured shock tube (CST) configuration which utilizes a quasi-steady state and quasi-one dimensional shock-free supersonic flows to deliver uniform particle velocity and spatial distribution. They have shown clinically effective, needle-free, pain-free administration and applicable to a large number of macromolecular drugs, particularly therapeutic proteins and vaccines (79).
1.6.4 MICRONEEDLES:

Microneedle arrays are applied to the skin surface so that they penetrate the upper epidermis (which contains no nerves) far enough to create a physical pathway and thus allow drug delivery, but are too short to reach the pain receptors in the dermis (87). The microarrays can be fabricated in many different ways, for example individual silicon needles measuring approximately 150μm in length and 80μm base diameter fabricated onto arrays of approximately 3 x 3mm (approximately 400 needles). Alternatively they can be metallic needles with hollow centres (5-70μm) through which drug could be administered, or biodegradable polymers or self-dissolving thread-like microneedles that dissolve in the skin to create channels (86, 88-89). Microneedle technology has been predominantly directed towards macromolecule delivery (90). Martanto et al. showed decreased glucose levels following insulin administration for 10min through a 105 stainless steel solid microneedle array (91). Similar results have been reported on mice and rats with polymer based self-dissolving microneedles and hollow microneedles (92-93). There have been concerns regarding the safety, reversibility and needle strength, however it has been shown to be a successful delivery method that offers painless, flexible and

Figure 1.7: (Left) Schematic diagram of microneedles in the skin (85), (a) section of a 20 by 20 array of microneedles, (b) close-up view of a microneedle tip (86).
convenient means of enhanced skin delivery (94-96). Many companies such as 3M have pursued interest in this technique and it is likely these will be available in the near future.

1.6.5 PHOTOMECHANICAL WAVE

![Schematic diagram of basic photomechanical wave delivery device](image)

Figure 1.8: Schematic diagram of basic photomechanical wave delivery device (97)

Photomechanical waves (PMW’s) or laser generated stress waves are the pulsed pressure waves produced by a blation of a material target (polystyrene) by Q-switched or mode-locked lasers which are thought to cause transient stratum corneum lipid permeabilization. Transdermal delivery of oligonucleotides, DNA (98), microspheres (100nm) (99) and gene delivery for targeted gene transfection has been reported. Tarawaka et al report successful gene delivery for skin grafts (rat skin) (100-101) They also investigated PMW induced structural changes using histological evaluation and TEM to identify morphological changes of the skin cell membrane and organelles concluding that PMW provided minimally invasive non-viral gene transfection technique (102).
1.6.6 ELECTRICAL CURRENT ASSISTED PERMEATION

1.6.6.1 ELECTROPORATION

Electroporation or electro-permeabilization involves the application of pulsed electric fields across a cell to produce transient (reversible) or permanent (irreversible) permeabilization of the cell membrane. Irreversible electroporation is an important method of sterilization in the food industry and is becoming an important minimally invasive tissue ablation technique in medicine (104). Typically a high voltage in the order of 100-1000V is applied as a pulse to a membrane for a short period of time (micro- or milli-seconds). These pulses create transient aqueous pores in lipid membranes that permit DNA to be introduced into bacterial cells. The same principle is applied to transdermal delivery of molecules that then largely permeate through these aqueous pores in the stratum corneum barrier (105-109).

Three possible enhancement mechanisms have been proposed for electroporation: formation of transient aqueous pores in the lipid lamellae, that provide a direct pathway through the stratum corneum barrier which could be the result of localised heating of the lipids causing a phase transition (110-111); iontophoretic delivery of

Figure 1.9: Schematic diagram of electroporation setup (103)
the ionized form of the drug molecule; and simple diffusion via the aqueous pores that are open for a short period of time after pulsing (112-114). Electroporation of the skin showed enhanced permeation in the order of $10^{-10}^{4}$ fold increase in flux for molecules in the size range of up to 40kDa (14). Prausnitz et al reported therapeutic levels of heparin, 100-500µg/cm².h following electroporation (115). The technique has been evaluated for vaccine and DNA delivery (expression of topically applied plasmid DNA), liposomes, nanoparticles and microspheres. Zhao et al reported delivery of a model peptide vaccine into mouse skin to generate a cytotoxic T lymphocyte response (116). Recently Landstrom et al demonstrated electroporation enhanced delivery of bleomycin in human subjects (n=6) for local tumors (basal cell carcinoma and squamous cell carcinoma) of the head and neck. The technique showed better results than surgical removal of the tumor and postoperative radiotherapy which can also be associated with complications such as facial nerve damage or damage to the parotid duct. However since the electro-chemotherapy was carried out under general anesthesia it is therefore compared more with invasive surgical procedure (117). There have been many questions regarding the safety of electroporation and pain associated with muscle spasms due to the high electric field. The recent use of microelectrodes that restrict the electroporation field to the SC layers (118) may address these issues and if enhancement can be achieved comfortably and this equipment can be miniaturised it may emerge as a promising transdermal delivery system.

1.6.6.2 MAGNETIC ENERGY

Magnetophoresis is an emerging transdermal drug delivery technique that uses magnetic field energy to enhance the permeation of molecules. Early reports on use of magnetic field energy involved magnetite incorporated drug particles carried to the target site using powerful static magnetic field. Some examples of such particles include chemotherapeutic and radioisotope agents combined with magnetite, iron, nickel, cobalt, neodymium-iron-boron or samarium-cobalt injected into blood stream and concentrated at the require site (119-120). Murthy et al reported enhanced skin permeation of drugs molecules that exhibited magnetic properties such as benzoic acid, salbutamol sulphate and terbutaline sulphate (121-123). He recently reported
the enhanced transdermal delivery of local anaesthetic, lidocaine which is a diamagnetic substance using magnetic field strength of 30, 150 and 300 mT (milli Tesla) (124).

1.6.6.3 ELECTROMAGNETIC FIELD (EMF) ENERGY

EMF energy displays both the characteristics of electrical and magnetic field energy. Pulsed electromagnetic fields (PEMF) both repetitive and/or single pulses at low frequencies has been shown to produce weak direct electric current effects on tissues that are capable of stimulating cellular levels, growth, differentiation and repair of cells and tissue. The potential effect of EMF on human health depends widely on the intensity and frequency of the fields. Early reports on the use of PEMF on healing disjoined fractures date back to 1972 (125). Since then various studies on the effect of PEMF energy on various cell types (fibroblasts, endothelial cells and keratinocytes) have been reported (126-128). In addition there have been positive effects of PEMF on wound healing (129-131) improving chronic skin ulcers (132-134) stimulation of collagen (135) and bone growth (136), photodynamic effects on cancer cells (137), and cutaneous microvasculature (138).
1.6.6.4 SONOPHORESIS (ULTRASOUND WAVES)

Figure 1.10: Schematic diagram of ultrasound mediated transdermal delivery with acoustic bubbling effect on skin layers (103).

Sonophoresis or phonophoresis is defined as the use of ultrasonic energy to enhance the topical or transdermal delivery of drugs. Earliest reports on the use of soundwaves date back to the 1950s when it was used in combination with steroids, analgesics or anti-inflammatory drugs to treat muscular and arthritic conditions (139). Ultrasound is an acoustic vibration propagating in the form of longitudinal compression waves at frequencies beyond the human auditory range of 0.02MHz (Figure 1.10).

Ultrasound devices used in transdermal studies range between low frequency (20 kHz) to several MHz (up to 16 MHz) frequency, however recent studies report that low-frequency ultrasound is more effective in delivering drugs and macromolecules than high-frequency ultrasound (140). Various extensive studies on the mechanism of skin permeation enhancement produced by ultrasound suggest a combination of indirect effects, such as mechanical impact due to the collapse of cavitation bubbles in solution because the bubbles grow and oscillate within the ultrasound transducer.
and the skin, resulting in disruption of the structure of lipid bilayers in the stratum corneum (141-142). The cavitation effect created by bubble formation in sonophoresis enables concentration of the ultrasound energy at the target site. A forced convection flow is generated in the presence of ultrasound due to cavitation (143) and combination of shockwaves and microjet patterns of inertial cavitations (144-145).

Cavitation based low-frequency ultrasound was approved by the US FDA for local anaesthesia using EMLA cream (lidocaine and prilocaine) (146), (147). Animal studies are reported for delivery of insulin, heparin, tetanus toxoid and vaccine (148). Low-frequency ultrasound has also been used in glucose monitoring for diabetes by utilising its ability to extract interstitial glucose. Handheld low-frequency ultrasound devices are available for cosmetic purposes in delivering vitamins (C and A) and in some cases as low-profile light weight cymbal transducers that could be integrated as patch delivery systems for insulin delivery (149-150). In the present work we have investigated two commercially available low-frequency ultrasound devices (US FDA approved Sonoprep® and patent pending Iozyme®) that are used for clinical and cosmetic purpose respectively. A more detailed description on the ultrasound mediated skin penetration enhancement has been presented in chapter 4.
IONTOPHORESIS

Iontophoresis may be defined as the transfer of charged molecules or ions across a biological membrane under the influence of an electric current. The technique was introduced in the 1740s as a treatment for arthritis (151), but it was in the 1930s that iontophoresis gained momentum in the treatment of hyperhidrosis (excessive sweating) and transport of drug molecules into the skin (152). Since then, iontophoresis has been used extensively in various fields such as diagnosis of cystic fibrosis by iontophoretically administering pilocarpine (153), treatment of hyperhidrosis of the palm and sole through tap water iontophoresis (154), and fluoride ion penetration into teeth in dentistry (155), local anaesthesia in skin and the ear canal (156) and ophthalmology and otolaryngological indications (157). The focus of iontophoresis skin delivery research has now shifted towards controlled systemic delivery, particularly for large macromolecules, peptides and proteins (158). A commercial iontophoretic patch for patient controlled analgesia has been available since 2006 (Iosys, Alza Mountain View, CA, USA). Similarly Lid oSite® lidocaine HCl/Epinephrine topical iontophoretic patch system for local anaesthesia has been used prior to blood draws, venipunctures and superficial dermatological procedures (Vyretis, Inc, Fair Lawn, NJ) Activation of iontophoresis via a button on

Figure 1.11: Schematic diagram of iontophoretic setup (103)
the patch increases delivery of fentanyl to control pain in acute postoperative patients (159). Other such miniature devices include one with a drug reservoir connected to a constant voltage, printed battery unit that delivers drug till the battery runs out (160). Similarly a low-cost microprocessor controlled delivery using single use iontophoresis for delivery of a cyclovir for treating herpes labialis has also been reported (161). Iontophoresis can be used as a non-invasive method of extracting endogenous substances termed reverse iontophoresis. This is utilised in the Glucowatch Biographer® a wrist watch device that provides glucose monitoring for diabetes (162).

Electrorepulsion is known to be the main mechanism by which iontophoresis exerts its enhancement effect on ionised solutes, though other factors including increased stratum corneum permeability in the presence of an electric current flow, and electroosmosis (movement due to convection flow) in the case of uncharged and larger water soluble molecules, are also known to contribute (14, 158, 163-165). The induction of electric current can cause temporary alterations in the barrier properties of the skin, leading to changes in its permeability. The isoelectric point of the mammalian skin is around pH 3-4, which means when a solution with pH lower than 3 is placed in contact with the skin, it would carry a net positive charge and vice versa for pH higher than 4 (166-167). Therefore at physiological pH the skin has a negative permselectivity and during iontophoresis electroosmotic movement of water into the body is observed from the anode (positively charged electrode) to the cathode (negatively charged electrode).

Identification of the pathways of transport during iontophoresis has been extensively investigated with suggestions that follicular or appendageal pathway are predominant (168). Further Turner et al demonstrated the evidence of current pathway to be appendageal route and not intercellular lipid route using TEWL and skin impedance measurements post-iontophoresis (169-170). The use of pilocarpine in the diagnosis of cystic fibrosis is in itself a suggestion that the electric current is travelling down the sweat glands. Due to the similarities between the absorption and transport kinetics of charged compounds following iontophoresis or passive delivery through dermis (in vitro and in vivo), suggestions on a similar rate-limiting mechanism and therefore the presence of an “aqueous” iontophoretic pathway have emerged. These aqueous pathways may include the skin appendages (hair follicle, sweat glands)
and/or transient pores that open and close momentarily and/or hydrated polar region of the bipolar lipid lamimates that exist between the corneocytes (171-172). Iontophoretic flux of potassium ferricyanide was viewed using an electro-chemical microscope coupled with video microscopy: the results indicated that 40-60% of the total transport was due to appendages and that the permeation was localised (173-174). The extent of i ontophoretic transport as a function of molecular weight of the permeant with visual quantification of i ontophoretic pathways (follicular versus non-follicular) using fluorescent labelled poly-L-lysine (PLL) were studied by Turner et al. The authors suggested that i ontophoretic delivery via a specific path would depend either upon the physicochemical properties of the permeant, the depth within the skin (i.e., stratum corneum versus viable epidermis versus dermis) or the experimental conditions. The low molecular weight PLL’s followed follicular pathway whereas with higher molecular weight PLL’s, an intrinsic affinity was found with non-follicular pathway (175). Similarly the transport of hydroquinone (an uncharged molecule) also followed the appendageal pathway (176). Parameters that affect the design of an i ontophoretic delivery system include the electrode type, current density, pH of the system, type of permeant molecule (positive or negative), size of the permeant molecule and the presence of competing ions (177). An expanded description on the mechanism and electrochemistry of i ontophoresis is provided in Chapter 5.

1.7 SYNERGISTIC APPROACH TO PERMEATION ENHANCEMENT

While all skin penetration enhancement techniques have the potential to enhance transdermal delivery of drugs, combination with other enhancers, both chemical and physical have been more effective. There are multiple positive benefits as the required amount or intensity of the individual techniques is reduced which means reduced severity to the skin (such as irritation) plus enhanced delivery. Listed in Table 1.1 are some of the examples of successful synergistic approaches to skin permeation enhancement.
Table 1.1: SYNERGISTIC TRANSDERMAL DELIVERY

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Drug molecule</th>
<th>Effect and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Iontophoresis + Chemical enhancers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iontophoresis (0.25 and 0.5mA/cm^2) + Ethanol, Polyethylene glycol 600, R- (+)-Limonene and Azone®</td>
<td>Sumatriptan</td>
<td>Both the enhancers individually enhanced the skin permeation of Sumatriptan. Synergy found only between Iontophoresis and Azone with a 2 fold increase in transdermal flux and no significant difference with the other chemical enhancers (178).</td>
</tr>
<tr>
<td>Iontophoresis (0.1mA/cm^2) + 1hr pre-treatment with 5% fatty acids (oleic acid, linoleic acid, decanoic acid, lauric acid) in propylene glycol</td>
<td>Micloidine hydrochloride</td>
<td>All of the chemical enhancers showed synergy with iontophoresis with enhancement ratios of 66.5, 75.1, 49.8 and 58.5 for oleic acid, linoleic acid, decanoic acid, lauric acid respectively. 5% oleic acid with iontophoresis achieved therapeutic levels of miclodrine hydrochloride (179).</td>
</tr>
<tr>
<td><strong>Sonophoresis + Chemical enhancers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonophoresis (20kHz) + 1% sodium lauryl sulphate (SLS)</td>
<td>Mannitol</td>
<td>3-fold increase in penetration with SLS alone, 8-fold with sonophoresis alone and 200-fold when combined. The threshold of ultrasound energy reduced from 141 J/cm^2 to 18 J/cm^2 (180).</td>
</tr>
<tr>
<td>Method</td>
<td>Compound</td>
<td>Enhancement Details</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
<td>------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sonophoresis High frequency (1-3MHz) and low frequency (20kHz) + 1% oleic acid and 1% dodecylamine</td>
<td>Testosterone (solid lipid microparticles)</td>
<td>4.8-fold enhancement ratio with pre-treatment of low frequency ultrasound at 2.5 W/cm² followed by 30 min with dodecylamine as compared to 2.4-fold with dodecylamine alone. No synergy with oleic acid and both high and low frequency ultrasound (181).</td>
</tr>
<tr>
<td>Microneedle + chemical enhancers</td>
<td>Docetaxel</td>
<td>Pre-treatment with microneedles enhanced the transdermal flux of Docetaxel and reduced the lag time by 70% (182).</td>
</tr>
<tr>
<td>Iontophoresis + Sonophoresis</td>
<td>Heparin</td>
<td>56-fold increase in transdermal flux as compared to 3-fold with sonophoresis and 15 fold with iontophoresis (183).</td>
</tr>
<tr>
<td>Sonophoresis (300kHz 5.2 W/cm² and 5.4 duty cycle) + Iontophoresis (0.32 ±0.03mA/cm²)</td>
<td>Benzoic acid, lidocaine, hydrocortisone, indomethacin, Timolol malate, vitamin B₁₂, etc</td>
<td>For compounds with mol. wt.&gt;1000 combined sonophoresis and iontophoresis showed synergy with 177-fold enhancements with mol. wt.&lt;500 only 30-fold for neutral compound as ultrasound pre-treatment increased the electroosmotic flow increased (184).</td>
</tr>
</tbody>
</table>
1.8 AIMS AND OBJECTIVES

The focus of the thesis is to assess the effectiveness of physical skin penetration enhancement techniques that utilised various energy sources to enhance the skin penetration of drug molecules. Four different energy based technologies were chosen for investigation, two of which were new and two were well established and successfully commercialised technology.

The studies conducted on physical skin penetration enhancement technologies are as elaborated below:

- **Aim of the first study** was to investigate skin permeation enhancement of naltrexone hydrochloride by a novel electromagnetic energy based (PEMF-Dermaporation) device. To understand the mechanism of skin penetration enhancement due to PEMF dermaporation, a preliminary *in vitro* multiphoton tomography- fluorescent lifetime imaging microscopy study was also conducted.

- **The second study aims at investigating the permeation of urea under the influence of a novel magnetic (ETP) energy based technology.** Using optical coherence tomography the effects of ETP technology induced skin hydration due to urea penetration was also conducted.

- **Aim of the third study was to investigate skin permeation of 5-aminolevulenic acid and curcumin dye under the influence of low-frequency ultrasound (Sonophoresis) energy.** Multiphoton tomography- fluorescent lifetime imaging microscopy was utilised to evaluate the effects of low-frequency ultrasound on human skin. The permeation of curcumin dye *in vivo* due to low-frequency ultrasound was also conducted, and

- **The fourth study investigates skin permeation of various peptides (both therapeutic and cosmetic) and a peptide prototype molecule using small current (iontophoresis) energy.** Various parameters such as pH of the donor solution, the background electrolyte in the receptor solution, concentration of the donor solution and the direction of current, that affect the skin permeation of these peptides in the presence of small electric current were investigated.

Table 1.2 below provides a summary of the compounds investigated in this PhD research with a brief list of their uses.
<table>
<thead>
<tr>
<th>COMPOUND NAME</th>
<th>USES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naltrexone hydrochloride</td>
<td>- Opioid antagonist</td>
</tr>
<tr>
<td></td>
<td>- Used as an adjunct in treating alcohol dependence.</td>
</tr>
<tr>
<td>Urea</td>
<td>- Moisturiser</td>
</tr>
<tr>
<td></td>
<td>- Penetration enhancer by skin hydration.</td>
</tr>
<tr>
<td>5-aminolevulinic acid</td>
<td>- PDT- Photodynamic therapy for treating non-melanoma cancers.</td>
</tr>
<tr>
<td>Dipeptide</td>
<td>- A model peptide with no known therapeutic effects.</td>
</tr>
<tr>
<td>$\alpha$-Ala–$\alpha$-Trp</td>
<td></td>
</tr>
<tr>
<td>Tetrapeptide</td>
<td>- Model synthetic peptide</td>
</tr>
<tr>
<td>$\alpha$-Ala–$\alpha$-Ala–$\alpha$-Pro–$\alpha$-Val</td>
<td>- Peptidic mimic for inhibiting human neutrophil elastase HNE for use in psoriasis management.</td>
</tr>
<tr>
<td>Acetyl-hexapeptide-3</td>
<td>- Synthetic cosmetic peptide.</td>
</tr>
<tr>
<td>Ac-Glu–Glu–Met–Gln–Arg–Arg-NH$_2$</td>
<td>- Used in Anti-aging creams due to Anti-wrinkle properties.</td>
</tr>
<tr>
<td>Triptorelin acetate</td>
<td>- Analogue of LHRH, Gonadotropin-releasing hormone agonist.</td>
</tr>
<tr>
<td>$p$-Glu–His–Trp–Ser–Tyr–[$\delta$-Trp]–Leu–Arg–Pro–Gly-NH$_2$</td>
<td>- Used in hormone responsive cancers such as prostate and breast cancer.</td>
</tr>
</tbody>
</table>
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Effect of Pulsed electromagnetic field energy (PEMF) on transdermal delivery of Naltrexone Hydrochloride
2.1 BACKGROUND

Electromagnetic field (EMF) energies are present around us with all electrical and electronic equipment said to generate some frequency of EMF energy. The properties of EMF are exploited in many disciplines, for example x-rays and laser therapies in medical imaging are high-frequency EMF radiation. EMF energy displays both the characteristics of electrical and magnetic field energy and has been shown to have a range of effects on body tissues as discussed in chapter 1. However the use of EMF as a skin penetration enhancement technique has received little attention until the recent development by a Perth based company (OBJ Ltd, Australia).

2.2 PEMF – DERMAPORTATION TECHNOLOGY

Dermaporation is a novel transdermal drug delivery technology that generates a pulsed electromagnetic field (PEMF) to enhance the movement of substances through the skin. The technique utilizes a time varying electromagnetic field that is believed to interact with the skin to enhance transdermal delivery. According to the manufacturer (OBJ Ltd) at the heart of the Dermaporation system is a low-energy time varying quasi-rectangular electromagnetic pulse packet. The Dermaporation technology generates a series of repeating quasi-rectangular waves of electromagnetic energy with peak maximum field strength of between 5 mT (milli Tesla) (Figure 2.1). The electromagnetic pulse is propagated through the energizing of a small spirally wound monofilament air-filled coil which is placed externally to the donor compartment of a Franz type diffusion cell so that the energizing coil was 7 mm above the skin surface (Figure 2.3). The dermaporation system utilizes a secure microprocessor smart card technology with automatic CRC (Cyclic Redundancy Check) data integrity testing and systems integrity testing to ensure the quality and repeatability of the field characteristics between experiments. The dermaporation system used in this experiment has no user alterable controls. The technique is non-invasive and has been demonstrated to be painless in a volunteer study (1). Enhanced human epidermal permeation of 5-aminolevulinic acid (2) and a dipeptide (3) using the dermaporation system utilized in this study has been previously reported.
2.3 AIM

The aim of the present study was to investigate the effect of PEMF provided by a Dermaporation system on the skin penetration of naltrexone hydrochloride (NTX) using an in vitro human epidermis diffusion model. A comparison of the effect of the PEMF on NTX permeation across human epidermis and a silicone membrane (polymethylsiloxane PDMS) was incorporated to provide an insight into the mechanism of the PEMF enhanced delivery. In addition a preliminary experiment utilising multiphoton tomography – fluorescent lifetime imaging microscopy (MPT-FLIM) was conducted to visualise the stratum corneum penetration of 10 nm gold nanoparticle applied to human epidermis with and without PEMF to investigate the mechanism of enhancement.

Figure 2.2: Chemical structure of naltrexone (NTX)
Naltrexone (NTX: Figure 2.2) is a small hydrophilic molecule (Mol. weight: 377.4 g/mol, log P: 1.92 and pKa: 8.13). It is a potent competitive opioid antagonist that has been used in several countries to assist in the maintenance of a drug-free state in the management of opioid addiction (4) and as an adjunct in the treatment of alcohol dependence (5-6). Alcohol use stimulates opioid receptors and releases endorphins in the brain; NTX is said to reduce the incentive to drink and decrease craving by blocking these receptors. Following conventional oral administration, naltrexone undergoes extensive first-pass metabolism in the liver resulting in oral bioavailability estimates in the range of 5-40% (7-8). Adverse effects reported in oral therapy include abdominal pain, nausea, vomiting and can cause dose related hepatocellular injury (9). Consequently an alternative NTX delivery route that would decrease the required drug dosage could benefit the therapeutic outcome, particularly in already hepato-compromised alcohol or opioid dependent patients. Buccal delivery (10-11) with chemical and electrical enhancement and injectable sustained release depot devices (12-13) has been investigated. However the latter cannot be easily discontinued if the patient requires opiate analgesia for pain. Transdermal delivery may offer a route to circumvent the liver-related problems by allowing lower doses to be administered whilst also providing the convenience of easy discontinuation when required, however passive delivery is not sufficient to provide therapeutic NTX levels.

2.4 MATERIALS AND METHODS

2.4.1 MATERIALS

All the chemicals and reagents listed below were used as supplied: naltrexone hydrochloride (NTX.HCl) (> 98% purity, SALARAS s.p.A, Como, Italy) was a gift from Go Medical, Perth; m ethanol and acetonitrile HPLC solvents from JT Baker, USA; orthophosphoric acid, Ajax Finechem (Australia); sodium hydroxide, analytical grade, Merck Pty Ltd. (Australia). Phosphate buffered saline solution pH 7.4 (PBS) was prepared according to the United States Pharmacopoeia. Silicone membrane (polydimethyl siloxane membrane PDMS: 0.005” non-reinforced sheets) was obtained from Specialty Manufacturing Inc, MI; USA. Gold nanoparticles (10 nm diameter) were obtained from the National Institute of Standards and Technology, USA.
2.4.2 HPLC SYSTEM AND OPERATIONS

The HPLC system (Agilent 1100) consisted of a binary pump (G1312A), 1100 thermostat autosampler (G1313A) and degasser (G1379A) equipped with 1100 photo diode array detector (G1315B). Separation of NTX.HCl was achieved on a C$_{18}$ (150 mm x 4.6 mm) Alltech column with 5 μm particle size. Peak integration was undertaken using a personal computer equipped with Chemstation revision A 08.01 Software. All NTX.HCl chromatographic standards were prepared by dissolving NTX.HCl in PBS (which is also used as the diffusion experiment receptor fluid), diluted with PBS and stored at 4 °C until required. The mobile phase consisted of acetonitrile: water with 10 mM orthophosphoric acid (adjusted to pH 3 with sodium hydroxide) (15:85). NTX.HCl was eluted at the retention time of 4.6 min at ambient temperature at a flow rate of 1 mL/min with a 20 µL injection volume and detection wavelength of 210 nm. The assay was fully validated prior to analysis of experimental samples.

2.4.3 HPLC ANALYSIS AND VALIDATION

The validation parameters for the assay included linearity, precision, inter- and intra-day repeatability, LOD and LOQ:

2.4.3.1 LINEARITY

Stock solution of NTX.HCl prepared in PBS was diluted in PBS to give 6 different working standards of concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56 µg/mL). 20 µL injections of these standards were analysed by HPLC. A calibration curve was obtained by plotting the concentration of NTX.HCl versus peak area. The linearity (quoted as $R^2$) was determined by linear regression analysis which was calculated by the least squares regression method.

2.4.3.2 PRECISION

The precision of the assay was determined by injecting two working standards, 1.56 µg/mL and 6.25 µg/mL of NTX.HCl six times onto the HPLC. The coefficient of variance was quoted as the percentage of standard deviation in peak areas upon the average peak area for these two concentrations.
2.4.3.3 INTRADAY AND INTERDAY REPEATABILITY

The intraday and interday repeatability was assessed by injection of 1.56 µg/mL and 6.25 µg/mL of NTX.HCl standards six times at different times in a day and 3 times on 3 different days respectively and the coefficient of variance was quoted as the percentage of standard deviation in peak areas upon the average peak area for these two concentrations.

2.4.3.4 LIMIT OF DETECTION (LOD) AND QUANTIFICATION (LOQ)

The limit of detection was calculated as greater than 3 times the baseline noise level. A blank solution of PBS was injected 6 times followed by standards (100, 50, 25, 12.5, 6.25, 3.125, 1.56 µg/mL) of NTX.HCl used for the calibration curve. The LOD was calculated by the following formula:

\[
LOD = 3 \times \frac{\text{average (n=6) peak height of noise}}{\text{slope (in peak height) of calibration curve}}
\]

The LOQ was calculated as 10 times the baseline noise level. The LOQ was calculated by the following formula:

\[
LOQ = 10 \times \frac{\text{average (n=6) peak height of noise}}{\text{slope (in peak height) of calibration curve}}
\]

2.4.4 IN VITRO SKIN DIFFUSION STUDIES

2.4.4.1 PREPARATION OF HUMAN EPIDERMAL MEMBRANE

Ethical approval for using human skin was obtained from the Human Research Ethics Committee of Curtin University prior to the study. Full thickness human skin (abdominal section) excised from three female patients (44, 39 and 59yrs) following abdominoplasty surgery at Perth hospitals was used for the present study. The skin samples were stored at -20°C immediately after obtaining until use. Skin was prepared by removing the subcutaneous tissue by dissection then isolating the epidermal membranes from human skin by the heat separation method (14) where the full thickness human skin was immersed in water at 60°C for 1 min. The epidermal membrane was then teased off the dermis, placed onto aluminium foil
with the stratum corneum layer facing upward, air dried for 15 min and then stored at −20°C (for not more than 2 months) until required. For diffusion experiments with artificial membrane (polydimethyl siloxane membrane PDMS: 0.005” non-reinforced sheets) the sheets were cut in squares using scissors and soaked overnight (18 h) in MilliQ water prior to the experiment. When mounted in the Franz cells a cross sectional area of 1.18 cm² was available for diffusion.

2.4.4.2 IN VITRO DIFFUSION STUDIES

Figure 2.3: Vertical Franz type diffusion cell with dermaporation (PEMF coil).
In vitro permeation studies across human epidermis or PDMS membrane were performed using Pyrex glass Franz-type diffusion cells (enabling permeation across skin sections of cross sectional area 1.18 cm²); receptor volume approximately 3.5 mL. The membrane was placed between the donor and receptor compartment of the cell and allowed to equilibrate for 1 h with PBS in the receptor compartment that was stirred continuously with a magnetic stirrer. PBS (1 mL) was placed in the donor and receptor compartments of the cell which was placed in a water bath maintained at 37±0.5°C. The membrane integrity was determined by visual inspection of the skin over a bright light and by electrical resistance (kΩ), capacitance (nF) and impedance (kΩ) measurements using a digital portable LCR meter (TH2821/A/B, Changzhou Tonghui Electronic Co., Ltd, China). TH2821B is a microprocessor-controlled portable meter with low power consumption. It was operated at 1 kHz with maximum voltage of 300 mV root-mean-square (rms) in the parallel equivalent circuit mode. The measurements were taken by immersing the stainless steel probe leads in the donor and receptor compartments (15). Membranes exhibiting an electrical resistance less than 20 kΩ were rejected from the study. The diffusion cells were emptied, receptor compartments refilled with fresh preheated PBS at 37±0.5°C and 1 mL of 0.45% N TX.HCl in PBS placed in the donor compartment that was then occluded. PEMF coils were placed around the exterior of

Figure 2.4: A typical Dermaportation (PEMF) unit in experimental setup
the donor compartment (Figure 2.3) and energy applied for 4 h, whilst passive cells had no external PEMF energy applied. This was taken as time 0 and PEMF energy initiated on the active cells immediately. At different time points, aliquots from the receptor phase were withdrawn from the sampling arm and replaced with fresh pre-heated (at 37°C) PBS over an 8 h period (Figure 2.4). The total NTX concentration (NTX base and ions) permeating the skin into the receptor solution samples obtained from individual experiments was determined by validated HPLC analysis. At time 8 h the donor and receptor fluids were recovered, the cell disassembled and the epidermal membrane examined on a light microscope for obvious tears (any cells with torn membranes were rejected). Experiments were repeated for both the PEMF and passive applications 4 times for PDMS membrane and 13 times for human epidermal experiments. The cumulative amount of drug permeated through the epidermis and silicone membrane to the receptor compartment was plotted as a function of time. The area under the NTX.HCl cumulative permeation versus time curves (AUC) for PEMF and passive delivery was calculated (Sigma plot 8.0) and expressed as µg.h/mL. Enhancement ratios based on AUC were determined to compare NTX permeation in the presence of the PEMF with passive diffusion.

2.4.5 MPM-FLIM ANALYSIS OF NANOPARTICLE PENETRATION

Human stratum corneum/epidermis was isolated from full thickness skin by heat separation method detailed above. The resulting stratum corneum/epidermis was used to assess PEMF assisted penetration of 10 nm gold nanoparticles. A droplet of 20 µl containing a well-characterized solution of 10 nm gold nanoparticles at 51.56±0.23 mg/g Au (National Institute of Standards and Technology, USA) was placed onto the stratum corneum of the human skin in vitro. The skin was exposed for 30 minutes prior to rinsing with 100 ml saline followed by MPM-FLIM analysis. A Dermainspect instrument with a Mai Tai laser at 740 nm and 850 nm was used to excite the gold nanoparticles. Second harmonic generation was quantified at lifetimes of 0-250 ps using a time-correlated photon counting module and analysed using SPCimage software (Becker and Hickl G mbH). Gold nanoparticle positive pixels were quantitated and graphed in untreated/unexposed and treated samples; two skin samples were treated with gold nanoparticles where one was exposed to the
PEMF device and the other was not exposed to the PEMF device. The MPM-FLIM technique is described in detail in chapter 4. The preliminary MPM-FLIM experiment for this chapter was undertaken by Dr Tarl Proatt at the University of Queensland.

2.5 STATISTICAL ANALYSIS

Differences in the permeation of NTX.HCl between the PEMF and passive application were analysed for statistical significance (p < 0.05) using the repeated measures ANOVA (implemented with the SAS software program using Proc Mixed). The data consist of measurements at 12 time periods on 12 samples (except that the 5 hour time point was only available for 9 of the samples). Because of the skewness in the concentrations, the regression model was applied to the log-transformed data (in order to obtain the p-values for comparison of treatments). The repeated measures on each sample were taken into account, and the model essentially stated that the concentration was a function of the time delay from start and the treatment. The overall p-values were highly significant for both the treatment differences and the differences between times.

2.6 RESULTS AND DISCUSSION

2.6.1 CHROMATOGRAPHY

NTX was analysed by UV detection using HPLC. NTX eluted at 4.6 min and the elution was clear of any interfering peaks. A typical chromatogram of NTX is shown in Figure 2.5. The linearity obtained by the HPLC method was $R^2 = 0.999$ over the range of the calibration curve (1.56-100 µg/mL) for NTX standard solutions. A calibration curve was obtained by plotting the peak area versus concentration of the standards injected. The coefficient of variance (CV) for precision of the method, determined by the relative standard deviation ($n = 6$) was 1.52% for 6.25 µg/mL and 0.51% for 1.56µg/mL. The interday and intraday repeatability of the method was 1.40% and 2.69% for 6.25 µg/mL, 0.68% and 1.36% for 1.56 µg/mL respectively. These were within the acceptable criteria for intraday repeatability which is R SD<
5%. The limit of detection (LOD) calculated as greater than three times the baseline noise level in the assay was 134 ng/mL. The limit of quantification (LOQ), calculated as greater than 10 times the baseline noise level in the assay was 449 ng/mL.

![Figure 2.5: HPLC chromatogram of NTX standard (6.25µg/mL).](image)

### 2.6.2 IN VITRO NTX PERMEATION WITH PEMF APPLICATION

The in vitro permeation profiles of NTX across human epidermis and silicone membrane are presented in Figures 2.6 and 2.7 respectively. The permeation parameters include, mean cumulative amounts that permeated the membrane, flux or the rate of permeation and AUC or area under the cumulative permeation versus time curve given in Table 2.1. A comparison of the cumulative amount of NTX penetrating the epidermis to the receptor solution versus time was plotted for passive (no PEMF) and PEMF applications. NTX penetration increased significantly compared to passive application over the time period of the experiment (p < 0.0001). The results indicated an increase in the mean cumulative permeation of NTX over 8 h in cells where PEMF was applied, as compared to cells without PEMF application.
Energy-based skin permeation enhancement of NTX has not been previously reported. However, Stinchcomb’s group have investigated prodrugs as a means of skin delivery (9, 16). In this case, approximately 2 to 7-fold enhanced flux of lipophilic alkyl ester prodrugs compared to NTX base was achieved using NTX-3-acetate compared to NTX base in mineral oil in this experimental series (9). These highly oil soluble prodrugs provided a higher NTX flux and underwent significant metabolic conversion in the skin. Similarly, a duplex “Gemini” prodrug approach was studied where two molecules of NTX were bonded together by a carbonated ester (17). The prodrug was hydrolysed into two NTX moieties via skin enzymes, appearing as mainly NTX in the receptor compartment. A 2-fold increase in flux compared to NTX base was achieved. Transdermal delivery of NTX across microneedle-treated skin has recently been reported with clinically relevant systemic drug levels achieved in humans (18).
Figure 2.6: Cumulative amount of NTX (µg/cm²) permeated through human epidermis to the receptor compartment by PEMF-Dermaportation (●) and passive (○) application (ANOVA p < 0.001). PEMF applied 0-4 h only as indicated by line on x axis. Data represent mean ± SEM, n=13 cells using skin from three human subjects.

In contrast to the human epidermal data, comparison of the cumulative amount of NTX penetration across silicone membrane (Figure 4) shows only a minor difference between the PEMF and passive applications. In this study PEMF energy was applied for the entire experimental period (0-8 h) and the cumulative amount of NTX permeating to the receptor compartment (mean ± SEM) was 104.9 ± 4.6 and 112.8 ± 5.1µg/cm² for passive and PEMF respectively. PDMS membranes provide a hydrophobic reproducible barrier that allows water passage equivalent to transepidermal water loss during permeation studies. This offers a simple barrier to mimic the hydrophobicity of the stratum corneum but is not subject to potential stratum corneum lipid perturbation by skin penetration enhancement techniques. For example, comparison between permeation of a solute across human skin and PDMS has been used to investigate the effects of co-solvents (19). The comparison between the human epidermis and PDMS membrane data (Table 2.1) suggests that the mechanism of action of the PEMF may involve an interaction with the epidermal structure to provide enhanced permeation rather than a predominately drug repulsion.
effect due to magnetic energy as minimal enhancement was seen with the synthetic membrane.

Figure 2.7: Cumulative amount of NTX ($\mu$g/cm$^2$) permeated through silicone membrane to the receptor compartment by PEMF-Dermaporation (●) and passive (○) application (mean ± SEM, n=4 cells; ANOVA p > 0.05). PEMF applied 0-8 h as indicated by line on x axis.

Studies reported on the biophysical effects of other magnetic energy applications and other energy based skin permeation enhancement techniques may provide insights into the mechanism of PEMF skin permeability enhancement. There have been previous reports of the creation of cell membrane pores by magnetic particles (20) and a number of other biophysical effects of magnetic energy (21-22).

Electromagnetic waves of both high and low-voltage pulse have been shown to perturb phospholipid layers due to electroporation and depending on the voltage and duration of pulse, molecular transport could be transcellular or intercellular. The pore size induced by electromagnetic electroporation depends on the duration and number of pulses used (23). Typically electroporation involves higher energy, short duration pulses and therefore may not be directly relevant to PEMF effects on skin. It could also be hypothesized that application of PEMF may increase or induce thermal transition of lipids, resulting in the skin. During this rearrangement period the porosity of the skin may increase temporarily resulting in
high permeation seen particularly during the first 1.5 h of our study with epidermal membrane (Figure 2.6). Following this initial restructure a constant permeation becomes apparent which would relate to the steady NTX permeation increase post PEMF. Silva et al showed that lipids in the stratum corneum can undergo thermal transition at temperatures below 0(20 - 40°C) (24). However, during in vivo studies volunteers did not report any sensation of warming of the skin due to PEMF application therefore the relevance of thermal effects may be minimal. Movement of water molecules due to osmosis under the influence of the applied electromagnetic field could also be considered as a possible mechanism of enhancement. This mechanism has been described for iontophoresis (25).

Recently Murthy et al reported magnetophoresis for the transdermal delivery of a diamagnetic drug (lidocaine). The magnetic field strength used in his study was much higher (30-300 mT) to what is reported in this work. The authors showed that effects of magnetophoresis was dependent on the drug molecule as its predominant mechanism follows magnetorepulsion (moving of the magnetic drug particle away from the magnetic field and towards the deeper layers) and magnetohydrokinesis (movement of water molecules in the presence of magnetic field). Using ATR-FTIR they showed no change in the layers of the skin due to magnetic field energy. In the same study the enhancement ratio in transdermal flux for lidocaine hydrochloride and lidocaine base increased linearly with increasing magnetic field strength from 3 to 8.9 and 1.3 to 3.9 (26).
Table 2.1 Permeation characteristics of human epidermal and synthetic PDMS membrane under passive and PEMF-Dermaportion treatments, (values are mean ± SD &SEM).

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>TREATMENT AND TIME</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0-8H</td>
<td>0-4H</td>
<td>4-8H</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>DP</td>
<td>P</td>
<td>DP</td>
<td>P</td>
</tr>
<tr>
<td>Mean cumulative permeation (µg/cm²)</td>
<td>80.2±150.5, 43.4</td>
<td>406.9±474.9, 137.1</td>
<td>60.6±123.4, 35.6</td>
<td>319.8±334.9, 96.7</td>
<td></td>
</tr>
<tr>
<td>Flux (µg/cm².h)</td>
<td>9.7±18.7, 5.4</td>
<td>44.1±51.9, 14.9</td>
<td>14.0±29.7, 8.4</td>
<td>69.8±74.0, 21.3</td>
<td>4.9±7.1, 2.0</td>
</tr>
<tr>
<td>AUC (µg.h/mL)</td>
<td>411.7</td>
<td>2329.0</td>
<td>131.7</td>
<td>859.5</td>
<td>1469.4</td>
</tr>
<tr>
<td>Enhancement Ratio (Based on AUC)</td>
<td>5.6</td>
<td>6.5</td>
<td></td>
<td></td>
<td>5.2</td>
</tr>
</tbody>
</table>

PDMS MEMBRANE

| Mean cumulative permeation (µg/cm²) | 104.9±9.1, 4.5 | 112.8±10.1, 5.1 | 54.6±4.7, 2.1 | 61.0±7.6, 2.2 |
| Flux (µg/cm².h)                     | 12.9±1.1, 0.5   | 14.2±1.6, 0.8  | 13.6±1.0, 0.6 | 14.7±1.5, 0.7 |
| AUC (µg.h/mL)                       | 432.2            | 484.9         | 114.9         | 124.8         |
| Enhancement Ratio (Based on AUC)    | 1.1               | 1.0           | 1.1           |               |
2.6.3 MPM-FLIM ANALYSIS OF NANOPARTICLES PENETRATION

A preliminary experiment was undertaken on ex-vivo human skin exposed to PEMF energy to visualise its influence on the permeation of 10nm gold nanoparticles (n= 1, 1). Initial experiments identified the major lifetime contribution of 10nm gold nanoparticle second harmonic generation as <250 ps (Figure 2.8, Panel a). This lifetime range has minimal activity (~4x10^5 pixels per 224x224 µm field of view) in untreated human skin and gold nanoparticle treated skin without exposure to the PEMF device (Figure 2.8, Panel b). Gold nanoparticle treated human skin exposed to the PEMF device had 200 times more gold nanoparticle positive pixels than the non-exposed (no PEMF), but gold nanoparticle treated group (Figure 2.8, Panels b to h). The images in Panels c to h show no major differences in stratum corneum/epidermis microanatomy between the treatment groups, indicating no obvious tissue damage. This suggests that the PEMF generated magnetic field facilitates 10 nm gold nanoparticle penetrations through the human stratum corneum. Furthermore, these data illustrate that the channels through which the nanoparticles move must be larger than or of the size of 10nm diameter of these rigid particles.

The presence of pores in the SC layers supports the notion that the existence of aqueous pore pathway for hydrophilic molecules is a well-established model for transdermal delivery (27-30). Mitragotri et al suggested the presence of pores as imperfections or defects in the SC lipid bilayers. In addition other authors have classified these imperfections or defects as grain boundaries, lattice vacancies, multi-molecular voids due to missing lipids, defects due to steric constraint between keratinocytes in the intercellular lipid bilayers or simple phase separation with evidence by x-ray diffraction (31) and atomic force microscopy (32). Depending on the nature or type of the defect, the pore size induced by grain boundaries, lattice vacancies and phase separation in the SC layers may span in the length of 1-10nm and multi-molecular voids may range in several nanometres (33). Further there is evidence of pore formation due to small and heavy electric voltage as shown by iontophoretic delivery of small polar charged molecules and large oligopeptides (34-36). Further investigation of the possible pore size distribution and range of pore formation due to PEMF energy needs to be undertaken.
Figure 2.8: MPM-FLIM analysis of nanoparticle penetration enhancement by PEMF (dermaporation). Panel a shows the lifetime profile of 10 nm gold nanoparticle second harmonic generation. The primary peak between 0-250ps was used to indicate the presence of gold nanoparticle (AuNP) within the stratum corneum (white bars) and epidermis (black bars). The presence of gold nanoparticle positive pixel was quantified in both untreated (--) and treated (AuNP) human skin were analysed by MPM-FLIM (Panel b). The treated group contained one unexposed and one exposed (PEMF-dermaporation piece of skin.
Figure 2.9: Typical lifetime images (0-250 ps) for gold nanoparticle (AuNP) in Panels c to h and the treatment shown to the left. Panel’s c to h is pseudo-coloured according to lifetime (bar in Panel c) where 0 is blue to 250 is red. The background levels of the stratum corneum/epidermis can be seen in Panels c and d. The major lifetime contribution of gold nanoparticle second harmonic is teal/green/yellow and can be seen particularly in the treated/PEMF exposed samples (Panels g and h).
2.7 CONCLUSION

Applied electromagnetic field enhanced the skin penetration of NTX. The enhanced NTX delivery due to PEMF-dermaporation was found at higher levels across human epidermis as compared to silicone membrane, therefore we hypothesize the mechanism of enhancement to be due to potential interactions between PEMF energy and epidermal structures to form transient pores through which drug and water can diffuse more readily. A preliminary MPM-FLIM experiment demonstrated that PEMF enhanced the stratum corneum penetration of 10 nm particles suggesting that the channels through which the nanoparticles move must be at least 10 nm in diameter as these are rigid particles. Further studies are required to determine the precise mechanism of enhancement by this electromagnetic field technology.
2.8 REFERENCES


CHAPTER 3

Effect of magnetic film array technology (ETP) on skin permeation of Urea: preliminary *in vitro* and *in vivo* assessment.
3.1 BACKGROUND

Dry skin is the most common dermatological problem and is widely treated by topical application of moisturizers of many different compositions. Rawlings and Matts have provided excellent reviews on the role of water in the skin, dry skin and moisturisation (1-3). Water content in and water loss from the stratum corneum play important roles in the hydration of the outer stratum corneum layers to maintain skin flexibility, and in providing sufficient water to facilitate enzyme reactions involved in stratum corneum maturation, corneodesmolysis and desquamation (4). A complex mixture of low-molecular-weight, water-soluble compounds known as Natural Moisturising Factors is present in the stratum corneum to assist in moisture retention (5). Dry skin can be induced by a number of factors including: low environmental temperature and humidity; abrupt changes in conditions associated with modern indoor-climate controlled environments; soap washing causing loss of lipid and natural moisturizing factors from the stratum corneum; aging and genetics (6).

Blank demonstrated that stratum corneum containing less than 10% water content is brittle (7). If untreated a “dry skin cycle” is established which will lead to scaly skin with increased hardness and brittleness (2). This occurs because the superficial dehydration of the stratum corneum induces release of inflammatory mediators, hyperproliferation and disruption of epidermal differentiation. However the “dry skin cycle” can be reversed by intervention with suitable moisturizing agents.

Humectants, occlusives and emollients are the most commonly used moisturizer components for management of dry skin (8). Other moisturizing agents include bilayer-forming lipids including ceramides and phospholipids, hydroxyacids and agents that induce epidermal differentiation and lipogenesis, such as ligands for the peroxisomal proliferator-activated receptor (e.g. linoleic and other long chain fatty acid), niacinamide and vitamin C. Rawlings and Matts recently reviewed the range of moisturizers, their properties and mechanisms of action (6).
Urea (carbamide) is an odourless and colourless crystalline solid (Figure 3.1). It is a slightly hygroscopic substance with good water solubility and weak alkaline properties. It is prone to hydrolysis. Urea is used as a hydrotrope in dermatology as a hydrating agent for the treatment of psoriasis, neurodermatitis, ichthyosis and other hyperkeratotic skin conditions. It has been used as a humectant in moisturizing creams since 1943 (9) and is a natural component of the stratum corneum Natural Moisturizing Factor (NMF). Moisturizers containing urea have been reported to improve stratum corneum barrier function, reduce trans-epidermal water loss (TEWL), increase skin capacitance and reduce irritation (10-11). As a moderate keratolytic substance, urea influences the stratum corneum keratinocytes with species-specific percutaneous absorption rates (12).

Oclusive formulations such as petroleum jelly, oils and occlusive dressings act as an occlusive film on the skin surface to reduce transepidermal water loss and thereby hydrate the skin. Many moisturizers contain combinations of agents to optimize the hydrating effect of the product on the skin. In a trial involving over 200 patients suffering from various skin disorders, urea was suggested as an effective moisturizer and an enhancer of hydrocortisone penetration into the skin (13). In a study on the percutaneous absorption of progesterone, the most efficient skin penetration enhancer besides Azone was urea in polyethylene glycol bases. The diffusion was enhanced 2.5-fold compared with the pure base (14). A nonirritating chemical enhancer system containing ethanol, menthol, camphor, methyl salicylate and urea in a hydrogel was shown to strongly enhance the skin penetration of the leuprolide (15). The modest skin penetration enhancing activity of urea results from a combination effects of increasing the water content of the stratum corneum (therefore possible consequent effects on stratum corneum bilayer lipids) together with modifications of
the intracellular keratin (keratolytic activity). However, the application of urea and its derivatives as penetration enhancers is limited by their inadequate chemical stability, the proteolytic properties and skin irritation (12). Due to marginal penetration enhancing properties, modified analogues of urea have been synthesized in attempts to combine the water-holding and keratolytic properties of urea with moieties that have shown enhancement activity such as C12 alkyl chains. Wong and co-workers synthesized series of cyclic urea analogues and found them to be as potent as Azone for enhancing the permeation of indomethacin across shed snake skin and hairless mouse skin (16).

Over the past decade Optical Coherence Tomography (OCT) has emerged as a high-resolution optical diagnostic imaging modality widely used in ophthalmology (17-18) and dermatology (19-21). Although the spatial resolution (typically 5-20 microns) of OCT is not as good as that of histology, it enables non-invasive, in vivo imaging of the internal tissue microstructures. This resolution is much higher than most of the current clinical diagnostic technologies such as X-ray, computed tomography (CT) or magnetic resonance imaging (MRI). In addition, unlike X-ray and CT, OCT does not use ionising radiation, instead using light waves in the near infrared. While this significantly reduces the penetration depth to a few millimetres (approximately 3 mm) due to the optical scattering of tissue, it is sufficient to reveal images of the stratum corneum, epidermis, upper dermis, hair follicles as well as sweat glands, where most skin pathologies and conditions occur, including skin cancers (19, 21-23). Crowther et al recently demonstrated a strong, positive correlation in measurement of stratum corneum thickness between OCT and confocal Raman spectroscopy, with the latter technology offering higher resolution for thinner stratum corneum sites (≈15 µm)(24).

The operation of OCT is based on interference of light from a low coherence broadband light source. The working principle is analogous to ultrasound imaging (19, 25), being based on the reflection of signal from the tissue but utilising light rather than acoustic waves. Unlike ultrasound, no direct tissue coupling is required for OCT imaging. As the tissue sample consists of different types of cells, organelles and microstructures, each with different reflective index, the incoming light beams are reflected and backscattered from different boundaries within the tissue. Thus
OCT is an important tool in the monitoring of transdermal delivery of drugs that alter the skin structure.

### 3.2 AIM

The aim of the current work was to evaluate the effect of a novel un-powered magnetic film array technology (ETP type 008) on the permeation of urea into and through the epidermis in vitro; the consequent hydration effect was determined in vivo. In vitro permeation of urea through human epidermis was evaluated in a Franz cell diffusion system. In vivo determination of the hydration effects of the administered urea was determined by OCT in human volunteers. Urea was administered as a simple measured dose of gel with occlusive film (control or passive) or with occlusive film with the additional magnetic film array placed externally to the occlusive (active or treatment).

### 3.3 MATERIALS AND METHODS

#### 3.3.1 MATERIALS

All the chemicals and reagents listed below were used as supplied: Urea gel was supplied as a 5% w/w urea in VersaBase Gel (PCCA, Huston, TX) as supplied from Compounding on Oxford, Perth, W.A.; phosphate buffered saline solution pH 7.4 (PBS) was prepared according to the United States Pharmacopoeia. P-dimethylaminobenzaldehyde (DMAB) was purchased from BDH Laboratory Chemicals Group, Poole, England. Ethanol was obtained from CSR Distilleries Group, Australia and concentrated sulphuric acid from Lab Scan Asia Co Ltd, Bangkok, Thailand. Passive occlusive material used for in vitro and in vivo consisted of a polymer film of similar thickness and cut to the same dimensions as the active magnetic polymer material. In addition, all in vitro cells and in vivo sites were further occluded with Parafilm M polymer film.
3.3.1.1 MAGNETIC FILM ARRAY TECHNOLOGY (ETP)

The magnetic film array material consisted of 35 mm x 35 mm sections of unpowered flexible array matrix (ETP Type 008), a proprietary enhanced transdermal delivery array film developed by OBJ Limited, Perth, Western Australia. According to the manufacturer, the magnetic film array (ETP Type 008) is a thin flexible polymer matrix containing multiple magnetic elements arranged to produce complex 3-dimensional magnetic gradients. The material has a peak magnetic field strength of 40 mT. However, the arrangement and distribution of alternating poles across the surface of the material results in a total magnetic gradient of 2 T/m².

3.3.2 METHODS

3.3.2.1 SPECTROSCOPIC ANALYSIS

Urea quantification was based on the analytical method of Knorste et al. (26): a modified derivatisation using p-dimethylaminobenzaldehyde (DMAB) to generate a coloured compound. The derivatisation of urea found in skin diffusion samples employed mixing equal volumes of sample solution and the DMAB reagent and measuring the absorbance of the coloured compound with a UV spectrophotometer. The DMAB reagent (4% w/v) was prepared in conc. sulphuric acid (4% v/v) in alcohol (95%). Typical derivatisation involved mixing 200µL of the urea sample (standard or unknown) with 200µL of the DMAB reagent, then waiting for 10 min to observe a colour change from white to strong yellow. The absorbance of the coloured derivatised solution was measured at 420 nm using a UVmini-1240 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, NSW, Australia) against an appropriate reagent blank (receptor solution processed as for skin permeation receptor solution samples). The assay was quantified for linearity, accuracy, lowest limit of detection and quantification.

3.3.2.1.1 LINEARITY

Stock solution of urea was prepared in PBS by diluting the 5% urea gel in PBS to give 5 different working standards of concentrations (125, 62.5, 31.25, 15.6, 7.8 µg/mL). 200µL of these standards was derivitized according to the procedure described earlier and analysed by UV spectrophotometer. A calibration curve was
obtained by plotting the concentration of urea versus absorbance. The linearity (quoted as $R^2$) was determined by linear regression analysis which was calculated by the least squares regression method.

### 3.3.2.1.2 ACCURACY

The accuracy of the assay for *in vitro* skin diffusion studies was determined by measuring the absorbance of two separate samples of PBS which were in contact with human epidermis for 24h at 37ºC and spiked with urea standards (15.6 µg/mL and 62.5 µg/mL) to give final concentration of 7.8 µg/mL and 31.25 µg/mL of urea. Each spiked sample and standard was measured six times for absorbance and the percentage difference between each standard and the corresponding spiked sample was calculated.

### 3.3.2.1.3 LIMIT OF DETECTION (LOD) & LIMIT OF QUANTIFICATION (LOQ)

The lowest limit of detection and quantification were calculated based on the formula mentioned below, by determining the standard deviation and slope of calibration curve of the standard solutions of urea in PBS.

$$\text{LOD} = 3 \times \text{Standard deviation in absorbance} / \text{slope of the calibration curve}.$$  
$$\text{LOQ} = 10 \times \text{Standard deviation in absorbance} / \text{slope of the calibration curve}.$$  

### 3.3.2.2 IN VITRO SKIN DIFFUSION STUDIES

#### 3.3.2.2.1 PREPARATION OF EPIDERMAL MEMBRANE

Epidermal membranes were obtained from human skin sourced from Perth hospitals (abdominal region following abdominoplasty surgery; three female donors 50 yrs, 39 yrs and 44 yrs) with ethics approval from the Human Research Ethics Committee of Curtin University. Epidermal membranes were obtained by the heat separation method (27) as described in Chapter 2.
3.3.2.3 **IN VITRO DIFFUSION STUDIES**

![Figure 3.2: Picture of in vitro setup showing placement of ETP film](image)

In vitro diffusion studies across human epidermis were performed using Pyrex glass Franz-type diffusion cells (enabling permeation across epidermal membranes of cross-sectional area $1.18 \text{ cm}^2$); receptor volume approximately $3.5 \text{ mL}$. The membrane was placed between the donor and receptor compartment of the cell and allowed to equilibrate for $1 \text{ h}$ with PBS in the receptor compartment that was stirred continuously with a magnetic stirrer. Membrane integrity was determined as described in chapter 2 with the same rejection criteria used. The diffusion cells were emptied, receptor compartments refilled with fresh preheated PBS at $37\pm0.5^\circ\text{C}$. Urea gel ($0.5 \text{ g}$) was placed in the donor compartment. Sections of magnetic film array, cut to a size suitable for insertion into the donor compartment of the Franz type cell were suspended above the gel (Figure 3.2), whilst passive cells had non-magnetic polymer film of similar dimensions placed above the gel. All cells were also occluded by sealing the top of the donor compartment of the cell with Parafilm. Aliquots from the receptor phase were withdrawn from the sampling arm and replaced with fresh preheated (at $37^\circ\text{C}$) PBS over a $2 \text{ h}$ period. The total urea content permeating the
epidermal membrane to the receptor solution samples obtained from individual experiments was determined by spectroscopic analysis. At time 2 h the donor and receptor fluids were recovered, the cell disassembled and the skin epidermal membrane examined for obvious tears (any cells with torn membranes were rejected). Experiments were repeated 9 times for magnetic film array enhanced (active) and 8 times for passive (control) diffusion experiments. The cumulative amount of urea permeating through the epidermis to the receptor compartment (µg/cm²) was plotted as a function of time (h) and the steady state flux (µg/cm².h), permeability coefficient (cm/min) and lag time (min) were calculated.

3.3.2.4 EXTRACTION FOR UREA FROM EPIDERMIS

The content of urea in the epidermis post diffusion studies was evaluated. To validate the extraction procedure a standard solution (1mg/mL) of urea was allowed to equilibrate with the pieces of epidermal membrane for 8 h. The epidermal membranes were then removed, followed by three water washes to remove any excess urea present on the exterior of the membrane. The epidermal membranes were then patted dry with tissue paper before immersing in 1mL of PBS in a glass vial and stirred continuously for 1 h. The vials were finally vortex mixed at 2500rpm for 2 min before taking 200µL of this solution and analysing for urea content by appropriate derivatisation as described. This extraction procedure was carried out on all the skin pieces at the end of diffusion experiment.

3.3.2.5 STATISTICAL ANALYSIS

Magnetic film application and passive application were analysed using a linear mixed effects model. Data were transformed using the square-root of the measures to stabilise the variance. The within-cell correlation over time was accommodated using a first-order autoregressive correlation structure. Post hoc comparisons between conditions at each time point were evaluated using the Mann Whitney U test. Permeation parameters were calculated from the data: steady state flux, permeability coefficient, lag time and cumulative amount of urea permeated at 2 h. Comparison of
the parameters obtained for active and control administration were compared by unpaired t tests.

3.3.2.6 IN VIVO HYDRATION: OPTICAL COHERENCE TOMOGRAPHY ASSESSMENTS

![Figure 3.3: Examples of optical coherence tomography images of human skin](image)

Direct measurement of penetration of urea into the skin in vivo is difficult thus OCT was utilised to measure the physical changes in the skin properties. OCT measurements were carried out by Dr. Wallace’s group at the University of Western Australia. A commercial swept-source FD-OCT system (Thorlabs, Newton, NJ, USA) that comprised of a broadband, high-speed frequency swept laser and a fibre-based Michelson interferometer with a balanced detection scheme was used to perform depth profiling and 3D image reconstruction at video rates. The system had a handheld probe to enable in vivo scanning of the skin. The light source had a centre wavelength of 1325 nm and a full width at half maximum bandwidth of 100 nm. The average output power to the skin was 10 mW with the maximum axial scan rate of 16 kHz. Axial resolution was 12 µm and transverse resolution was 15 µm. Maximum imaging depth was 3 mm with a maximum imaging width of 10 mm. The scanning process involved axial and transverse scanning of the tissue sample. An axial or depth scan (A-scan) was obtained by translating the reference arm length, resulting in localized interference fringes with amplitudes related to the sample reflectivity (19).
Adjacent A-scans were combined to produce B-scans (i.e. two-dimensional image), which depict the tissue’s cross-sectional subsurface structures.

The study was conducted on a human volunteer (male, 55 years old) with a healthy skin type under ethics approval from the University of Western Australia. The OCT measurements were conducted on two regions of skin. The first region on the inside right forearm had 5% urea gel topically applied in conjunction with occlusive Parafilm with the addition of an external ETP Type 008 (active). The second region in a similar position on the left arm, had the same amount of 5% urea gel applied and then occluded using Parafilm and a passive polymer sheet of the same dimensions as the ETP Type 008. OCT images were acquired at 0, 30 and 60 min in triplicate. Four sites were measured from each region resulting in 72 images which were used to determine changes in epidermal thickness in response to penetration of the active ingredient, urea. The epidermal thickness was calculated from OCT images by boundary differentiated pixel counts of the region encompassed by the stratum corneum and dermal/epidermal boundary from each image. This was then colored using a primary fill and the thickness averaged over 5 mm widths across the image (Figure 3.2). The average thickness was calculated for each site at each time point and the percentage increase in thickness calculated for times 30 and 60 min relative to time 0. The error on the thickness measurement is 10-15 µm.

3.4 RESULTS AND DISCUSSION

3.4.1 UREA ANALYSIS

Spectrophotometric analysis was carried out for quantitative analysis of urea that permeated the skin. Upon derivatisation with 4% D MAB reagent a colored derivatised compound was formed in the presence of urea. All calibration curves of urea standards showed good linearity in the concentration range of 7.8-125 µg/mL ($r^2 = 0.99; n=5$). The limit of detection (LOD) and limit of quantification (LOQ) of the assay were 0.83 and 2.5 µg/mL respectively. The assay method permitted the detection of 1.56 and 6.25 µg/mL of urea with 93.4% and 93.1% accuracy respectively.
3.4.2 IN VITRO SKIN DIFFUSION STUDY

Figure 3.4: Cumulative amount of urea (µg/cm²) permeating human epidermis to the receptor compartment during application of 5% urea gel with ETP008 (●) versus passive occluded (○) conditions (mean ± SEM; n=9 and 8 cells respectively).

The in vitro permeation profiles of urea across human epidermis are presented in Figure 3.4 and permeation parameters are given in Table 3.1. The results were compiled from 9 active cells (occlusion plus magnetic film) and 8 control cells (occlusion plus control film). A comparison of the cumulative amount of urea penetrating the epidermis to the receptor solution versus time was plotted for passive and magnetic array enhanced applications (Figure 3.3). The permeation parameters calculated were mean cumulative amount of urea permeation represented as µg/cm², the area under the cumulative amount of urea permeation versus time curve (AUC) as µg.min/mL, the flux or the rate of permeation as a function of time expressed in µg/cm².h, the lag time of permeation and the enhancement ratio.
The mean cumulative permeation of urea over 2 h for ETP application was 89.54±7.34 as compared to passive occlusion application 20. 83±2.02 µg/cm²; (mean±sem). Based on the unpaired t test this increase in permeation was significant (p<0.0001). A linear mixed effects model analysis showed a significant difference between groups (F₁,₁₅ = 19.92: p = 0.0005), a significant difference over time (F₁₁₄ = 156.45: p < 0.0001) and a significant group * time interaction (F₁₁₄ = 23.02: p < 0.0001). There was a significant difference between groups at all time points (p < 0.02). Mann-Whitney U values for each comparison are included in Table 3.2. All other permeation parameters showed significantly enhanced urea permeation by magnetic application (based on unpaired t tests). The lag time was reduced from 40.58 ± 3.98 to 21.13 ± 6.27 min (p<0.02), whilst steady state flux increased from 0.24 ± 0.03 to 0.75 ± 0.06 µg/cm².h (p<0.0001) by administration of urea with the magnetic film array (Table 3.1). The efficacy of solvent extraction of urea from the epidermal membrane post diffusion experiments was 86% (n=3). The amount of urea remaining in the epidermis after 2h application period was (5.43 ± 0.74) µg/mL for passive occlusion and (6.99 ± 0.70) µg/mL (mean ± sem) for magnetic film application.
Table 3.1: In vitro skin permeation parameters for urea following administration as 5% gel with ETP verses passive occluded application (values are mean ± SD, SEM).

<table>
<thead>
<tr>
<th>Parameters Mean ± SD,SEM</th>
<th>0-120 min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive</td>
<td>ETP</td>
</tr>
<tr>
<td>Mean cumulative permeation(µg/cm²)</td>
<td>20.83 ± 5.72, 2.02</td>
<td>89.54 ± 23.23, 7.34</td>
</tr>
<tr>
<td>Steady state flux (µg/cm².min)</td>
<td>0.24 ± 0.08, 0.03</td>
<td>0.75 ± 0.20, 0.06</td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>40.58 ± 11.25, 3.98</td>
<td>21.13 ± 18.80, 6.27</td>
</tr>
<tr>
<td>Permeability coefficient (cm/h)</td>
<td>(9.61 ± 2.8, 1.0) x 10^{-6}</td>
<td>(2.98 ± 0.6, 0.23) x 10^{-5}</td>
</tr>
</tbody>
</table>

3.4.3 IN VIVO HYDRATION: OPTICAL COHERENCE TOMOGRAPHY ASSESSMENTS

Examples of OCT images are provided in Figure 3.3 and 3.6. Over all measurements, OCT showed that 5% urea gel under passive occlusion increased the relative epidermal thickness by 3% and 6% at time points 30 and 60 minutes respectively compared with time 0. Under active ETP occlusion, the relative change in epidermal thickness increased by 16% and 11% at 30 and 60 min respectively. These results are displayed in Figure 3.5; the errors of the relative change were calculated from the error in measuring the epidermal thickness. Thirty minutes after topical application of the urea gel the OCT measurements revealed an over 5-fold increase in epidermal thickness using the active ETP occlusion compared to passive occlusion. The effect
was less marked at 60 minutes but there was still an almost 2-fold difference in the relative epidermal thickness comparing the active and passive occlusion.

Figure 3.5: Difference in epidermal thickness at time point 30 and 60 min relative to 0 min due to administration of 5% urea gel with passive and active (ETP 008) occlusion giving the percentage change (mean ± error: calculated from the error in measuring the epidermal thickness, which are 10-15 µm; 72 OCT images).
Figure 3.6: Examples of optical coherence tomography images of human skin at 60 min post application of urea gel with passive occlusion and ETP 008 magnetic film array occlusion

Table 3.2: In vitro skin permeation: statistical analysis of difference in urea permeation over time for magnetic versus passive administration Mann-Whitney U analysis.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.021</td>
<td>0.021</td>
<td>0.006</td>
<td>0.006</td>
<td>0.002</td>
<td>0.001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

3.4.4 DISCUSSION

A comparison of the cumulative amount of urea penetrating the epidermis to the receptor solution versus time was plotted for passive and magnetic film array applications. In all cases cells were occluded and non-magnetic polymer film of similar dimensions was used as control to ensure that any increase in urea penetration was due to the magnetic field energy in the array film and not simply the occlusive
effect of magnetic film in proximity to the gel. Urea penetration was significantly increased by magnetic film array compared to passive occlusion over the time period of the experiment (p < 0.001). All permeation parameters showed significantly enhanced urea permeation in the presence of the magnetic field. The extraction of urea from the epidermal membrane post permeation studies showed small (less than 2 fold) difference in the amount of urea retention from passive occlusion versus ETP magnetic array occlusion. This could be due to the increased permeation of urea across the epidermal membrane due to ETP magnetic array as compared to passive occlusion.

As described in chapter 2 MPT-FLIM showed skin permeation of the nanoparticles with the pulsed electromagnetic field with no evidence of passive permeation. This suggested that the magnetic energy could induce channels within the stratum corneum of at least 10 nm in diameter. Modest skin penetration enhancements of urea are due to its keratolytic properties and increased hydration by creating hydrophilic diffusion channels within the skin. The 4-fold increased penetration of urea in the presence of a magnetic film could further substantiate our previous findings that pore or channels are created within the skin in the presence of magnetic energy. It must be noted that these previous studies involved a range of different types of magnetic fields different in characteristics to the field array used in this study. Apart from being a hydrotrope urea is a penetration enhancer in itself, therefore an active magnetic film array (ETP) technology that increases urea penetration would benefit in treating hyperkeratotic skin conditions.

Direct measurement of penetration of urea into the skin in vivo is difficult thus OCT was utilised to visualise and measure the physical changes in the skin properties due to hydration. As expected, the epidermal depth was increased by hydration due to occlusion and the presence of the topically administered hydrating agent urea. OCT permitted monitoring of the time dependent effects on skin properties in response to hydration. In this study the application of 5% urea gel with occlusion increased epidermal thickness by 3% and 6% after 30 and 60 min respectively. However when urea gel was applied with the magnetic film (ETP008) the epidermal thickness increased by 16% after 30 min and 11% at 60 min. This suggests that the magnetic field array material increases hydration by enhancing the rate of urea permeation into the skin and confirms the in vitro epidermal penetration data obtained in the study. It
appears from the OCT measurements that the changes in the epidermal properties with magnetic area application maximize after about 30 min and then stabilize or even decrease over longer periods of time. Given the difficulty in making repeat measurements in exactly the same place (positioning accuracy equals the transverse resolution of the OCT imaging system which is 15 µm) this may be due to changes in tissue morphology due to skin appendages like hair follicles or sweat ducts. There is the added complication that the changes in the levels of hydration may also change the refractive index of the epidermis, here for all thickness measurements we have assumed the refractive index to be 1.4. Further studies with more subjects and multiple time points are required to fully understand the in vivo changes.

3.5 CONCLUSIONS

Urea gel was applied as a moisturizer. Its penetration across human epidermis was determined in vitro and epidermal hydration effect visualized in vivo by OCT. Administration with a novel magnetic film technology provided enhanced skin penetration of urea and increased epidermal hydration when compared to administration under an occlusive film. The practical benefits of an un-powered, thin and flexible magnetic film array that is suited to fabrication as a drug patch or cosmetic mask with enhanced skin delivery warrants further investigation.
REFERENCES


4 CHAPTER 4

Effect of sound waves (Sonophoresis) on human skin permeation of 5-Aminolevulinic acid \textit{in vitro} and Curcumin dye \textit{in vivo} visualization with Multiphoton Tomography imaging
4.1 BACKGROUND

Sonophoresis or phonophoresis (sonication) is defined as the use of sound waves or ultrasound, beyond 20 kHZ frequency to enhance the topical and transdermal delivery of drugs through skin. Ultrasound is an acoustic vibration propagating in the form of longitudinal compression waves at frequencies beyond the human auditory range of 0.02MHz. The intensity parameter, measured in Watts/cm² is the quantity of energy conveyed by the ultrasound wave when it passes through a given site. Therapeutic ultrasound is normally generated by a transducer that converts electricity into ultrasound using the piezo electric principle (1).

A transducer is predominantly a crystal such as quartz, and/or polycrystalline materials such as lead-zirconate-titanium (PZT) or barium titanate. The pressure applied on a transducer has rapidly alternating potential across opposite faces of a piezoelectric crystal, this induces corresponding alternating, dimensional changes and thereby convert electrical voltage into vibrational energy or ultrasound waves that is emitted from the surface of the transducer. The ultrasound waves generated by the transducer are longitudinal and propagate away from the front face in the direction in which the transducer is pointing or the direction of oscillation, and retains a specific waveform and constant frequency (2).

The ultrasound beam or waveform has two distinctive regions: the near field also known as Fresnel zone and the distant or far field also known as Fraunholer zone (Figure 4.1). The near field is a cylindrical beam of spatially fluctuating acoustic energy, caused by the constructive and destructive interface of the ultrasonic wave and the distant field is a diverging beam that exhibits a central acoustic energy in the beam, which smoothly falls off at either side. The length of the near field is dependent on the diameter of the treatment head and the wavelength (1).
Figure 4.1: Diagrammatic representation of the output from a large transducer used in sonophoresis (figure adapted from Meidan, 2003) (1). A represents the transducer; B, cylindrical beam in the near field; C, diverging beam in the far field; D, distance from the transducer; E and F are the ultrasound energy.

Based on the frequency range of sound or acoustic waves, ultrasound conditions are classified into two distinct sets and their reported uses include (a) Low-frequency or power ultrasound (20-100 kHz) as used in dentistry and (b) High-frequency ultrasound (1-10 MHz) used for diagnostic scanning. To transfer acoustic energy to the body tissue, it is necessary to use a contact medium as air is a poor medium for ultrasound propagation. Currently available contact media includes oils, water/oil emulsions, aqueous gels, surfactant solutions and ointments.

The four known parameters such as frequency (Hz), intensity (W/cm²), duty cycle (ms) and distance of the transducer from skin have shown to be critical for safe and efficacious sonophoresis. A wide range of frequencies (0.02-16 MHz) (3-11), intensities (0.1-3 Watts/cm²) (12-13), duty cycles (continuous or pulsed) (14-17) and transducer distances (18) have been investigated for a variety of drug molecules including low/high molecular weight peptides and proteins across various skin types and synthetic membranes.

Since the 1990s transdermal transport by low-frequency (f<100 kHz) has been found to be more effective than that of high frequency ultrasound (9-10, 19). Low-frequency sonophoresis has been applied as both pre and simultaneous treatment regimens (6-7, 20-23). In pre-treatment a short application of ultrasound permeabilizes the skin for several hours during which drug molecules could...
permeate. In simultaneous treatment enhanced permeation of drug molecules occurs due to (a) skin permeabilization and (b) induced convection flow. The transdermal transport due to simultaneous ultrasound has been shown to decrease when the acoustic waves are turned off (23). Tachibana et al (7-8, 24) reported the application of low-frequency sonophoresis on transdermal transport of lidocaine and insulin across hairless rat skin in vivo. Mitragotri et al (9, 25) showed that a application of even lower ultrasound frequencies (20 kHz) enhanced the transdermal transport of various low-molecular weight drugs and high molecular weight proteins across human skin in vitro.

The biological effects of ultrasound on body tissue are classified as (a) thermal, (b) cavitation, and (c) acoustic streaming. Streaming effects are important in high-frequency applications while cavitation effects occur predominantly with low-frequency ultrasound (2). The mechanism of skin permeation enhancement by sonophoresis is said to be a combination of indirect effects. Mechanical impact due to the collapse of cavitation bubbles in solution which results in disruption of lipid bilayers (23, 26) and a forced convection flow due to cavitation (27). Significant research has been focused on understanding the permeation pathways through skin during sonophoresis (11, 28-34).

In the present work I investigated two commercially available ultrasound devices (Sonoprep® and DF Ionzyme®) which are used for clinical and cosmetic purposes respectively. Sonoprep® generates low frequency (55 kHz), high-intensity (15 W/cm²) ultrasound and has been used as a pre-treatment sonophoresis. The instrument has been successfully applied for rapid anaesthesia (EMLA cream with lidocaine and prilocaine) (35-36), topical vaccination (antigenic proteins of tetanus toxoid and candida albicans) (37), transdermal insulin (human recombinant insulin), and transdermal heparin (Low molecular weight heparins) (38). The Environ® DF Ionzyme® is a patent pending facial treatment device that can deliver both low frequency sonophoresis (21 kHz) and iontophoresis. The device is designed to provide alternating or single energy (ultrasound and electric current) to enhance skin penetration of cosmetic creams. The instrument has been used successfully to deliver cosmetic creams made by Environ® that contain vitamin C, A and growth factors (39-40). The current chapter investigates effects of sonophoresis on human skin using multiphoton tomography-fluorescent lifetime imaging microscopy. The
epidermal permeation of 5-aminolevulinic acid in vitro and curcumin in vivo across human skin were conducted.

4.2 AIMS AND OBJECTIVES

The aim of the study was to investigate sonophoretic delivery of curcumin as a model drug across human skin in vivo using MPT-FLIM analysis and 5-ALA across human skin in vitro using Franz type diffusion cells. Two commercially available ultrasound devices (Sonoprep® and DF Ionzyme®) were used as the source of low-frequency (55 kHz and 21 kHz) sonophoresis. Using MPT-FILM, the effects of low-frequency sonophoresis on human skin in vivo was also investigated.

4.3 COMPOUNDS INVESTIGATED AND THEIR PROPERTIES

4.3.1 CURCUMIN

Figure 4.2: Chemical structure of Curcumin

Curcumin is a low molecular weight (368.3 g/mol) lipophilic polyphenol molecule (Figure 4.2) derived from the rhizome of the turmeric (Curcuma longa) plant. About 2–8% of turmeric by weight is curcumin. Curcumin and some of its analogues are responsible for the bright yellow colour of turmeric. Curcumin is used in the food industry as a additive (E100) for food colouring and flavouring. Chemically, curcumin is (1E, 6E)-1, 7 -bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (41-43). Numerous molecular targets have been identified for curcumin such as growth factors, growth factors receptors, transcription factors, cytokines, enzymes
and gene regulating proliferation and apoptosis. Based on the direct and/or indirect interactions of curcumin with these target sites, pharmacological properties of curcumin such as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-proliferative and anti-apoptotic have been identified. A detailed review on the target interactions of curcumin and the subsequent pharmacological effects of curcumin has been presented by Aggarwal 2009 (44). Various reports have been published on the potential therapeutic applications of curcumin against several chronic diseases including cancer of the pancreas, inflammatory, neurological conditions such as degenerative brain disorders, glioma multiforma and Alzheimer’s disease, cardiovascular and skin diseases such as psoriasis (45-52).

Absorption and fluorescence spectroscopic properties of curcumin have been found to be useful to quantitatively estimate the uptake of curcumin to different cells and also to evaluate its intracellular localization. Fluorescence spectroscopy has been used to monitor preferential localization of curcumin in the membrane of cells. Curcumin fluorescence has been employed to follow the unfolding pattern and structural changes in proteins as they preferentially bind to membrane lipids, and cellular proteins. The absorption spectrum of curcumin is slightly red shifted and the fluorescence spectrum shows specific changes on binding to biomolecules. Knowledge of the binding constants and probable binding locations of curcumin in macromolecular systems, have been useful for understanding the structural changes in some of the hosts and in evaluating the pharmacological effects at the molecular level (53). In the present study we have used curcumin as a fluorescent dye or a marker. Penetration of curcumin under the influence of low-frequency sonophoresis was visualised in vivo using MPT-FLIM.
4.3.2 5-AMINOLEVULENIC ACID

![Chemical structure of 5-Aminolevulinic acid](image)

Figure 4.3: Chemical structure of 5-Aminolevulinic acid

5-aminolevulenic acid hydrochloride (5-ALA) is a low molecular weight; 167.9 g/mol, hydrophilic molecule (Figure 4.3). It is a naturally occurring intermediate of heme biosynthesis (54). In the first step of the heme biosynthetic pathway 5-ALA is formed from glycine and succinyl CoA. The synthesis of 5-ALA is regulated by the amount of heme in the cell. The rate limiting step in the formation of heme is the incorporation of iron into PpIX (Protoporphyrin IX) and takes place in the mitochondria under the action of the enzyme ferrochelatase (Figure 4.4).
Figure 4.4: Biosynthetic pathway for heme. Fluorescing and photosensitizing compounds are enclosed in rectangular boxes, with protoporphyrin IX highlighted. The 5-ALA/heme feedback control is indicated in dotted lines and the principal biosynthetic pathway of 5-ALA induced PpIX is indicated in large arrow (55).

By adding exogenous 5-ALA, PpIX may accumulate because of the limited capacity of ferrochelatase. Porphobilinogen deaminase is another enzyme of the heme synthesis pathway. The activity of porphobilinogen is higher in some tumors and that of ferrochelatase is lower, therefore PpIX accumulation is selective. PpIX is an efficient photosensitizer. Photodynamic therapy involves systemic administration of a tumor-localizing photosensitizer and its subsequent activation by light of an appropriate wavelength to create a photochemical reaction causing photodamage to the tumor cells. 5-ALA is thus used as a photosensitizing prodrug in photodynamic therapy (PDT) to produce PpIX in the treatment of nonmelanoma skin cancers like basal cell carcinoma, squamous cell carcinoma, actinic keratoses and Bowen’s disease. Currently ALA-PDT is carried out with topical cream (20%) formulations;
However, one of the challenges with successful PDT is sufficient delivery and retention of 5-ALA at the target site, which is usually deeper layers in the skin requiring longer incubation time of the tissue with 5-ALA. Being hydrophilic, its passive diffusion is limited and hence local bioavailability of 5-ALA is normally insufficient for therapeutic effect \((56-57)\). Both chemical and physical penetration enhancement techniques have been investigated to improve the skin penetration of ALA and have found considerable success \((58-60)\). In the current chapter, we have investigated skin permeation of 5-ALA using low frequency sonophoresis (Sonoprep®) that could ultimately aid in attaining better anti-tumor action with PDT.

### 4.4 MULTIPHOTON TOMOGRAPHY-FLUORESCENT LIFETIME IMAGING MICROSCOPY (MPT-FLIM)

**Figure 4.5:** A schematic representation of the principle of Multiphoton tomography - a fluorescent molecule simultaneously absorbs 2 photons and each photon provides half the energy normally required to excite the fluorophores into the electronic state from the ground state \((61)\)
Figure 4.6: Examples of endogenous fluorophores in the different layers of human skin.

MPT is a relatively new quantitative visual tool that has evolved into a non-invasive method for the direct visualization of tissue structures with sub-cellular resolution (62-63). MPT enables simultaneous monitoring of the morphology and movement of solute transport in the tissue and has the advantage of sensitive detection of endogenous fluorophores including second harmonic generation (SHG) produced by asymmetrical components like collagen. MPT been used in visualising skin (64-65), liver (66) and other organs (67-68). MPT fluorophore excitation is achieved by the non-linear absorption process in which two near-infrared (NIR) photons of approximately half the one-photon excitation energy are simultaneously absorbed by the fluorescent molecule (Figure 4.5). The use of NIR laser radiation avoids thermal heating, photobleaching and out-of-focus fluorescence/SHG excitation by low one-photon excitation. This approach excites the intrinsic or endogenous (Figure 4.6) and extrinsic or exogenous fluorophores which are generally excited by ultraviolet (UV) light. Due to poor UV penetration depth, endogenous fluorophores in deep tissue areas could not be imaged with single photon techniques. However, two-photon excitation with NIR laser pulse allows imaging of endogenous fluorophores in the mitochondrial cells in the basal cell layer and even intradermal cells. In the present work MPT was employed to investigate the effects of ultrasound on human skin in vivo and to visualise the sonophoretic permeation of a fluorescent dye (curcumin) across human skin in vivo.
4.5 MATERIALS & METHODS

4.5.1 CHEMICALS

All chemicals and reagents listed below were used as supplied: 5-ALA (A3785 ~98%), fluorescamine (F9015 ≥ 98%) and curcumin ((E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) were purchased from Sigma-Aldrich (Sydney, NSW Australia). $^{14}$C-ALA (55 mCi/mmol) from American Radiolabelled Chemicals Inc (St Louis, MO, USA). Phosphate buffered saline was prepared according to United States Pharmacopeia and purified deionised Milli-Q water from Millipore (North Ryde, NSW Australia), A quasonic gel from Parker Laboratories Inc., (Fairfield, New Jersey, USA).
4.5.2 INSTRUMENTATION AND CONDITIONS:

4.5.2.1 SONOPREP®- ULTRASONIC SKIN PERMEATION INSTRUMENT

![Diagram of the Sonoprep instrument unit](image)

The Sonoprep instrument unit (Eco Therapeutics, Franklin, MA USA) consists of a control console, an ultrasonic handpiece, a reference electrode, a 12 volt battery charger, a coupling medium cartridge and a cleaning kit. The Sonoprep device delivers ultrasonic energy to the skin through an aqueous ultrasound coupling medium. The tip of the device includes a cylindrical ultrasonic horn inside a housing that positions the horn 7.5 mm above the skin. The housing is filled with the coupling medium, which consists of phosphate buffer saline and 1% sodium dodecyl sulphate.

Figure 4.7: (a) Sonoprep ultrasound generating device by Sontra Medical Corporation, (b) Ultrasonic cavitation of fluid by activated handpiece at surface of target skin site (36).
The treated site has an internal diameter of 1.0 cm and cross sectional area of 0.8 cm\(^2\) (diameter). The energy (12 watts RMS) is delivered to the ultrasonic horn by an ultrasonic control system at a frequency of 55 kHz; axial ultrasonic motion at the tip creates cavitation bubbles that rapidly expand and contract in the coupling medium at the skin surface (38).

4.5.2.2 ENVIROM\textsuperscript{®} DF IONZYME\textsuperscript{®}- ULTRASONIC SKIN PERMEATION INSTRUMENT

![DF Ionzyme ultrasound generating device by Environ Skincare (Pty) Ltd.](image)

The DF Ionzyme device (Environ Skincare (Pty) Ltd, Western Cape, South Africa) consists of a hand held probe that delivers ultrasonic waves and a separate probe for iontophoresis. The energy application is carried out using a foot pedal attached to the unit. The device contains an individual switch system for the choice of energy to be used. An ultrasound transmission gel (Aquasonic 100) was used as a medium prior to sonication.
4.5.3 *IN VIVO SONOPHORESIS WITH SONOPREP® AND DF IONZYME®*

For ultrasound application with Sonoprep®, prior to ultrasonic application, a locator ring is placed on the skin which covers 0.8 cm² skin area. The coupling medium cartridge is placed in the Sonoprep hand piece and the subject grasps the ‘reference electrode’. When the hand piece is pressed down on the site locator ring, it automatically delivers the coupling medium and activates. Upon optimum skin permeabilization which is identified by a skin conductivity feedback mechanism the instrument stops sonication. On average we observed the application time to be around 9-45 seconds depending on the skin condition and location. For ultrasound application with DF Ionzyme® once the area of application was marked the aquasonic gel was applied to the area, the sonication probe was placed carefully on the gel making sure not to touch the skin surface. The device was activated with the foot pedal to deliver sonication for 60 seconds, which was timed. For *invivo* curcumin dye penetration studies, the areas marked as passive and ultrasound (see protocol below) each received 50µL of 0.1% w/v curcumin in mineral oil. The area was left undisturbed for 1 hour and excess dye was wiped off with tissue prior to imaging.
4.5.4 MULTIPHOTON TOMOGRAPHY-FLUORESCENT LIFETIME IMAGING MICROSCOPY (MPT-FLIM)

Figure 4.9: A Schematic representation of the internal components of a Multiphoton microscope.

The MPT imaging was carried out on a DermaInspect system (JenLab GmbH, Jena, Germany), that was illuminated with an ultrashort (85 femtosecond pulse width) pulse mode-locked 80-MHz Titanium Sapphire MaiTai laser (Spectra Physics, Mountain View, USA) with a tuning range of 710-920 nm. A T CSPC (time correlated single photon counting) 830 detector (Becker and Hickl, Berlin, Germany) was integrated into the tomography system to enable FLIM measurements (figure 4.7). A HF110 filter wheel was used to switch between the following emission filters: BG39 (350-650 nm), FDIG (500-580 nm) and FDIB (320-520 nm). The objective lens was a Plan-Neoflaur oil-immersion (40x/NA-1.3) and the space between the invivo adaptor and the objective lens was filled with index-matching oil for high (40x) magnification objectives (69).

4.5.5 IMAGING PROTOCOL & FLIM ANALYSIS

Prior to commencement of the imaging the forearms of the subjects were cleaned with soap and the area blotted dry with a tissue. Markers were used to identify the area of imaging and treatment as control (no curcumin and no ultrasound), passive
(curcumin and no ultrasound) and ultrasound (with curcumin). TEWL measurements using a TM 210 Tewameter (Courage + Khazaka, Cologne, Germany) were recorded for baseline readings on those areas. Ultrasound was applied to the selected area according to the procedure detailed in the previous section. Post-ultrasound TEWL measurements were recorded accordingly. The imaging of the areas were conducted in two series; firstly a z-stack on the area (treated with ultrasound and that of control) at every 5 μm depth, thus covering from 0 -25 μm was taken with excitation wavelength set at 740 nm to investigate the effects of ultrasound on human skin. Secondly, for curcumin penetration studies after application of curcumin and 1h penetration time, the areas marked as passive and ultrasound treated were imaged similarly at 740 nm covering 0-25 μm depth.

MPT imaging involved tightly focusing ultra short pulsed excitation laser light onto the skin using a high-NA oil-immersion microscope objective. The excited fluorescence was collected by the same imaging optics and reflected to a detection arm by a dichroic mirror that was transparent for the excitation radiation in the IR (infrared) spectral range, and reflective for UV and visible. In the detection arm, the fluorescence was split into an intensity detection path and FILM detection path. Both detection paths included optical filters which contained broad spectrum filters to block the IR excitation light and capture spectral contents of the tissue auto-fluorescence. The FLIM path detectors comprised of four channels terminated by four fast-response optical detectors, photomultiplier tubes (PMT), suitable for single photon events for fluorescence lifetime analysis. The four detection channels have pre-selected filters: with band pass of 350-450 nm (channel 1), 450-515 nm (channel 2), 515-620 nm (channel 3) and 620-670 nm (channel 4). An image was formed by recording detector signals versus the focal spot position in the sample. Two images from the two channels could be overlaid at the post processing stage.

The FLIM analysis was undertaken using SPC 830 2.9. The module builds up a photon distribution over the scan coordinates, x and y and the time in the fluorescence decay, t (70). Signal from the four single photon counting detectors working in different wavelength intervals are processed simultaneously. Fluorescence lifetime analysis was done by the SPC image data analysis package (Becker and Hickl). The image analysis involved measuring the fluorescent decay matrix (\( \tau_1 \) and \( \tau_2 \)) of free and protein bound NAD (p) H and redox ratio (\( a_1 \) and \( a_2 \)) changes to characterise the metabolic activity of the cells pre- and post-ultrasound.
treatment at 740 nm (channel-1) and for penetration of curcumin dye according to its emission wavelength at 740 nm (Channel-3).

4.5.6 QUANTITATIVE ANALYSIS

4.5.6.1 PREPARATION OF RADIOLABELLED STANDARDS AND SAMPLES

The donor solution consisting of 5-ALA in PBS was spiked with $^{14}$C-5-ALA (~1µCi/mL) to achieve 0.5 µCi/cell. A standard with a known quantity of 5-ALA and $^{14}$C-5-ALA was mixed with scintillation cocktail (Emulsifier safe, Perkin Elmer, Waltham, MA, USA) and analysed for radioactivity in the Packard Liquid Scintillation Counter (LSC: Canberra, CT, USA). The disintegrations per min were converted to amount in µg/mL by mathematical transformation.

4.5.7 IN VITRO PERMEATION STUDIES

4.5.7.1 PREPARATION OF MEMBRANE

Ethical approval for using human skin was obtained from the Human Research Ethics Committee of Curtin University prior to the study. Briefly, the subcutaneous tissue was removed by dissection (71) from skin samples (abdominal region following abdominoplasty surgery at Brisbane hospitals). For experiments involving full thickness skin, upon removal of subcutaneous fat the skin was stored at -20°C (for not more than 2 months) until required.
Permeation studies across full thickness human skin were performed using Pyrex glass Franz-type diffusion cells (enabling permeation across skin sections of cross sectional area 1.18 cm²; receptor volume approximately 3.5 mL; Figure 4.10). The membrane was placed between the donor and receptor compartment of the cell and allowed to equilibrate for 1 h with PBS in the receptor compartment that was stirred continuously with a magnetic stirrer. 1 mL PBS was placed in the donor compartment of the cell which was then placed in a water bath and maintained at 37±0.5°C. The membrane integrity was then determined by electrical resistance (kΩ) measurement using an LCR multimeter (TH2821/A/B, Changzhou Tonghui Electronic Co Ltd, Jiangsu Province, China). The measurements were taken by immersing the silver-silver chloride electrode attached to the probe lead tips, one each in the donor and receptor compartments (72). Membranes exhibiting an electrical resistance less than 20 kΩ were rejected from the study.

Using a Tewl meter (Tewameter TM 210, Courage and Khazaka GmbH Electronic, Koln-Germany) probe the T EWL measurements were taken for all the skin pieces pre- and post-ultrasound application. In all groups (passive or no sonication and sonication) the donor compartment was then filled with 0.5 mL of 10% 5-ALA solution (600 mM aqueous solution) in PBS spiked with ¹⁴C-ALA (~1 µCi/mL) to...
achieve 0.5 µCu/cell following the pre-treatment time. Samples were collected at different time points over 0-8 h and analysed for 14C-ALA content using a liquid scintillation counter as mentioned above.

### 4.5.7.3 IN VITRO SONOPHORESIS

For *in vitro* experiments, each skin piece was treated with ultrasound for varied application time as detailed above. TEWL measurements were recorded pre and post ultrasound application using the Tewlmeter. Since the Sonoprep machine is designed for *in vivo* application we modified it to use on an *in vitro* experimental setup. The skin pieces for ultrasound treatment were placed on a copper plate surrounded by PBS solution. The operator held the reference electrode in one hand which was in contact with this PBS solution surrounding the skin to ensure contact while the ultrasonic hand piece (held in the other hand) was placed on the skin piece and activated as described above. The circuit was completed due to the PBS solution acting as the conducting medium around the skin. The skin pieces were prepared for the diffusion study as described above. Permeation of 5-ALA across sonicated or treated and passive or untreated skin was measured over 8 h. The data was plotted as mean cumulative amount permeating to the receptor compartment versus time. Transdermal flux and permeability coefficient were calculated over the linear portion of the plot.

### 4.5.8 STATISTICAL ANALYSIS

For the 5-ALA *in vitro* permeation data the statistical analysis was carried out with SPSS 16.0 for Windows (SPSS Inc., Chicago IL). The Mann-Whitney U Test (1-tailed) was used to determine significant differences between all parameters.

### 4.6 RESULTS AND DISCUSSION:

There is considerable evidence in favour of ultrasound mediated skin permeation enhancement for a range of drugs and macromolecules, both using *in vitro* models and *in vivo* studies but direct correlation using an instrument designed for clinical application has been problematic. To our knowledge MPT was used to reveal some
effects of ultrasound on human skin (*invivo*) for the first time. A bi-exponential fit was used to generate fluorescence decay curves and determine the lifetime components $\tau_1$ and $\tau_2$ with the corresponding intensity coefficients $a_1$ and $a_2$ on single points within the image. When the tissue is excited at 740 nm and 350-450 nm EM, the $\tau_1$ and $\tau_2$ fluorescence lifetime decay histogram correlates with free and protein-bound NAD (p) H respectively. All the fluorescent lifetime components calculations are based on stratum granulosum layer. The fluorescent lifetimes components for free NAD (p) H is around 300 ps and that of protein bound-NAD (p) H is 2000-3000 ps with the emission peak of 450 nm that is measured in Channel 1. The results are presented in Figures 4.11-4.16. The fluorescent lifetime components of the cells in the stratum granulosum layer pre- and post sonophoresis are presented in panel a which shows a plot of lifetime versus pixel frequency (mean ± SEM) and panel b which represents examples of images used for calculating these lifetime components.
Figure 4.11: Panel (a) shows the fluorescence lifetime profile of free NAD (p) H from the cells in the stratum granulosum layer in the untreated or control area and that of the Sonoprep® treated area, in vivo, n=3 (mean ± SEM). Panel (b) are some examples in vivo human skin images of areas used for calculating the lifetime profiles. All images are 214x214 µm.
Figure 4.12: Panel (a) shows the fluorescence lifetime profile of protein bound NAD (p) H from the cells in the stratum granulosum layer in the untreated or control area and that of the Sonoprep® treated area, *in vivo n*=3, (mean ± SEM). Panel (b) are some examples of *in vivo* human skin images from areas used for calculating the lifetime profiles. All images are 214x214 µm.
Figure 4.13: Panel (a) shows the fluorescence lifetime profile of free NAD (p) H from the cells in the stratum granulosum layer in the untreated or control area and that of the DF Iozyme® treated area, *in vivo*, n=3, (mean ± SEM). Panel (b) are some examples of *in vivo* human skin images from areas used for calculating the lifetime profiles. All images are 214x214 µm.
Figure 4.14: Panel (a) shows the fluorescence lifetime profile of protein bound NAD (p) H from cells in the stratum granulosum layer in the untreated or control area and that of the DF Iozyme® treated area, in vivo, n=3, (mean ± SEM). Panel (b) are some examples of in vivo human skin images from areas used for calculating the lifetime profiles. All images are 214x214 µm.
Figure 4.15: Panel (a) shows the redox ratio state from the cells in the stratum granulosum layer in the untreated or control area and that of the Sonoprep® treated area, in vivo, n=3, (mean ± SEM). Panel (b) are examples of in vivo human skin images of area used for calculating the redox ratio. All images are 214x214 µm.
Figure 4.16: Panel (a) shows the redox ratio state from cells in the stratum granulosum layer in the untreated or control area and that of the DF Iozyme® treated area, \textit{in vivo}, \( n=3 \), (mean ± SEM). Panel (b) are examples of \textit{in vivo} images from area used for calculating the redox ratio. All images are 214x214 µm.

The components \( \tau_1 \) and \( \tau_2 \) for all the subjects in the study for both the sonophoresis devices did not show any changes post ultrasound treatment indicating no change in
protein binding (Figure 4.11, 4.12, 4.13 and 4.14 (panel a)). This indicated that both the ultrasound devices (Sonoprep® and Ionzyme®) did not cause any cellular changes in the epidermal cells. However, when the corresponding intensity coefficients were used to measure the Free: Bound NAD (p) H ratio \((a_1/a_2)\) component to determine the metabolic activity of the cells treated with ultrasound, a shift in peak ratio from 4.0 for control or no ultrasound to 2.5 for ultrasound treated cells \((n=3, \text{ mean}±\text{SEM})\) indicated increased metabolic activity in the mitochondria of the epidermal cells (Fig 4.15 and 4.16 (panel a)). This is normally associated with a disruption in the barrier properties of the skin. We suspect that ultrasonic waves during sonophoresis disturbed the cells and during their recovery phase the metabolic activity of the cells is increased due to rapid enzymatic activity (73-77). Under the conditions tested, both the commercially available ultrasound devices (Sonoprep® and DF Ionzyme®) showed similar effects on skin.

Both histological and microscopy studies on both human and animal skin exposed to ultrasound under various conditions have been reported. Levy et al (14) performed histological studies on hairless rat skin which was exposed to therapeutic ultrasound (1 MHz, 2 W/cm2) using haematoxylin and eosin and reported no damage to the skin post ultrasound application. Mitragotri et al performed histological studies of hairless rat skin exposed to low-frequency ultrasound (20 kHz) and found no damage to the epidermis and underlying tissue (10). Paliwal et al used confocal microscopy and transmission electron microscopy (TEM) to study the effects of low-frequency sonophoresis on full thickness pig (Yorkshire) skin and used quantum dots as tracers to visualise the sonophoretic permeation pathway. The authors reported no damage or destructive effects on the morphology of the stratum corneum and epidermal structures using histology and with TEM ultra-structural changes were only seen in stratum corneum of the LTR’s (localised transport region). Using TEM images, changes in the ultra-structure were found to be profound in the deeper layers from stratum granulosum onwards. The authors showed an increase in lacunar spaces and the frequency of their occurrence of these spaces. Lacunar spaces are the domains embedded within the lipid bilayers and are related to sites of subjacent corneodesmosome degradation (78). The authors quantified the increase in lacunar spaces and their frequency post ultrasound treatment indicating the presence of a transient three-dimensional network that was capable of transporting macromolecules (28).
Fig 4.17: Mean pixel intensity due to curcumin dye penetration through human skin over a depth of 0-25µm. Comparison of control or no ultrasound and no curcumin (○), untreated or passive curcumin (◊), treated or iozyme ultrasound with curcumin (–●–) and sonoprep ultrasound with curcumin (– ––) (mean±SEM; n=3, 1, 3 respectively)

The in vivo skin permeation studies with curcumin are presented as the pixel intensity versus depth of penetration plot (Figure 4.17). The emission wavelength of curcumin was between 460-650 nm depending on the medium (53) which could be analysed with (515-620 nm) EM filters when the tissue was excited at 740 nm and eliminate auto-fluorescence from endogenous fluorophores (350-515 nm) EM. The curcumin signal increased substantially in ultrasound treated skin (both Sonoprep and I onzyme) as compared to untreated or passive diffusion and control or no ultrasound and no curcumin area (Table 4.1). Curcumin penetration post sonication from both ultrasound devices was variable (Figure 4.16). Low-frequency sonophoresis effects have been heterogeneous in this study, which may be due to the fact that sonophoretic cavitation is not confined homogeneously on the area of application. Low-frequency sonophoresis is reported to create LTR’s (localised transport region) and penetration of dyes such as sulforhodamine B (hydrophilic)
from ultrasound treated areas showed clear difference in skin colour and on LTR’s and non-LTR’s in previous studies (79).

Table 4.1: *In vivo* Curcumin dye penetration, pixel intensity data (mean ± SD, SEM) for sonophoresis and passive diffusion, (n = 3, 1, 3).

<table>
<thead>
<tr>
<th>Depth in µm</th>
<th>Control (no ultrasound and no dye)</th>
<th>Passive (no ultrasound with dye)</th>
<th>Post Sonoprep®</th>
<th>Post DF Iozyme®</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18.7±11.0, 6.3</td>
<td>39.6±9.9, 5.7</td>
<td>25.2±8.5, 4.9</td>
<td>54.4±38.0, 21.9</td>
</tr>
<tr>
<td>5</td>
<td>19.6±9.1, 5.2</td>
<td>33.1±8.9, 5.0</td>
<td>43.5±1.4, 0.8</td>
<td>64.4±61.5, 35.5</td>
</tr>
<tr>
<td>10</td>
<td>15.9±4.2, 2.4</td>
<td>32.0±15.0, 8.6</td>
<td>35.3±11.6, 6.7</td>
<td>62.3±47.0, 27.1</td>
</tr>
<tr>
<td>15</td>
<td>15.0±3.9, 2.3</td>
<td>33.5±14.2, 8.2</td>
<td>34.6±15.6, 9.0</td>
<td>57.2±28.0, 16.1</td>
</tr>
<tr>
<td>20</td>
<td>18.8±8.5, 4.9</td>
<td>31.7±14.9, 8.6</td>
<td>40.9±26.9, 15.5</td>
<td>74.2±71.8, 48.4</td>
</tr>
<tr>
<td>25</td>
<td>17.6±10.3, 5.9</td>
<td>14.5±6.7, 12.7</td>
<td>43.7±42.5, 24.55</td>
<td>19.4±3.6, 2.1</td>
</tr>
</tbody>
</table>

Curcumin permeation was found to be consistently increasing from the stratum corneum layer to the stratum basale for both the sonophoresis devices. Sonophoresis by Iozyme® showed curcumin pixel intensity from 0-20 µm and the signal decreased at deeper layers (25 µm), whereas sonophoresis by Sonoprep® showed low and steady increase in curcumin pixel intensity from 0-25 µm (Figure 4.17) suggesting that the channels created due to sonophoretic cavitation by Sonoprep® reached deeper layers therefore little curcumin signal was found on the surface. This difference in permeation could also be explained by the presence of a penetration enhancer such as surfactants. Sonoprep® contains 1% sodium dodecyl sulphate in its coupling medium. Sonophoresis in the presence of surfactants is said
Kushner et al. reported a dual channel two-photon microscopy study to visualise the permeation pathways of sulforhodamine B, a hydrophilic dye, and rhodamine B hexyl ester, a lipophilic dye through exercised human skin in vitro. The enhanced sonophoretic permeation profiles of both the dyes showed heterogeneity due to the presence of LTR and non-LTR. The authors concluded that the ultrasound treated skin was greatly perturbed in the LTR’s. The presence of a chemical enhancer such as ethanol and sodium lauryl sulphate tested with ultrasound played a significant role in the transdermal enhancement. The LTR of ultrasound-treated and ultrasound/sodium lauryl sulphate-treated areas for both the dyes showed transcellular pathways (31). The TEWL measurements taken before and after sonophoresis showed an average (n=3 and 7, mean ± SEM) more than 100% increase clearly indicating perturbation of the cells in the area (Table 4.2). Although the both the ultrasound used in our study was painless, some subjects encountered mild thermal sensation at the end of sonication with DF Iozyme and faint erythema the following day with Sonoprep.

Table 4.2: *In vivo* mean± SD (3 measures) TEWL measurements pre and post ultrasound treatment n= 3.

<table>
<thead>
<tr>
<th>N</th>
<th>Control</th>
<th>Sonoprep</th>
<th>Percentage Increase</th>
<th>Iozyme</th>
<th>Effect as Percentage Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.0</td>
<td>32.5±0.2</td>
<td>270.8</td>
<td>16.6±0.5</td>
<td>138.3</td>
</tr>
<tr>
<td>2</td>
<td>14.5</td>
<td>39.8±0.5</td>
<td>274.4</td>
<td>18.3±0.3</td>
<td>126.2</td>
</tr>
<tr>
<td>3</td>
<td>16.6</td>
<td>38.6±0.4</td>
<td>232.5</td>
<td>21.2±0.5</td>
<td>127.7</td>
</tr>
</tbody>
</table>
4.6.1 QUANTITATIVE ANALYSIS OF 5-ALA

Quantitative analysis of 5-ALA was performed by liquid scintillation counting. All standards and samples were prepared at room temperature and the sample volume was 200 µL. The radiolabelled ALA samples were analysed on a Packard Liquid Scintillation Counter (LSC: Canberra, CT, USA). Prior to radiolabelled analysis 5-ALA samples and standards were quantified on a validated HPLC method (80) to confirm the analysis.

4.6.2 INVITRO DIFFUSION STUDIES

Fig 4.18: Cumulative amount of 5-ALA (µg/cm²) permeating full thickness human skin to the receptor compartment under the influence of ultrasound (●) and passive (○) (mean ± SEM; n=7 and 6 cells respectively).

To correlate the in vivo findings of curcumin skin permeation, a preliminary invitro 5-ALA penetration across human skin was conducted. Excised human skin was pre-treated with ultrasound prior to the administration of 5-ALA solution and the subsequent diffusion study carried out over 0-8 h. The cumulative amount of 5-ALA that permeated the full thickness human skin to the receptor solution over time was plotted for sonophoresis and passive diffusion (Figure 4.18). The results indicated a significant enhancement in the amount and rate of 5-ALA permeating full thickness
human skin under the influence of ultrasound when compared to no passive diffusion (p<0.001). The estimated transdermal flux was calculated over the entire experimental period (0-8 h) was 54.8±8.0 µg/(cm².h) and the corresponding permeability coefficient was (4.1±0.6)×10⁻⁴ cm/h with sonication. There have been very few publications on ultrasound mediated ALA skin delivery. Using high frequency ultrasound in the range of 1.1 MHz and 3W/cm² intensity, Ma et al have reported enhanced skin permeation of ALA across BALB/c nude mice bearing WiDr human colon adenocarcinoma. The authors used a 20% oil-in-water emulsion and applied it over the surface of the tumour for 30 min to 3 h. Ultrasound (pulsed and/or continuous) was applied for 5-10 min prior to ALA application and in some cases immediately after ALA application. Using fluorescence microscopy ALA-induced PpIX was measured and PpIX production in tumor cells was recorded within 1 h with 5 min ultrasound application as compared to 3 h in normal clinical practice. There was no significant increase in PpIX production post ultrasound permeation. Ultrasound treatment was able to increase the permeation of 5-ALA and availability of ALA in the tumour site (81). The ultrasound treated 5-ALA induced PpIX was presented with 1 h of light irradiation due to the difference in experimental conditions and direct comparison of our results with the aforementioned work is difficult.

Table 4.3: Invitro 5-ALA permeation data (Mean ± SD, SEM) for sonophoresis and passive diffusion, (n = 6, 7).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Passive</th>
<th>Ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cumulative permeation (µg/cm²)</td>
<td>0.0±0.0</td>
<td>452.5±173.2, 65.4</td>
</tr>
<tr>
<td>Flux (µg/cm².h)</td>
<td>0.0±0.0</td>
<td>54.8±21.1, 8.0</td>
</tr>
<tr>
<td>Permeability Coefficient (cm/h)</td>
<td>0.0±0.0</td>
<td>(4.1±0.6)×10⁻⁴</td>
</tr>
</tbody>
</table>

Whilst ultrasound pre-treatment showed substantial increase in ALA permeation the enhancement was highly variable. As explained in the previous section, although low-frequency sonophoresis is an efficient skin penetration enhancement technique its effect is heterogeneous. However, the data presented in Table 4.3 show that pre-
treatment sonophoresis groups showed significantly (p<0.001) greater skin penetration of 5-ALA than passive diffusion. This result demonstrates enhanced skin penetration effects of low frequency ultrasound for this polar molecule. The TEWL measurements taken pre and post sonophoresis also indicated disruption in the cellular structures (Table 4.4).

Table 4.4: *In vitro* mean± SD (3 measures) TEWL measurements pre and post ultrasound treatment n= 7.

<table>
<thead>
<tr>
<th>N</th>
<th>Control</th>
<th>Sonoprep</th>
<th>Effect as Percentage Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.7</td>
<td>23.7±0.5</td>
<td>202.5</td>
</tr>
<tr>
<td>2</td>
<td>13.8</td>
<td>18.0±0.7</td>
<td>130.4</td>
</tr>
<tr>
<td>3</td>
<td>11.2</td>
<td>19.3±0.5</td>
<td>172.3</td>
</tr>
<tr>
<td>4</td>
<td>13.1</td>
<td>18.5±0.3</td>
<td>141.2</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>17.7±0.5</td>
<td>141.6</td>
</tr>
<tr>
<td>6</td>
<td>12.3</td>
<td>20.4±0.7</td>
<td>165.8</td>
</tr>
<tr>
<td>7</td>
<td>11.5</td>
<td>29.6±0.6</td>
<td>257.3</td>
</tr>
</tbody>
</table>

4.7 CONCLUSIONS

Low-frequency sonophoresis was able to enhance the skin penetration of two low molecular weight compounds; a polar molecule (5-ALA) *in vitro* and a non-polar molecule (curcumin) *in vivo*. Our results are supported by previous reports that show low-frequency ultrasound is an efficient physical skin penetration enhancement technique. Both devices showed enhanced drug delivery when compared to passive drug exposure. The Sonoprep® uses 55 kHz sonophoresis to deliver low and high molecular weight compounds. DFI Onzyme® uses 21 kHz sonophoresis to deliver cosmesuticals on the surface of the skin. MPT-FLIM was used for the first time to
investigate the effects of sonophoresis on NAD (p)H in human skin, *in vivo*. These data revealed increased metabolic activity in the epidermal cells after ultrasound application. This may be due to a breach in the barrier properties of the skin. Fluorescence lifetime imaging is a fairly new technique and its applications are being explored for a better understanding. Further works with the technology is underway to optimise its use in permeation studies.
REFERENCES


5 CHAPTER 5

Effect of Small Current (Iontophoresis) on the Human Skin Permeation of a Peptide-like Molecule and Various Peptides

Preliminary Invitro Studies
5.1 BACKGROUND

Peptide and protein drugs, termed biopharmaceuticals or bi drugs, represent a significant and growing portion of the overall pharmaceutical market (1). Some of the most frequently marketed biopharmaceuticals include monoclonal-antibody-based products for the treatment of cancer and autoimmune diseases, therapeutic vaccines, insulin for diabetes treatment, human growth hormone for supplementation in hormone deficiency, and interferon A for treatment of hepatitis B and C. The widespread application of peptide and protein therapeutics is restricted due to their own physicochemical properties such as high molecular weight and hydrophilicity, which lead to poor oral bioavailability, poor transfer across biological membranes, and low stability in the bloodstream (2-3). Several biopharmaceuticals have undergone extensive research and development towards alternative administration routes, such as transdermal, pulmonary, nasal, modified oral and buccal delivery, particularly peptide hormones, such as insulin, vasopressin, calcitonin and luteinizing hormone-releasing hormone (LHRH), and some small protein molecules, such as human growth hormone and interferon α (4).

The transdermal route has the potential to be an efficient delivery option for proteins and peptide drugs as it avoids gastric degradation and hepatic first-pass metabolism and has relatively low proteolytic activity (5). However, the application of transdermal delivery to a wider range of drugs, including therapeutic proteins and peptides, is limited due to the significant barrier properties of the skin as discussed in Chapter 1. In addition to the size and complexity of their molecular structure, peptide and protein molecules are mostly hydrophilic and often exist in a charged state. Therefore passive skin diffusion is unlikely to be effective and there is a need to apply an effective technique to enhance skin transport. Physical and chemical penetration enhancers have been extensively investigated for enhanced skin permeation of proteins and peptides as described in Chapter 1. Iontophoresis is one of the electrical based physical skin penetration enhancement techniques. It is a century-old technique that involves the use of a mild electrical current (up to 0.5mA/cm²) to drive charged and uncharged molecules across the skin (6). The technique is painless and non-invasive, and delivers consistent quantities of drug through skin. The positive or negative drug ions act as charge carriers across the high impedance stratum corneum, such that the drug is rapidly delivered into the epidermis (7).
Proteins and peptides are usually charged at physiological pH or can be rendered charged by altering pH and therefore ideal candidates for iontophoresis. Transdermal iontophoretic delivery has been demonstrated for a number of peptides including cyclosporin, nafarelin, arginine vasopressin (AVP), LHRH, octreotide, calcitonin and insulin (8).

**Figure 5.1: A schematic representation of anodal iontophoresis (9)**

A basic iontophoretic device consists of a power supply connected to an electrode (anode and a cathode), which connects separately to the skin via a contacting medium (Figure 5.1). The preferred materials for electrodes are silver/silver chloride (Ag/AgCl) as they do not cause any pH change in the contact medium and are versatile and reversible (10-12). During passive permeation the unionised form of the drug molecule is better absorbed than its ionised form (13). However in iontophoresis the electrical potential acts as an external force that pushes the ionized drug molecules across the membrane. In order to push a negatively charged drug across an epidermal membrane it is placed in contact with the negative electrode (cathodal iontophoresis) from which it is repelled and gets attracted towards the opposite electrode placed elsewhere in the body. Anodal iontophoresis is used for positively charged drug molecules. The electrochemistry of an iontophoretic circuit is such that there is oxidation at the anode and reduction at the cathode. Oxidation at
the Ag/AgCl anode results in the loss of a Cl\(^-\) from the solution, which is balanced either by pushing a cation (Na\(^+\), Drug ion\(^+\)) through the skin or by the arrival of an anion from beneath the skin (Cl\(^-\)). Equally, reduction at the cathode releases a chloride ion, an extra negative charge, which is neutralised either by the electromigration of an ion (Cl\(^-\) or drug\(^-\)) through the skin or by the arrival of a cation from beneath. Therefore the number of electrons flowing through the external circuit is a direct reflection of the amount of ionic charge flowing through the skin. Similarly when an electrical potential gradient is established across a membrane, ions on either side will migrate in the direction governed by their charge (14). The sum of the flux of individual ions must equal the current applied by the power source; in other words, there is competition among all the ions present to carry the charge. The physicochemical characteristics such as electrical mobility and concentration of the ion concerned and the properties of the media through which the ion is moving, determine the probability of the ion being the major carrier, and eventually being efficiently transported across the skin (10, 15).

Iontophoretic transport is a complex behaviour and there have been a number of detailed analyses of the mechanistic aspects of iontophoresis and iontophoretic enhancement (16-25). Electrorepulsion or electromigration is known to be the main mechanism by which iontophoresis exerts its enhancement effect on ionised solutes, but other factors including increased stratum corneum permeability in the presence of an electric current flow, and electroosmosis (movement due to convection flow) in the case of uncharged larger water soluble molecules, also contribute (7, 26-29). In 1940, Abramson and Gorin deduced an equation to correlate iontophoretic flux to electric mobility, electroosmosis and simple diffusion (30). According to the Nernst-Plank equation, the flux \(J_{inp}\) of a non-ionic, i.e., across a membrane under the influence of an electric field is due to three components: a diffusion component \(J_p\), an iontophoretic (electromigration) component \(J_e\) and an electroosmotic (convection flow) component \(J_c\), given by the equation below.

\[
J_{inp} = J_p + J_e + J_c \quad \text{Eq: (5.1)}
\]

\[
J_p = K_s D_s \quad \text{Eq: (5.2)}
\]

\[
J_e = C_i \quad \text{Eq: (5.3)}
\]
\[ J_c = kC_s I_d \quad \text{Eq: (5.4)} \]

\( K_s \) is the partition coefficient of the drug molecule between the donor solution and the stratum corneum, \( C \) and \( C_i \) are the concentration of the donor solution and of the ionic species, \( i \), in skin tissue, \( dC/hs \) is the concentration gradient across the skin, \( dE/hs \) is the electrical potential gradient across the skin, \( D_s \) is the diffusivity of the drug molecule across the skin, \( D_i \) is the diffusivity of the ionic species \( i \) in the skin, \( I_d \) is the current density, \( Z_i \) is the valency of the ionic species, \( k \) is the proportionality constant, \( F \) is the Faraday constant, \( T \) is the absolute temperature and \( R \) is the gas constant (26, 31). Iontophoretic flux can be given based on transport number of an ion in the solute to be transported, by Faraday’s law that links, at steady state, the amount of charge exchanged at the electrode to the mass transfer of ions through the membrane. Faraday’s law relates the electromigration flux (Je) to the applied current:

\[ J_e = \left( I \times t_i \right) / \left( z_i \times F \right) \quad \text{Eq: (5.5)} \]

Where \( I \) is the intensity of current passed through the circuit, \( I_i \) is the current carried by the ion \( i \), \( t_i \) and \( z_i \) are the transport number and valence of ion \( i \) respectively. Transport number is the fraction of the total charge transported by the ion \( i \) and is given by:

\[ t_d = \frac{c \times z \times \mu}{c \times z \times \mu} \quad \text{Eq: (5.6)} \]

where \( c, z \) and \( \mu \) refer to concentration, valency, and mobility of either the drug \( d \) or the ion \( i \) in the system respectively (18). Two main parameters that control the iontophoretic flux are applied current and transport number can be identified from these equations. Current application can be controlled by the power supply, but transport number depends on the physicochemical properties and concentration of the drug and other ions present in the system (14). Transport numbers are between 0 and 1 and measure the efficacy of drug transport. The transport numbers of \( \text{Na}^+ \) and \( \text{Cl}^- \), during saline iontophoresis were \( \approx 0.6 \) and \( 0.4 \), respectively, indicating the skin’s cation permselectivity (32-34). With the assumption that no drug can carry current more efficiently that these two endogenous ions, the values of
the transport numbers of cationic and anionic species never exceed 0.6 and 0.4 respectively. Transport numbers of low molecular weight drugs are approximately 0.05-0.20 and much less for large molecular weight peptides and proteins (35). In summary, parameters that affect the design of an iontophoretic delivery system include the electrode type, current density, pH of the system, type of permeant molecule (positive or negative), size of the permeant molecule and the presence of competing ions (36).

5.2 AIMS AND OBJECTIVES

The aim of this study was to investigate the application of iontophoresis to the delivery of a small peptide-like model compound (5-aminolevulinic acid), a model dipeptide (Ala-Trp or AT), a therapeutic tetrapeptide (Ala-Ala-Pro-Val or AAPV), a cosmetic hexapeptide (Argerline acetate or Acetyl-hexapeptide-3 or EEMQRR) and a therapeutic decapeptide (Triptorelin acetate or EHWSYWLRPG) across human skin in vitro using side-by-side diffusion cells. The objectives of the study plan included investigating the following parameters:

- **pH**: Peptides are often ionised in aqueous solutions, however the fraction of the ionic species would depend on the pH of the solution, hence it’s important to understand the optimum pH of a peptide solution for efficient iontophoretic delivery.

- **Background co-ions and counterions**: Depending on the presence of co-ions and counterions in a peptide solution, competition arises among the primary charge carriers of the applied current. Hence it’s important to understand the extent of co-ions and counterions concentration as opposed to peptide concentration in the donor formulation, to maximise efficient iontophoretic enhancement. Therefore the study plan included investigating the influence of various concentrations of co-ions and counterions in the peptide donor solution.

- **Concentration**: It is important to determine if the skin permeation enhancement due to iontophoresis was concentration dependent as this could aid in maximising iontophoretic delivery of the peptide by simple scale-up, therefore the effect of peptide concentration on the donor solution was evaluated.

- **Direction of current**: Depending on the primary charge carrying ion (cationic or anionic or non-ionic) of a molecule anodal or cathodal iontophoresis could be
applied to determine the efficient iontophoretic enhancement, hence the effect of direction of applied current on iontophoretic delivery of peptides was determined.

Based on the findings from individual iontophoretic permeation studies, a correlation on the influence of increasing molecular weight on iontophoretic transport of molecules was drawn.

5.3 COMPOUNDS INVESTIGATED AND THEIR PROPERTIES

5.3.1 5-AMINOLEVULINIC ACID (5-ALA):

ALA is a small molecular weight (131.1 g/mol), highly polar, hydrophilic (pKa 4.0 and 8.4) molecule with a basic peptide like structure (carboxylic and primary amine group, Figure 4.2), its zwitterionic (net charge zero) at physiological pH, rendering ALA a good model peptide prototype molecule. It is the intermediate in heme biosynthesis, and is also the precursor of protoporphyrin IX (PpIX). PpIX is strongly fluorescent and undergoes photobleaching rapidly producing singlet oxygen molecules. Consequently, ALA is used as an endogenous photosensitizer in photodynamic therapy (PDT) for a range of nonmelanoma skin cancers like basal cell carcinoma, squamous cell carcinoma, actinic keratoses and Bowen’s disease, and in photodiagnosis (37). As described in chapter 4, one of the problems associated with effective ALA-PDT is its homogenous delivery at the target site (its penetration depth) (38). This chapter presents iontophoretic delivery of ALA across human epidermal membrane. In an attempt to understand the mechanism of iontophoretic enhancement the transdermal delivery of D-Glucose (an electroosmotic marker) along with ALA has also been presented.
5.3.2 $\ell$-ALA–$\ell$-TRP (ALANINE–TRYPTOPHAN):

![Chemical Structure of $\ell$-Ala–$\ell$-Trp](image)

Ala-Trp is a model dipeptide molecule (molecular weight 275.9 g/mol), hydrophilic (Alanine pKa 2.34 and 9.69, Tryptophan pKa 2.28 and 9.39, log P 0.61 ± 0.52) and zwitterionic (net charge zero) at physiological pH (Figure 5.2). The native dipeptide has no known therapeutic activity and was chosen as a small molecular weight model compound.

5.3.3 $\ell$-ALA–$\ell$-ALA–$\ell$-PRO–$\ell$-VAL (ALANINE–ALANINE–PROLINE–VALINE):

![Chemical Structure of $\ell$-Ala–$\ell$-Ala–$\ell$-Pro–$\ell$-Val](image)

AAPV is a small molecular weight (355.4 g/mol), hydrophilic (Log P -0.45 ± 0.65) model tetrapeptide. Its molecular structure fits the P–P$_1$ subsites of elastase and
inhibits human neutrophil elastase (HNE) (Figure 5.3). HNE or human leukocyte elastases (HLE) are broad spectrum serine protease derived from the azurophilic granules present in human neutrophils and macrophages. The enzyme acts as a pro-inflammatory agent, by causing degradation of a broad spectrum of matrix components (extracellular) including elastin and has the ability to generate chemotactic factors to cleave antithrombin I/II (39). HNE thus has physiological functions in human host defence mechanism against tissue injury (in lungs, arteries, skin, collagen, fibrin, fibronectin, cartilage proteoglycans, cytokines, platelet IIb/IIIa receptor and cadherins) caused by bacterial infections and extracellular matrix remodelling following tissue injury. HNE is implicated in emphysema, adult respiratory distress syndrome, glomerular nephritis, rheumatoid arthritis, periodontitis, atherosclerosis and in malignant tumours (40-42). With respect to the skin, elevated levels of active HNE are detected in psoriatic lesions, atopic dermatitis, allergic contact dermatitis wherein disassociation of keratinocytes leads to spongiosis (dermatitis), spongiform pustules (psoriasis) and scaling (39). Epithelial tissue is often protected by excessive proteolysis by HNE and other protease by proteinase inhibitors e.g. α1-protease inhibitor, α2-macroglobulin. These are locally produced at the site of tissue injury and by the liver which generates saturating quantities of α1-antitrypsin, a serine protease inhibitor, in the circulation. Elafin and secretory leukocyte protease inhibitor are members of the four-disulphide core family with anti-HNE activity that exhibit up-regulation in response to inflammatory stimuli (43). Under normal conditions the proteolytic activity of HNE is controlled by some endogenous inhibitors (e.g. α1-protease inhibitor, α2-macroglobulin). However, in acute inflammatory disorders an imbalance between HNE and its natural inhibitors leads to abnormal tissue destruction and disease development. HNE inhibitor imbalance can be restored by administering natural inhibitors (α1P) or a low molecular weight peptidic inhibitor. Peptidic HNE inhibitors have a common hydrophobic peptide sequence which partially mimics certain amino acid sequences found in elastin. The sequence Ala-Ala-Pro-Val fits the P-P1 subsites of elastase and hence has shown to competitively inhibit (HNE) with Ki approximately 1.5 × 10^{-3}M (44). The current chapter proposes an effective skin delivery technique (Iontophoresis) for enhanced transdermal application of a peptidic HNE inhibitor (AAPV), which might emerge as a therapeutic product for a range of inflammatory disorders associated with the skin, such as eczema and psoriasis.
5.3.4 AC-GLU-GLU-MET-GLN-ARG-NH₂ (ACETYLHEXAPEPTIDE-3):

![Chemical Structure of Ac-Glu–Glu–Met–Gln–Arg–Arg-NH₂](image)

**Figure 5.4: Chemical Structure of Ac-Glu–Glu–Met–Gln–Arg–Arg-NH₂**

Acetyl hexapeptide-3 is a synthetic peptide (Mol. Wt. 888.6 g/mol and log P -4.5 ± 0.95) that belongs to the group of neurotransmitter-affecting peptides which have been incorporated into cosmeceutical products for anti-ageing properties (Figure 5.4). Physiologically, the formation of wrinkles appears to be partly due to excessive stimulation of the muscle fibres in the face (45-46). One of the strategies to reduce the intensity of wrinkle lines is to down-regulate muscle action either directly or by attenuating the activity of the innervating neurone (45, 47-48).

Neurotransmitter inhibitor peptides inhibit acetylcholine release at the neuromuscular junction and have a curare-like effect. Currently botulinum neurotoxin type A (BTX-A) is the only US-FDA approved subcutaneous and intramuscular injection for facial wrinkles (49). Seven types (A–G) of botulinum toxin (single-chain polypeptides) target peripheral cholinergic neurons where they selectively proteolyse synaptosome associated protein of 25 kDa (SNAP-25), syntaxin 1 and synaptobrevin, the soluble N-ethylmaleimide-sensitive factor at tachment protein receptor (SNARE) proteins.
responsible for transmitter release, to cause neuromuscular paralysis but of different durations. Type A toxin proteolytically degrades the SNAP-25 protein, a type of SNARE protein. The SNAP-25 protein is required for the release of neurotransmitters from the axon endings. Botulinum toxin specifically cleaves these SNAREs, and to prevent neurosecretory vesicles from docking and/or fusing with the nerve synapse plasma membrane and releasing their neurotransmitters (50), (51).

Less invasive topically applied small molecules that mimic the action of BTX are being investigated and developed. Acetyl hexapeptide-3 (Argireline®) is a synthetic peptide that is marketed as a component of eye care products and designed from the N-terminal end of the protein SNAP-25 that interferes with the SNARE ternary complex and inhibits Ca2+-dependent catecholamine release from chromaffin cells (52), (53). In a open label vehicle-controlled trial, 10% acetyl hexapeptide-3 and placebo creams were applied twice daily on 10 women and demonstrated a nearly 30% vs. 10% improvement in periorbital rhytids after 30 days as measured by silicone replica analysis respectively (52). Patent applications have been filed for transdermal delivery of cosmetic peptide involving a combination of neurotransmitter inhibiting peptide mimics with acetylhexapeptide-3, using a micro-device such as a battery operated iontophoresis kits for home application as an anti-wrinkle kit (54-55). In the current chapter we present enhanced iontophoretic delivery of this cosmetic hexapeptide. To optimise iontophoretic delivery of the cosmetic hexapeptide the effects of peptide concentration, solution pH and background co-ions and counterions were investigated.

![Chemical Structure of p-Glu–His–Trp–Ser–Tyr–[\textit{D}-Trp]–Leu–Arg–Pro–Gly-NH₂]

Triptorelin (Molecular weight: 1131.5 g/mol, pKa: 7.2, 9.5, 12 and Log P 0.3 ± 1.41) available as acetate or pamoate is a synthetic gonadotropin-releasing hormone (GnRH) agonist and a long-acting LHRH analogue (Figure 5.5). GnRH and its analogues are used as a fertility treatment and is reported to be effective in assisted reproduction in the treatment of hormone-responsive cancers such as prostate cancer or breast cancer, precocious puberty and estrogen-dependent conditions (such as endometriosis or uterine fibroids) (56). Triptorelin is marketed in the name of decepeptyl® as a slow release injection.

Transdermal iontophoretic delivery has been investigated for LHRH and its analogues (57-61). There has been reports on iontophoretic delivery of triptorelin with various current densities and in combination with chemical penetration enhancers and formulation modification into nanospheres (62), (63), (61). In the
Current chapter we present enhanced iontophoretic delivery of riptorelin acetate across human epidermal membrane at much lesser current density than reported and emphasized on optimizing the peptide formulation for efficient delivery.

The experiments conducted for the various compounds are summarised in Table 5.1.
Table 5.1: Summary of iontophoresis experiments for compounds investigated:

<table>
<thead>
<tr>
<th>Name of the compound</th>
<th>Study Plan</th>
</tr>
</thead>
</table>
| **5-ALA**            | 1. Anodal iontophoresis: 5-ALA (60mM) at pH 5.5  
                        2. Electroosmotic contribution: D-Glucose (56mM) at pH 5.5 |
| **Ala-Trp**          | 1. Anodal iontophoresis: Ala-Trp (3.6mM) at pH 7.4  
                        2. Effect of pH: Ala-Trp (3.6mM) at pH 5.5  
                        3. Effect of background electrolyte: Ala-Trp in Water versus receptor solution (3.6mM) at pH 5.5  
                        4. Effect of peptide concentration: Ala-Trp (36mM) at pH 5.5 |
| **AAPV**             | 1. Anodal iontophoresis: AAPV (28.4mM) at pH 7.4  
                        2. Effect of pH: AAPV (28.4mM) at pH 5.5 and 3.0  
                        3. Effect of background electrolyte: AAPV in Water versus receptor solution (28.4mM) at pH 7.4  
                        4. Effect of peptide concentration: AAPV (2.84mM) at pH 7.4  
                        5. Cathodal iontophoresis: AAPV (28.4mM) at pH 7.4 |
| **Acetyl-hexapeptide-3** | 1. Anodal iontophoresis: Acetyl-hexapeptide-3 (1.13mM) at pH 7.0  
                        2. Effect of pH: Acetyl-hexapeptide-3 (1.13mM) at pH 5.0  
                        3. Effect of background electrolyte: Acetyl-hexapeptide-3 in Water versus receptor solution (1.13mM)  
                        4. Effect of peptide concentration: Acetyl-hexapeptide-3 (11.3mM) at pH 7.0  
                        5. Cathodal iontophoresis: Acetyl-hexapeptide-3 (1.13mM) at pH 7.0 |
| Triptorelin Acetate | 1. Anodal iontophoresis: Triptorelin (0.9mM) at pH 7.0  
|                    | 2. Effect of background electrolyte: Triptorelin in Water versus receptor solution (0.9mM)  
|                    | 3. Effect of peptide concentration: Triptorelin (9mM) at pH 7.0 |
5.4 MATERIALS & METHODS

5.4.1 MATERIALS

All chemicals and reagents listed below were used as supplied: 5-aminolevulinic acid hydrochloride (5-ALA) (A3785 ~98%), L-Alanyl-L-Trptophan (05400 ≥ 99%), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid HEPES (H3375 ≥99.5% titration) and fluorescamine (F9015 ≥98%) were purchased from Sigma-Aldrich (Sydney, NSW, Australia). AAP V (AV-4-NH₂, P 100401-SY121097, ≥96% purity), Acetyl-hexapeptide-3 (Ac-ER-6, 54947, ≥95% purity) and Triptorelin acetate (HG-9, 5773-63-4, ≥98% purity) were purchased from GL Biochem Ltd (Shanghai, China), Phosphate buffer saline was prepared according to United States Pharmacopeia. Sodium chloride (NaCl) from Asia Pacific Speciality Chemicals Ltd (Seven Hills, NSW, Australia), analytical grade potassium chloride (KCl) and sodium hydroxide (NaOH) were purchased from Merck Pty Ltd. (Darmstadt, Germany) and boric acid was purchased from Ajax Finechem (Sydney, NSW Australia). An analytical grade acetonitrile was purchased from J T Baker (Philipsburg, NJ, USA) and trifluoroacetic acid (TFA) from Sigma-Aldrich (Sydney, NSW, Australia). Triethylamine (TEA) from Fluka chemika (Switzerland) and orthophosphoric acid from Ajax Finechem (Australia). Silver (Ag)-wire (99.99%, 1 mm diameter) was obtained from Sigma-Aldrich and purified deionised Milli-Q water from Millipore (North Ryde, NSW Australia).

5.4.2 METHODS

5.4.2.1 INSTRUMENTS AND CONDITIONS

All HPLC analysis was conducted on an Agilent 1100 series equipped with a quaternary pump (G1311A), a autosampler (G1313A), solvent degasser (G1312A) with a photo diode array detector/fluorescence detector (G1321A). Separation of the compounds was achieved on a Phenomenex Jupiter C18 300A column (5 µm, 150mm×4.6 m m) with a guard column (wide pore C 18). Peak integration was undertaken on a personal computer using Chemstation A08.01 software. The assay methods for each of peptide are as follows.
1. The elution of 5-ALA was performed on an isocratic mobile phase system (30:70 acetonitrile (0.1% TFA): water (0.1% TFA) which gave a retention time of 6.8 min with a flow rate of 1 mL/min and fluorescent detection at 395/480nm (excitation/emission). Add sarika paper reference.

2. The elution of Ala-Trp was performed with a gradient mobile phase system with a flow rate of 1 mL/min at 210 nm. Buffer A consisted of 0.045% TFA in water and buffer B was 0.036% TFA in acetonitrile. A linear gradient from 10% to 100% from 0-12 min eluted Ala-Trp at 9.2 min. Post run of 1 min brought the gradient back to 10%.

3. For the elution of the AAPV a gradient mobile phase system with a flow rate of 1 mL/min at 220 nm was used. The solvents consisted of Buffer A as water with 0.1% TFA and buffer B as acetonitrile with 0.1% TFA. A linear gradient from 5% to 20% from 0-6 min eluted AAPV at 4.4 min. Post run from 6-8 min brought back the flow of buffer B from 20% to 5%.

4. For the separation of the acetyl hexapeptide-3 an isocratic mobile phase system consisting of (90:10) water (0.05% TFA) and acetonitrile (0.05% TFA) was used with the flow rate of 1 mL/min. The retention time was 5.8 min at 210 nm.

5. The elution of the triptorelin was performed on an isocratic mobile phase system consisting of (75:25) water (20mM triethylamine and 50mM phosphoric acid) and acetonitrile. The retention time for the decapeptide was 4.3 min at 280 nm.

All standards and samples were prepared at room temperature and the injection volume was 20 µL for ALA and 50 µL for the other compounds. The radiolabelled samples were analysed on a liquid scintillation counter (Packard LSC: Canberra, CT, USA).

5.4.2.2 PREPARATION OF RADIOLABELLED SAMPLES:

For permeation studies with ALA the donor solution consisting of ALA and D-Glucose aqueous solution was spiked with 14C-5-ALA and 3H-D-Glucose (~1 µCi/mL) to achieve 0.5 µCi/cell. A standard with a known quantity of ALA, D-Glucose, 14C-5-ALA and 3H-D-Glucose was mixed with scintillation cocktail (Emulsifier safe, Perkin Elmer, Waltham, MA, USA) and analysed for radioactivity.
in a liquid scintillation counter (Packard LSC: Canberra, CT, USA). Disintegrations per minute were converted to amount in µg/mL by appropriate mathematical transformation.

5.4.3 QUANTITATIVE ANALYSIS

Quantitative analyses of all peptides were performed by reverse phase HPLC and liquid scintillation counting was also employed for ALA. Using the assay conditions described in the previous section, isocratic or gradient elution was used as required to optimise the chromatographic detection by UV absorption except in the case of ALA.

Pre-column derivatisation: 5-ALA belongs to the class of alpha amino ketones with a weak chromophoric carbonyl group; therefore it was unsuitable for quantification by ultraviolet (UV) absorption spectroscopy. Consequently, ALA solution in water was reacted with 0.1% fluorescamine solution and 0.1 M borate buffer for 10 min at room temperature to give a fluorescent compound based on a reported method. Briefly, a 200 µL unknown or reference sample containing ALA in water was derivatised with an equal volume of fluorescamine solution and 600 µL of 0.1 M borate buffer. ALA reacted with fluorescamine for 10 min at room temperature to form a fluorescent derivative that was assayed by HPLC using fluorescent detection. The assay was fully validated prior to analysis of samples from penetration studies. The HPLC assay validation parameters included linearity, accuracy, precision, inter and intraday repeatability, LOD and LOQ.

5.4.3.1 LINEARITY

Stock solutions for all of the peptides were prepared in water and diluted in receptor solution (20mM HEPES buffer + 137mM sodium chloride) respectively to give 6 different working standards. A calibration curve was obtained by plotting the concentration of each peptide versus their respective peak areas. The linearity (quoted as $R^2$) was determined by linear regression analysis, which was calculated by the least squares regression method.

1. For 5-ALA a pre-column derivatisation using fluorescamine in alkaline medium generated a fluorescent product that permitted 5-ALA quantification using
HPLC with fluorescence detection (64). Working standards of concentrations (25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.36 µg/mL) were prepared and 20 µL injections of these standards were analysed by fluorescence detection.

2. For the Ala-Trp working standards of concentrations (25, 12.5, 6.25, 3.125, 1.56, 0.78 µg/mL) were prepared and 50 µL injections of these standards were analysed by UV detection.

3. For AAPV working standards of concentrations (50, 25, 12.5, 6.25, 3.125, 1.56 µg/mL) were prepared and 50 µL injections of these standards were analysed by UV detection.

4. For a cetylhexapeptide-3 working standards of concentrations (50, 25, 12.5, 6.25, 3.125, 1.56 µg/mL) were prepared and 50 µL injections of these standards were analysed by UV detection.

5. For triptorelin acetate working standards of concentrations (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 µg/mL) were prepared and 50 µL injections of these standards were analysed by UV detection.

5.4.3.2 PRECISION

The precision of the assay was determined by injecting two working standards, six times onto the HPLC. The coefficient of variance was quoted as the percentage of standard deviation in peak areas upon the average peak area for these two concentrations.

The concentrations of working standards chosen for precision studies were as follows: 0.36 and 3.12 for ALA, 1.56 and 6.25 µg/mL for Ala-Trp, 3.12 and 12.5 µg/mL for AAPV, 6.25 and 25 µg/mL for a cetyl hexapeptide-3 and triptorelin acetate.

5.4.3.3 INTRA-DAY AND INTER-DAY REPEATABILITY

The intra-day and inter-day repeatability was assessed by injecting two working standards six times at different times in a day and three times on three different days respectively and the coefficient of variance was quoted as the percentage of standard deviation in peak areas upon the average peak area for these two concentrations. Concentrations were as for precision determine.
5.4.3.4 LIMIT OF DETECTION (LOD) AND QUANTIFICATION (LOQ)

The limit of detection was calculated as greater than 3 times the baseline noise level. A blank solution of vehicle was injected 6 times followed by working standards of each compound as used for the calibration curve.

The LOD was calculated by the following formula:

\[
LOD = 3 \times \text{average (n=6) peak height of noise} \div \text{slope (in peak height) of calibration curve}
\]

The LOQ was calculated as 10 times the baseline noise level. The LOQ was calculated by the following formula:

\[
\text{LOQ} = 10 \times \text{average (n=6) peak height of noise} \div \text{slope (in peak height) of calibration curve.}
\]

5.4.4 DONOR SOLUTION FOR PERMEATION STUDIES

All peptides were prepared either in water or in the receptor solution used for permeation studies (20mM HEPES + 137mM NaCl) and adjusted to appropriate pH using 0.1M or 0.01M sodium hydroxide (NaOH) and/or 0.1 or 0.01M hydrochloric acid (HCl).

5.4.5 CONDUCTIVITY TESTING OF DONOR SOLUTION

To measure the electrical conductivity of the donor solution prior to iontophoresis studies, each peptide solution (all 1% w/v) was prepared in purified water as described above. The pH of the solution was recorded, then adjusted to either 5.0-5.5 or 7.0-7.4. The conductance of each peptide donor solution was measured using a conductivity meter (CMD 230, Meter Lab, Radiometer Analytical, Pacific Laboratory Ptv. Ltd, Victoria, Australia) that was calibrated prior to testing with a standard 0.01M potassium hydroxide solution. The electrical conductance was expressed in S/cm (semens/cm).
5.4.6 EPIDERMAL PENETRATION STUDIES

5.4.6.1 PREPARATION OF MEMBRANE

Ethical approval for using human skin was obtained from the Human Research Ethics Committee of Curtin University prior to the study. The procedure followed for preparation of the membrane was as described in Chapter 2. When mounted on the side-by-side diffusion cells a cross-sectional area of 0.95 cm² was available for diffusion.

5.4.6.2 IN VITRO DIFFUSION STUDIES

![Example of side-by-side diffusion cells](image)

Figure 5.6: Examples of side-by-side diffusion cells used in permeation studies.

Permeation studies across human epidermis were performed using pyrex glass side-by-side diffusion cells, enabling permeation across skin sections of cross-sectional area 0.95 cm²; donor and the receptor volume 1.8±0.1 mL (Figure 5.6). The
membrane was placed between the donor and receptor compartment, the cell filled with HEPES (20mM) and NaCl (137mM) solution that was stirred continuously with a magnetic stirrer and allowed to equilibrate for 1 h in a water bath maintained at 35±0.5°C. The membrane integrity was then determined by visual inspection over a bright light and by electrical resistance (kΩ) measurement using a digital portable LCR multimeter (TH2821/A/B, Hangzhou Tonghui Electronic Co Ltd, Jiangsu Province, China). The measurements were taken by immersing the silver-silver chloride electrode attached to the probe lead tips, one each in the donor and receptor compartments (65). Membranes exhibiting an electrical resistance less than 20 kΩ were rejected from the study. The diffusion cells were emptied and the receptor compartments refilled with fresh preheated electrolyte solution (20mM HEPES + 137mM NaCl) at 35±0.5°C.

5.4.7 IONTOPHORESIS EXPERIMENTS

Preparation of Electrodes: Ag/AgCl electrodes are preferred over platinum to minimise pH changes caused by electrolysis of water in the solution surrounding the electrodes. Silver wire (1mm diameter, 6 cm long) was gently sanded with fine emery paper then dipped in 1 M KCl solution for 2.5 hour at 1 mA current. After 1 hour, the electrode that turns black/dark in colour is the AgCl electrode (cathode) whilst the silver electrode is the anode in the iontophoresis experiments.

All iontophoresis experiments were conducted across human epidermal membrane using side-by-side diffusion cells (Figure 5). The receptor compartment of the cells was filled with the electrolyte solution (20mM HEPES + 137mM NaCl) and the donor compartment with either

1. ALA (60 mM aqueous solution), ALA (60 mM) and D-Glucose (56 mM) in aqueous solution spiked with 14C and 3H ALA and D-Glucose respectively (~1 µCi/mL), or
2. Ala-Trp (3.6 and 36 mM) prepared in water and in receptor solution at pH 7.4 and 5.5, or
3. AAPV (2.84 and 28.4 mM) prepared in water and in receptor solution at pH 7.4, 5.5 and 3.0, or
4. acetyl hexapeptide-3 (1.13 and 11.3 mM) prepared in water at pH 7.4, 5.5 and at six different combinations (three of them with 20, 10, 5 mM HEPES + 137,
68.5, 34.2 mM NaCl respectively and three with 137, 68.5 and 34.2 mM NaCl) of components in receptor solution, all at pH 5.5-6.0 or

5. Triptorelin acetate (9 and 0.9 mM) prepared in water and in receptor solution at pH 7.4, 5.5.

Figure 5.7: Custom built iontophoresis unit manufactured by Art Electro NSW, Australia.

For anodal iontophoresis, active cells each received the silver/silver chloride electrodes that were placed in the donor and receptor compartment respectively and connected to a 0.4mA current supply (Figure 5.6, custom built by Art Electro NSW, Australia) and vice versa for cathodal iontophoresis. The iontophoretic current was supplied for 2 h unless otherwise stated. Passive diffusion cells were treated in the same way but without the placement of electrodes. Samples were collected at different time points over 2 and 1 hours respectively. The data was plotted as mean cumulative amount permeating to the receptor compartment versus time. Transdermal flux and permeability coefficient were calculated over the linear portion of the plot.

5.4.8 STATISTICAL ANALYSIS

The data for 5-ALA was analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago IL). The Mann-Whitney U Test (1-tailed) was used to determine significant differences between all parameters. The data for rest of the peptides were analyzed

5.5 RESULTS AND DISCUSSION

All the results from the in vitro permeation studies where a comparison is made between iontophoresis and passive diffusion are expressed as line graphs. The results from comparison studies other than passive diffusion are expressed as bar graph such that it’s easier to visualise the difference.

5.5.1 HPLC ASSAY VALIDATION

Assays for ALA and peptides were successfully developed or adapted from literature methods and validated prior to analysis of samples from the permeation experiments. All assays complied with the acceptable limits as stated by British Pharmacopeia. The validation parameters for individual peptides are stated in Table 5.2 and 5.3 and sample chromatograms are presented in Figures 5.8-5.12.
Figure 5.8: Sample HPLC Chromatogram of ALA, 12.5 µg/mL standard.

Figure 5.9: Sample HPLC Chromatogram of Ala-Trp, 25 µg/mL standard.
Figure 5.10: Sample HPLC Chromatogram of AAPV, 12.5 µg/mL standard.

Figure 5.11: Sample HPLC Chromatogram of Acetylhexapeptide-3 12.5 µg/mL standard.
Figure 5.12: Sample HPLC Chromatogram of Triptorelin acetate, 12.5 µg/mL standard.

Table 5.2: HPLC assay validation parameters for each peptide: Linearity.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Linearity Range (µg/mL)</th>
<th>Correlation coefficient (R²)</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>1.56-100</td>
<td>0.9995</td>
<td>35</td>
<td>117</td>
</tr>
<tr>
<td>Ala-Trp</td>
<td>0.78-25</td>
<td>0.9995</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>AAPV</td>
<td>1.56-50</td>
<td>0.9996</td>
<td>19</td>
<td>62</td>
</tr>
<tr>
<td>Acetylhexapeptide-3</td>
<td>1.56-50</td>
<td>0.9998</td>
<td>60</td>
<td>202</td>
</tr>
<tr>
<td>Triptorelin</td>
<td>0.78-50</td>
<td>0.9993</td>
<td>95</td>
<td>320</td>
</tr>
</tbody>
</table>
Table 5.3: HPLC assay validation parameters for each peptide: accuracy, precision, inter and intraday repeatability coefficient of variance (CV) calculated.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Precision</th>
<th>Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV %</td>
<td>CV %</td>
</tr>
<tr>
<td></td>
<td>0.36 µg/mL</td>
<td>3.12 µg/mL</td>
</tr>
<tr>
<td>ALA</td>
<td>0.38</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>1.56 µg/mL</td>
<td>6.25 µg/mL</td>
</tr>
<tr>
<td>Ala-Trp</td>
<td>0.34</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>3.12 µg/mL</td>
<td>6.25 µg/mL</td>
</tr>
<tr>
<td>AAPV</td>
<td>0.21</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>6.25 µg/mL</td>
<td>25 µg/mL</td>
</tr>
<tr>
<td>Acetylhexapeptide-3</td>
<td>1.61</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>6.25 µg/mL</td>
<td>25 µg/mL</td>
</tr>
<tr>
<td>Triptorelin</td>
<td>2.17</td>
<td>0.69</td>
</tr>
</tbody>
</table>
5.5.2 CONDUCTIVITY MEASUREMENTS FOR PEPTIDE DONOR SOLUTIONS

The electrical conductivity of the donor solutions are presented in Table 5.4 and Figure 5.13. All the peptide donor solutions tested for iontophoresis were found to have higher conductance compared to the vehicle (water) in which they were prepared. Proteins and peptide molecules are charged at physiological pH or can be charged by altering the pH to suit iontophoretic permeability. Conductivity measurements are an indicator of the appropriate pH where the peptide in the donor solution has maximum charge carrying capacity or capacity to conduct current. Various studies have been published on the use of conductivity measurements as predictors of transport efficiency of a charged species and to predict maximum drug flux (for anionic drugs) (66-67).

For 5-ALA, due to stability issue as explain elsewhere the optimum pH for iontophoretic studies was selected as 5.5. At pH 5.5, 5-ALA is expected to be more...
ionised (cationic) as this pH is just below its isoelectric point (pKa: 4.0 and 8.4). 5-ALA exists in a zwitterionic form (net charge zero) at pH 7.4. The Ala-Trp and AAPV were both in their base form. They are custom made peptides and their pKa values were not available in the literature. The conductivity measurements in these cases were used to identify the optimum pH for maximum electrical conductance. At pH 7.4 Ala-Trp was predominantly charged with greater electrical conductance than at pH 5.5. For AAPV the electrical conductivity did not shown any change when formulated at pH 5.5 or 7.4, but did decrease from its initial pH 2.5. Similar results were observed for acetylhexapeptide-3 and triptorelin acetate, the change in pH from 5.5 and 5.0 to 7.4 and 7.0 respectively did not affect their electrical conductivity.

Table 5.4: Electrical conductivity measurements for peptide solutions

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At Initial pH</td>
</tr>
<tr>
<td>5-ALA</td>
<td>1.0013 (2.3)</td>
</tr>
<tr>
<td>Ala-Trp</td>
<td>0.0040 (4.8)</td>
</tr>
<tr>
<td>AAPV</td>
<td>0.2810 (2.5)</td>
</tr>
<tr>
<td>Acetylhexapeptide-3</td>
<td>0.7663 (2.2)</td>
</tr>
<tr>
<td>Triptorelin acetate</td>
<td>0.1017 (4.8)</td>
</tr>
<tr>
<td>Water</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

5.5.3 STATISTICAL ANALYSIS

The readings demonstrated some skewness and heteroskedasticity (standard deviations appeared to become larger as the readings increased) so a logarithmic transformation was applied to the measurements before analysis. This ensured that a linear regression model would be appropriate to describe the readings over time. The primary purpose of fitting a model was to identify the statistical significance of differences in slope or intercept of the regression lines between certain pairs of cultures. A random effects regression model was used to describe the data, with the
cell number labelled as a random-effect (taking into account the correlations between observations from the same cell). The regression included terms for the slope and the intercept as well as a term to test whether the slopes were different for the two cultures being compared (the interaction between time and culture). A p-value < 0.05 was taken to indicate a statistically significant difference (between slopes or intercepts). Statistical analyses were performed using The Mixed Procedure in the SAS software package.

5.5.4 INVITRO PERMEATION STUDIES

5.5.4.1 IONTOPHORETIC TRANSPORT OF 5-ALA ACROSS HUMAN EPIDERMIS

The first set of experiments consisted of anodal iontophoresis of ALA (60 mM) applied as a hydrochloride salt at pH 5.5 in water to avoid any competing ions that would reduce its iontophoretic transport. The choice of pH of the donor solution was based on the isoelectric point of ALA (~6.0). At pH 5.5 ALA is expected to be ionised or predominantly cationic with pKa of 4.0 and 8.4. In addition, ALA exhibits chemical instability in aqueous solutions at physiological pH. During our preliminary experiments with pH 7.4, the solution turned a faint yellow colour, which upon storage at room temperature or at -20°C became a dark orange colour. Similar observations of colour change in aqueous ALA solutions at physiological pH and at 37°C have been documented, and the discolouration attributed to chemical instability of ALA resulting in the formation of condensation products such as dihydropyrazin (68-69). The authors addressed the problem by preparing ALA solutions (0.18 M) in phosphate buffer at pH 5 with storage on ice until use in their fluorescence diagnostic studies of human bladder tumours (69).
Figure 5.14: Cumulative amount of 5-ALA (µg/cm²) permeating human epidermis to the receptor compartment under constant current anodal iontophoresis (●) versus passive diffusion (○), (mean ± SEM; n=5 and 6 cells respectively)

Anodal iontophoretic permeation of 5-ALA across human epidermis was conducted over a period of 2 h and passive diffusion for 4 h. Figure 5.14 shows a plot of the cumulative amount of 5-ALA that permeated the human epidermis into the receptor solution over time for iontophoresis and passive diffusion. There was a significant enhancement in the rate of 5-ALA iontophoretic transport through human epidermis when compared to passive diffusion (p=0.004). The estimated transdermal flux calculated over the linear portion (0-2h) of the curve was 75± 6 nmol/(cm².h) or 12.5±1.0 µg/(cm².h) for iontophoresis and 5±2 nmol/(cm².h) or 0.8±0.4 µg/(cm².h) for passive delivery. This represents a 15-fold enhancement in the mean flux over 0-2h. The corresponding permeability coefficient was (130.0±11) ×10⁻⁵ cm/h and that of passive delivery (8.5±4.8) ×10⁻⁵ cm/h (Table 5.5). Applied current (0.38mA/cm²) normalised transdermal flux calculated over 0-2h was 34.8±2.9 µg/(mA.h) for iontophoretic 5-ALA.

The additional anodal iontophoresis experiments were carried out with the addition of D-glucose (56 mM) to 5-ALA (60 mM). D-Glucose is, like 5-ALA, highly water soluble, polar and of similar molecular size (molecular weight 180.16). It was
included in this study as a neutral marker to determine the influence of electroosmosis on the iontophoretic delivery of 5-ALA. Figure 5.15 shows the cumulative amount of 5-ALA and D-glucose, present together, that permeated the human epidermis into the receptor solution over time. The estimated transdermal flux calculated over the linear portion (0-2h) of the curve was 50±5 nmol/(cm².h) or 9.3±0.8 µg/(cm².h) for 5-ALA and 24±4 nmol/(cm².h) or 4.8±0.7 µg/(cm².h) for D-Glucose. The applied current normalised transdermal flux for 5-ALA was 21±1.9 µg/(mA.h) in the presence of the electroosmotic marker and that of D-glucose alone was 11±1.7 µg/(mA.h). The corresponding permeability coefficient for iontophoretic 5-ALA was $(52.0±11) \times 10^{-5}$ cm/h and that of D-Glucose $(27±4.1) \times 10^{-5}$ cm/h (Table 5.5).

![Figure 5.15: Cumulative amount of 5-ALA (■) and D-Glucose (□) (µg/cm²) permeating human epidermis to the receptor compartment under constant current anodal iontophoresis, (mean ± SEM; n=5 and 6 cells respectively)](image)

Previous reports have been published on the iontophoretic delivery of 5-ALA over a range of experimental conditions (70-74) Lopez et al (2001) reported the effect of pH (7.4, 5.5 and 4.0) on the iontophoretic delivery of 5-ALA. They reported that the pH at which 5-ALA was iontophoretically administered had little effect on permeation despite the fact that at physiological pH, 5-ALA exists as a neutral zwitterion and therefore the principal iontophoretic transport mechanism is electroosmosis. (75) At lower pH (5.5 and 4.0) the fraction of 5-ALA existing in cationic form will increase,
thus offering advantages with regard to the permselectivity of the skin to cations, and therefore increased electrorepulsive transport. However, the increase in electrorepulsive transport of 5-ALA at lower pH (pH 4.0) was offset by the decrease in electroosmotic flow as the skin lost its negative charge. Therefore lowering the pH to make the drug more cationic did not substantially increase 5-ALA delivery. In the same study, the authors report that at pH 5.5 (as used in the present study) the iontophoretic flux was approximately 30 nmol/(cm².h) from a 15 mM 5-ALA solution, a 6-fold increase compared to passive application of the same donor composition with a 6h application of 0.5mA/cm² constant current (73). In a separate study, anodal flux of 50.6±8.8 nmol/(cm².h) was achieved from a 15 mM 5-ALA (pH 7.4) solution when 0.5 mA/cm² constant current was applied for 6h (75). It should be noted that all these experiments were carried out using dermatomed pig ear skin (~700µm). Although the iontophoretic flux of 75±6 nmol/(cm².h) achieved in our study was comparable to the aforementioned studies, our current density (0.38 mA/cm²) and application time (2h) were different to the published data, we were able to show a 15-fold enhancement of 5-ALA flux over 0-2h (Table 5.5).

Table 5.5: Permeation parameters of 5-Aminolevulenic acid (5-ALA) and D-Glucose by Iontophoresis and passive diffusion, (Mean ±SD, SEM)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5-Aminolevulenic acid</th>
<th>D-Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive (0-2h)</td>
<td>Iontophoresis (0-2h)</td>
</tr>
<tr>
<td>Mean Cumulative amount µg/cm²</td>
<td>1.6±2.1,0.9</td>
<td>25.4±5.7,2.5</td>
</tr>
<tr>
<td>Flux µg/(cm².h)</td>
<td>0.8±1.1,0.4</td>
<td>12.5±2.3,1.0</td>
</tr>
<tr>
<td>Flux/I µg/(mA.h)</td>
<td>34.8±6.4,2.9</td>
<td>21.7±3.8,1.9</td>
</tr>
<tr>
<td>Permeability Coefficient cm/h</td>
<td>(8.5±0.1,4.8)×10⁻⁵</td>
<td>(130±25,11)×10⁻⁵</td>
</tr>
</tbody>
</table>
The results from D-Glucose permeability study concur with Lopez et al (2001), who demonstrated that the electrotransport of mannitol decreased substantially from pH 7.4 to pH 4.0 solutions due to the neutral charge of the skin, and therefore no electroosmosis. At pH 5.5 as used in our study, the skin is not completely neutralised and still has its permselectivity towards cations thereby favouring convective flow across the membrane. Therefore we conclude from our study incorporating D-Glucose, that the mechanism of iontophoretic 5-ALA electrotransport has roughly equal contribution from electrorepulsion and electroosmosis, under these conditions. A summary of all permeability parameters for 5-ALA and D-Glucose is presented in Table 5.5. The results these experiments demonstrated that 0.38mA/cm² iontophoresis for 0-2h was able to enhance the epidermal permeation of 5-ALA and D-Glucose.
5.5.4.2 IONTOPHORETIC TRANSPORT OF ALA-TRP ACROSS HUMAN EPIDERMIS

Four series of iontophoretic studies were carried out, to determine the effect of peptide concentration, pH and the presence of co-ions and counterions on the iontophoretic delivery of Ala-Trp.

The first set of anodal (0.38 mA/cm²) iontophoretic permeation studies compared the permeation of Ala-Trp, 3.6 mM in purified water at pH 7.4 across human epidermis in vitro for 1h versus passive diffusion of the dipeptide. The plot of cumulative amount of Ala-Trp that permeated the human epidermis to the receptor solution over time (Figure 5.16) indicates a significant enhancement in the amount and rate of Ala-Trp iontophoretic transport compared to passive diffusion (P=0.01). The estimated Ala-Trp iontophoretic transdermal flux and permeability coefficient calculated over 1h was 1.4±0.6 nmol/(cm².h) or 0.4±0.1 µg/(cm².h) and (0.4±0.1)×10⁻³ cm/h (mean ± SEM) respectively as compared to no passive permeation. The applied current (0.38mA/cm²) normalized transdermal flux for Ala-Trp calculated over 0-1h was 1.0±0.4 µg/(mA.h). The result from this study demonstrates that 0.38mA/cm²...
Iontophoresis applied over 1h enhanced the epidermal permeation of Ala-Trp in comparison to no passive permeation.

Figure 5.17: Cumulative amount of Ala-Trp at pH 7.4 (■) and Ala-Trp at pH 5.5 (■) permeating human epidermis to the receptor compartment under the constant current anodal iontophoresis (mean ± SEM; n=5 and 5 cells respectively)

The second set of iontophoretic permeation studies were conducted to establish the effect of pH and that determine whether the dipeptide delivery would increase if it was more cationic as has been previously demonstrated (76-77). A plot of the cumulative amount of Ala-Trp 3.6 mM in purified water at pH 5.5 that permeated the human epidermis to the receptor solution over time (Figure 5.17). A significant enhancement in the amount and rate of Ala-Trp iontophoretic transport compared to passive diffusion (P < 0.0001). The estimated Ala-Trp iontophoretic transdermal flux, applied current (0.38mA/cm²) normalised transdermal flux and permeability coefficient calculated over 1h were 36.1±11.2 µg/(mA.h), 54.2±0.1 nmol/(cm².h) or 13.0±2.8 µg/(cm².h) and (13.0±3.0)×10⁻³ cm/h (mean±SEM) respectively as compared to the passive permeation parameters 0.1±0.1 µg/(cm².h) and (3.0±2.7)×10⁻⁵ cm/h for flux and permeability respectively. The results from this study clearly indicated that lowering the pH of the peptide solution significantly (p < 0.0001) increases the anodal iontophoretic epidermal permeation of Ala-Trp.
In the third set of permeation studies the iontophoretic delivery of Ala-Trp, 3.6 mM prepared in receptor solution (20mM HEPES + 137mM NaCl) at pH 5.5 to determine the effect of the presence of co-ions and counterions. The comparison plot of cumulative amount of Ala-Trp in the presence of co-ions and counterions that permeated the human epidermis to the receptor solution over time is presented in Figure 5.18. The plot indicates a significant reduction (p<0.0001) in the rate of Ala-Trp iontophoretic transport compared to permeation of Ala-Trp in the absence of ions (Ala-Trp in water). The estimated Ala-Trp iontophoretic transdermal flux and permeability coefficient calculated over 1 h were 3.08±0.23 nmol/(cm².h) or 0.85±0.06 µg/(cm².h) and (0.05±0.01)×10⁻³ cm/h, (mean ±SEM) respectively. The applied current (0.38mA/cm²) normalised transdermal flux for Ala-Trp in the presence of co-ions and counterions was 2.3±0.1 µg/(mA.h) as compared to 36.1±11.2 µg/(mA.h) in the absence of co-ions and counterions. The results from this study indicate a decrease in Ala-Trp epidermal permeation in the presence of co-ions and counterions.

Figure 5.18: Cumulative amount of Ala-Trp in water (■) and Ala-Trp in receptor solution (■) (nmol/cm²) permeating human epidermis to the receptor compartment under the constant current anodal iontophoresis, (mean ± SEM; n=5 and 6 cells respectively)
The fourth set of permeation studies was to determine the concentration effect on the iontophoresis delivery of Ala-Trp. The comparison plot of cumulative amount of Ala-Trp (36mM) that permeated the human epidermis to the receptor solution over time when compared to Ala-Trp permeation from a lower concentration (3.6mM) is presented in Figure 5.19. The plot indicates a significant enhancement (p<0.0001) in the amount and rate of Ala-Trp iontophoretic transport. The estimated Ala-Trp iontophoretic transdermal flux, applied current normalised transdermal flux and permeability coefficient calculated over 1h was 188.4±41.1 nmol/(cm².h) or 51.9±µg/(cm².h), 144±31.4µg/(mA.h) and (5.5±1.2)×10⁻³ cm/h (mean ± sem) respectively. The result from this study indicates that iontophoretic epidermal permeation of Ala-Trp is concentration dependent.

All the experiments with Ala-Trp clearly demonstrated that iontophoresis enhances delivery of this dipeptide: the permeation parameters for Ala-Trp are presented in Table 5.6.
Table 5.6: Permeation parameters of Ala-Trp iontophoresis and passive diffusion, (mean ± SD, SEM)

<table>
<thead>
<tr>
<th>Treatment (0-1h) &amp; pH</th>
<th>Mean Cumulative Permeation µg/cm²</th>
<th>Flux µg/(cm².h)</th>
<th>Flux/I µg/(mA.h)</th>
<th>Permeability coefficient cm/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pass, 7.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ionto, 7.4</td>
<td>0.4±0.4, 0.1</td>
<td>0.3±0.4, 0.1</td>
<td>1.0±1.1</td>
<td>(0.4±0.4,0.1)×10⁻³</td>
</tr>
<tr>
<td>Pass, 5.5</td>
<td>0.02±0.04, 0.01</td>
<td>0.1±0.03, 0.01</td>
<td>0.02</td>
<td>(0.01±0.02,0.02)×10⁻³</td>
</tr>
<tr>
<td>Ionto, 5.5</td>
<td>16.0±8.8, 3.6</td>
<td>13.0±7.0, 2.8</td>
<td>36.1±19.5, 11.2</td>
<td>(13±7.4,3.0)×10⁻³</td>
</tr>
<tr>
<td>Ionto with co-ions &amp; counterions, 5.5</td>
<td>0.9±0.1, 0.06</td>
<td>0.8±0.1, 0.06</td>
<td>2.3±0.3, 0.1</td>
<td>(0.9±1.3,0.06)×10⁻³</td>
</tr>
<tr>
<td>Ionto with 36mM, 5.5</td>
<td>57.6±29.8, 12.1</td>
<td>51.9±27.8, 11.3</td>
<td>144±77, 31.4</td>
<td>(5.5±2.9,1.2)×10⁻³</td>
</tr>
</tbody>
</table>

Given that the dipeptide was zwitterionic at physiological pH, the principal mechanism of iontophoretic enhancement was expected to be electroosmosis. On the basis of the conductivity measurements, it was anticipated that the iontophoretic flux at pH 7.4 should have been higher as this pH showed highest electrical conductance. However, electrical conductance is a measure of the total charge carrying capacity of the ion and does not give the predominant charge of the molecule, therefore in this case conductivity measurements could not be used to predict the iontophoretic flux.

Iontophoretic flux of a ion depends on the predominant charge of the molecule and the corresponding electrode used to deliver the ion across the membrane. When the pH was reduced to 5.5, where the dipeptide is expected to be more cationic, anodal permeation dramatically increased suggesting the shift in the principle mechanism of
iontophoretic enhancement to electromigration (78). There is very little work reported on skin delivery of Ala-Trp. One study reported enhanced epidermal permeation of Ala-Trp across human epidermis by a electromagnetic physical enhancement technique. The flux and permeability coefficient of the dipeptide were 19.4 µg/(cm².h) and $19 \times 10^{-3}$ cm/h calculated over the linear portion of the cumulative amount versus time curve (0.33-2 h) from an applied concentration of 3.6 mM (79). The transdermal flux achieved with Ala-Trp, 3.6 mM at pH 5.5 with iontophoresis was comparable to that of the aforementioned study. However, the study design and protocols are different from one another.

The presence of sodium chloride, 137 mM in the donor solution decreased the Ala-Trp delivery by 20-fold. Similar observations were reported by Alba et al with the delivery of a positively charged dipeptide (D-[Arg]-Kyorophin) (80). The final series of experiments to determine the effect of Ala-Trp concentration indicated a 4-fold increase in iontophoretic permeation when the dipeptide concentration increased 10-fold. Alba et al reported similar observations when the influence of concentration (5-40 mM) on the iontophoretic delivery of D-[Arg]-Kyorophin was investigated. A linear relationship between the concentration and iontophoretic delivery, both electromigration and electroosmotic, suggested that peptide-peptide and peptide-skin interactions were of insufficient levels to impact electrotransport of the dipeptide.

There have been a number of iontophoresis studies using small model dipeptide molecules mainly focussed towards understanding the mechanistic aspects of the delivery technique. Alba et al reported the individual contribution of the electrotransport of a small peptide molecule D-Arg-Kyorophin (80). In another work Alba et al also investigated the effect of charge and molecular weight on the iontophoretic transdermal delivery of series of dipeptides (lysine containing and tyrosine containing blocked or unblocked partially). They demonstrated that the transdermal flux of these dipeptides and amino acids was determined by the charge/molecular weight ratio and that the ionic mobility of the charged species in a peptide would decrease with increasing molecular weight (6). Altenbach et al demonstrated the interaction of iontophoretic current on the epidermal permeation of a model dipeptide (Tyrosine-Pheylalanine). The dipeptide investigated is reported to be metabolised in the epidermal membrane into corresponding amino acids. The iontophoretic permeation of the electroosmotic marker (BAd-α-Glucose) was hen
applied alone was greatly reduced in the presence of the dipeptide and/or aminoacids suggesting possible permeability interactions in iontophoretic delivery between permeants or the products of their cutaneous metabolism (81).

### 5.5.4.3 IONTOPHORETIC TRANSPORT OF ALA-ALA-PRO-VAL ACROSS HUMAN EPIDERMIS

A similar set of four permeation studies was conducted with AAPV to determine the influence of pH, peptide concentration in the presence of co-ions and counterions and the direction of current.

![Cumulative amount (µg/cm²) of AAPV (Tetrapeptide) from pH solutions](image)

**Figure 5.20**: Cumulative amount (µg/cm²) of AAPV (Tetrapeptide) from pH 7.4 (●), pH 5.5 (▲) and pH 3.0 (■) solution permeating human epidermis to the receptor compartment under constant current anodal iontophoresis, (mean ± SEM; n=9, 3 and 3 cells respectively)

The first series of experiments consisted of a nodal iontophoresis of AAPV (28.4 mM) prepared in purified water with the pH of the solution was adjusted to 3.0, 5.5 or 7.4. All permeation studies were conducted for 0-2h with a constant current of 0.38mA/cm². The plot of cumulative amount of iontophoretic delivery of tetrapeptide from the three different pH solutions across the human epidermal membrane is presented in Figure 5.20. The transdermal flux, applied current normalised transdermal flux and corresponding permeability coefficient at pH 3.0 was 3.7±1.5
µg/ (cm².h), 1 0.4±4.2 µg/(mA.h) and (4.0±1.4) × 10⁻⁴ cm/h; at pH was 5.5 176.9±15.9 µg/ (cm².h), 490.1±44.1 µg/(mA.h) and (1.8±0.1) × 10⁻² cm/h; and at pH was 7.4 175.5±7.1 µg/ (cm².h), 484.5±19.7 µg/(mA.h) and (1.8±0.07) × 10⁻² cm/h respectively. No passive permeation was observed from any of the three donor pH solutions. The result from this study demonstrates that 0.38mA/cm² anodal iontophoresis significantly increased the epidermal permeation of AAPV. The pH of the donor solution plays an important role in effective iontophoretic delivery of this tetrapeptide with significant difference in iontophoretic permeation with pH change from 3.0 to 5.5 (p<0.0001) and 3.0 to 7.4 (p<0.0001).

Figure 5.21: Cumulative amount (µg/cm²) of AAPV (28.4 mM) in water ( ), AAPV (28.4 mM) with co-ions and counterions ( ) and AAPV (2.84 mM) with co-ions and counterions ( ) solution permeating human epidermis to the receptor compartment under the constant current anodal iontophoresis, (mean ± SEM; n=9, 3 and 4 cells respectively)

The second set of studies was conducted to determine the influence of co-ions and counterions on the iontophoretic delivery of the tetrapeptide. In addition, AAPV was presented as two concentrations (2.84 and 28.4 mM). The reason behind choosing two different concentrations was to determine if the influence of counterions and co-ions depended on the peptide concentration. Therefore whilst keeping the
concentration of counterions and co-ions constant, two different concentrations of the
tetrapeptide were investigated. The plot of cumulative amount of AAPV 28.4 mM in
water, 28.4 mM and 2.8 mM in presence of counterions and co-ions at pH 7.4 that
permeated human epidermal membrane with iontophoresis versus time is presented
in Figure 5.21. Flux and permeability coefficient reduced in the presence of co-ions
and counterions as compared to the above mentioned AAPV permeation in the
absence of co-ions and counterions. The flux and permeability coefficient of AAPV
at different concentrations (28.4 mM and 2.84 mM) were 98.7±11.9 µg/(cm².h),
5.06±0.5 µg/(cm².h) and (1.0±0.1) × 10⁻² cm/h, (5.3±0.4) × 10⁻³ cm/h respectively.
The applied current normalised flux was 273.1±33.0 µg/(mA.h) for 28.4 mM AAPV
and 14.0±1.1 µg/(mA.h) for 2.84 mM in the presence of co-ions and counterions.
The results from this study demonstrate that presence of counterions and co-ions in
the donor solution significantly (p<0.0001) reduces the nodal iontophoretic
permeation of AAPV.

Figure 5.22: Cumulative amount of AAPV (µg/cm²) permeating human
epidermis to the receptor compartment under the constant current cathodal
iontophoresis (■) versus anodal iontophoresis (□), (mean ± SEM; n=3 and 4
cells respectively)

The next set of experiments was designed to investigate the influence of the direction
of current in order to understand the predominant charge carrier in AAPV
iontophoretic delivery. For cathodal iontophoresis, AAPV 28.4mM in water at pH 7.4 was subjected to the same constant current (0.38mA/cm²) for 2h as used for anodal iontophoresis. The cumulative amount of AAPV that permeated the epidermal membrane with cathodal iontophoresis was compared with a nodal iontophoresis (Figure 5.22). The flux and corresponding permeability coefficients are 163.9±10.8 µg/ (cm².h) and (1.7±0.1) × 10⁻² cm/h for cathodal iontophoresis as compared to previous reported 176.9±15.9 µg/ (cm².h) and (1.8±0.1) × 10⁻² for AAPV (28.4mM) prepared in water at pH 7.4. Similarly, the applied current normalised flux for cathodal iontophoresis of AAPV was 454±29.9 µg/(mA.h) as compared to 484.5±19.7 µg/(mA.h) for a nodal iontophoresis. The results from this study demonstrate that iontophoretic epidermal permeation of AAPV was found to be similar (p=0.18) from both the directions (anodal and cathodal) of current.

All the experiments with AAPV clearly demonstrated that iontophoresis enhances delivery of this tetrapeptide; the respective permeation parameters for AAPV are presented in Table 5.7.
Table 5.7: Permeation parameters of AAPV iontophoresis and passive diffusion, (mean ±SD, SEM)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean Cumulative Permeation µg/cm²</th>
<th>Flux µg/(cm².h)</th>
<th>Flux/I µg/(mA.h)</th>
<th>Permeability coefficient cm/h</th>
</tr>
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<tbody>
<tr>
<td>Passive</td>
<td>No permeation data</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Iontophoresis</strong></td>
<td></td>
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<tr>
<td>pH 7.4</td>
<td>350.4±45.9, 175.5±21.4, 484.5±59.3, (1.8±0.20, 0.07)×10⁻²</td>
<td>15.3, 7.1, 19.7</td>
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<tr>
<td>pH 5.5</td>
<td>341.3±50.3, 176.9±27.6, 490.1±76.5, (1.8±0.20, 0.1)×10⁻²</td>
<td>29.1, 15.9, 44.1</td>
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<tr>
<td>pH 3.0</td>
<td>7.2±7.3, 2.9, 3.7±3.7, 1.5, 10.4±10.3, (0.04±0.03, 0.01)×10⁻²</td>
<td>4.2</td>
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</tr>
<tr>
<td>28.4 mM AAPV with co-ions and counterions</td>
<td>226.3±74.6, 98.7±29.2, 273.1±81.0, (1.0±0.3, 0.1)×10⁻²</td>
<td>30.4, 11.9, 33.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.84 mM AAPV with co-ions and counterions</td>
<td>9.8±1.7, 0.8, 5.06±1.0, 14.0±2.8, (0.5±0.1, 0.04)×10⁻²</td>
<td>0.5, 1.1</td>
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<tr>
<td>28.4 mM AAPV with cathodal Ionto</td>
<td>320.2±36.6, 163.9±18.7, 454±51.8, (1.7±0.10, 0.1)×10⁻²</td>
<td>21.1, 10.8, 29.9</td>
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</table>

In all the conditions tested no passive permeation of the tetrapeptide was detected. The results clearly demonstrated that constant current iontophoresis of 0.38mA/cm² for 2 h enhanced the transdermal delivery of the tetrapeptide at an optimum physiological pH range (7.4-5.5). The presence of co-ions and counterions reduced the rate of AAPV permeation by 56% or 2-fold when compared to the flux of AAPV applied as the same concentration in water (Table 5.7). This reduction was concentration dependent as well. When the same scenario was attempted at lower molar concentration of the tetrapeptide the reduction was more prominent. This was due to the difference in molar concentrations of the tetrapeptide, 2.8 mM as against
137 mM of NaCl. Since the tetrapeptide is zwitterionic at physiological pH, cathodal iontophoresis was going to reveal the predominant charge carrier and therefore determine the direction of ionic movement. The flux and the cumulative amount of the tetrapeptide that permeated by cathodal iontophoresis was similar to a nodal iontophoresis. This suggested approximately equal contributions of cations and anions as charge carriers or that the predominant pathway of iontophoretic delivery was electroosmosis. Further investigation using an electroosmotic marker would reveal the extent of electroosmotic contribution.

Other studies published in the literature include one by Toth et al who demonstrated N-terminal conjugation of this tetrapeptide with one, two and three lipophilic moieties to enhance the inhibitory potency of the tetrapeptide. The authors reported 10,000 fold increase in inhibitory potency against human HNE enzyme when administered intradermally (82). In another study chemical penetration enhancer such as aqueous ethanol 75% was used to enhance the passive delivery of the tetrapeptide. Authors reported no penetration across full thickness skin at 24h, however upon removal of stratum corneum layers by tape-stripping a flux of 73.39 µg/(cm².h) was achieved from a saturated solution of the tetrapeptide (83). Further chemical conjugations with short chain lipo-aminoacid (LAA) have been attempted to increase the lipophilicity of the tetrapeptide. The study involved in vitro skin diffusion across human epidermal membrane of the tetrapeptide conjugated with a racemic mixture (both D and L isomer) of a short chain LAA. The authors reported enhanced skin permeation with the D-isomer being better than L-isomer of the LAA. The cumulative amounts reported are 80 µg and 30 µg respectively from D- and L-isomer as compared to no passive permeation over 8 hours (84).

All the results from iontophoretic epidermal permeation of AAPV presented in this section of the chapter are shown to be higher in comparison to the aforementioned skin penetration enhancement techniques. However, direct comparison is problematic due to the difference in study design and protocols. Little information is known in terms of the charge, transport number or the ionic mobility of the tetrapeptide, therefore further investigations into these parameters would be a worthwhile topic of future development in the studies of this tetrapeptide. Overall we can conclude that further investigations of iontophoretic delivery of this peptidic
HNE inhibitor is warranted and could be a useful therapeutic option in the treatment of psoriatic lesions and other inflammatory disorders.

5.5.4.4 IONTOPHORETIC TRANSPORT OF ACETYL HEXAPEPTIDE-3 ACROSS HUMAN EPIDERMIS

The iontophoretic permeation studies for acetyl hexapeptide-3 were carried out in four series to determine the influence of pH, peptide concentration, direction of current and presence of co-ions and counterions.

Figure 5.23: Cumulative amount of acetylhexapeptide-3 (µg/cm²) permeating human epidermis to the receptor compartment under constant current Anodal iontophoresis (●) versus passive diffusion (○), (mean ± SEM; n=8 and 3 cells respectively)

In the first set of experiments consisted of anodal iontophoresis of acetyl hexapeptide-3, 1.13 mM prepared in purified water at pH 7.0. Figure 5.23 shows a plot of the cumulative amount of hexapeptide that permeated across human epidermis with anodal iontophoresis and by passive diffusion versus time. The iontophoretic flux calculated over 0-2h was 15.7±2.3 µg/(cm².h) and the applied current normalized flux was 43.7±9.2 µg/(mA.h). The corresponding permeability coefficient was (1.7±0.2) ×10⁻² cm/h. The transdermal flux and permeability
The coefficient for passive permeation of the hexapeptide was 2.3±0.5 µg/ (cm².h) and (2.5±0.5) ×10⁻³ cm/h respectively. The results from this study demonstrate that constant current anodal iontophoresis (0.38mA/cm²) applied over 0-2h significantly (p<0.001) increased the epidermal permeation of the hexapeptide.

Figure 5.24: Cumulative amount (µg/cm²) of Acetylhexapeptide-3 (1.13 mM) pH 7.0 (■) and pH 5.0 (□) solution permeating human epidermis to the receptor compartment under constant current anodal iontophoresis, (mean ± SEM; n=8 and 7 cells respectively)

The second set of experiments was conducted to determine the influence of pH of the donor peptide solution. A comparative plot of mean cumulative amount of hexapeptide, 1.13 mM in purified water at pH 5.0 and pH 7.0 that permeated with anodal iontophoresis is presented in Figure 5.24. The iontophoretic flux for hexapeptide at pH 7.0 as mentioned above, calculated over 0-2h was 15.7±2.3 µg/ (cm².h) and that at pH 5.0 were 2.6±0.3 µg/ (cm².h). The lowering of pH reduced the transdermal flux of the hexapeptide by 5-fold. Similarly the applied current normalised transdermal flux for hexapeptide at pH 5.0 was 12.7±3.7 µg/(mA.h) which is 3 times lower than pH 7.0. The corresponding iontophoretic permeability coefficient at pH 7.0 was (1.7±0.2) ×10⁻² cm/h and at pH 5.0 was (4.8±1.4)×10⁻³.
cm/h respectively. The transdermal flux for passive permeation of hexapeptide at pH 5.0 was 2.57±0.8 µg/(cm²·h) and the corresponding permeability coefficient was (2.7±0.9)×10⁻³ cm/h respectively. The results from this study indicated that lowering of pH in an attempt to make the hexapeptide more cationic did not increase the anodal iontophoresis of the hexapeptide.

![Figure 5.25: Cumulative amount (µg/cm²) of Acetylhexapeptide-3 (EEMQRR) 1.13 mM in water ( ), in Donor-1 (20mM HEPES + 137mM NaCl (■)), in Donor-2 (10mM HEPES + 68.5mM NaCl (□)), in Donor-3 (5mM HEPES + 34.2mM NaCl (■)), in Donor-4 (137mM NaCl (■)), in Donor-5 (68.5mM NaCl (□)) and Donor-6 (34.2mM NaCl (□)) solutions permeating human epidermis to the receptor compartment under the constant current anodal iontophoresis, (mean ± SEM; n=8,5,5,5,3,and 3 cells respectively)](image)

The third set of permeation studies was conducted to determine the influence of co-ions and counterions. Six different combinations of peptide (donor) solution were applied as described in materials and methods section and noted in Figure 5.25 above. The duration of anodal iontophoresis was chosen to be 0-1h based on the findings from initial iontophoresis studies with the hexapeptide. The plot of cumulative amount of the hexapeptide permeating the human epidermis from the six different donor solutions with anodal iontophoresis is presented in Figure 5.25. The transdermal flux of hexapeptide in the absence of co-ions and counterions was

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21.5±5.0 µg/ (cm².h) as compared to 8.88±2.2 µg/ (cm².h) in the presence of 20 mM HEPES buffer a nd 137 mM NaCl a nd 0.4±0.2 µg/ (c m².h) in t he pr esence o f 1 37 mM NaCl alone. The applied current normalised transdermal flux in the presence of 20 m M HEPES b uffer was 24. 6±6.3 µg/(mA.h) a nd 1. 3±0.3 µg/(mA.h) in t he presence of 137 mM NaCl alone, which is 2-fold and 33-fold lower than hexapeptide in the absence of co-ions and counterions respectively. The corresponding permeability coefficient were (2.3±0.5) × 10⁻² cm/h in the absence of co-ions and counterions and (9.3±2.4) × 10⁻³ cm/h with 20 mM HEPES buffer and 137 mM NaCl and (5.0±1.3) × 10⁻⁴ cm/h with 137 mM NaCl alone. A comparison among the different combinations of co-ions and counterions concentrations shows that the maximum cumulative amount of hexapeptide permeation (22.5±6.0 µg/ cm²) and transdermal flux (12.8±1.4 µg/ (cm².h)) was achieved with the lowest amount of HEPES and NaCl combination, which is 5 mM and 34.2 mM respectively (Table 5.7). The results clearly indicated that the presence of counterions and co-ions reduced the anodal iontophoretic flux of the hexapeptide and that the effect was concentration (counterions and co-ions) dependent.
Figure 5.26: Cumulative amount (µg/cm²) of Acetylhexapeptide-3 (1.13 mM) solution permeating human epidermis to the receptor compartment under constant current Anodal iontophoresis (■) and Cathodal iontophoresis (▲), (mean ± SEM; n=7 and 5 cells respectively)

The fourth set of permeation studies was carried out to determine the influence of the direction of current cathodal versus anodal iontophoresis. The comparison plot of cumulative amount of hexapeptide, 1.13mM prepared in purified water at pH 7.0 that permeated the human epidermis with the same constant current 0.38 mA/cm² cathodal iontophoresis versus anodal iontophoresis is presented in Figure 5.26. The flux, applied current normalised flux and permeability coefficient of the hexapeptide from cathodal iontophoresis calculated over 0-2h was 1.1±0.3 µg/(cm².h), 3.0±2.2, 0.8 and (1.2±0.3) ×10⁻³ cm/h respectively. The results from this study indicated that cathodal iontophoresis did not favour transdermal permeation of the hexapeptide and the transdermal flux was close to passive diffusion of the hexapeptide, suggesting the hexapeptide could be cationic.
Figure 5.27: Cumulative amount (µg/cm²) of acetyl hexapeptide-3 from 1.13 mM (■) and 11.3 mM (▲) solution permeating human epidermis to the receptor compartment under constant current anodal iontophoresis, (mean ± SEM; n=6 and 5 cells respectively)

The fifth set of permeation studies was carried out to determine the effect of peptide concentration on the anodal iontophoretic delivery of the hexapeptide. The plot of cumulative amount of hexapeptide, 11.3 mM and that of 1.13 mM at pH 7.0 permeating the human epidermis with anodal iontophoresis is presented in Figure 5.27. The flux and permeability coefficient of hexapeptide, 11.3 mM calculated over 0-2h for anodal iontophoresis were 41.0±6.1 µg/(cm².h) and (4.3±0.6) ×10⁻³ cm/h respectively. The flux and permeability coefficient of the hexapeptide, 11.3 mM by passive diffusion, was 4.4±2.5 µg/(cm².h) and (4.6±2.6) ×10⁻⁴ cm/h respectively. The results from this study indicated that anodal iontophoresis of the hexapeptide was concentration dependent. A 10-fold increase in donor concentration increased the passive flux of the hexapeptide by 2-fold. Similarly, the applied current normalized flux for 11.3 mM hexapeptide was only 2-fold higher than that of 1.13 mM hexapeptide, 113.6±17.0 µg/(mA.h) and 3.7±9.2 µg/(mA.h) respectively (Table 5.8).
All the experiments with a cetyl hexapeptide-3 clearly demonstrated that iontophoresis enhances delivery of this hexapeptide; the respective permeation parameters for the hexapeptide are presented in Table 5.8.
Table 5.8: Permeation parameters of acetyl hexapeptide-3 (EEMQRR) i ontophoresis (A- Anodal, C- Cathodal) and passive diffusion, (mean ± SEM), Donor-1 (20mM HEPES + 137mM NaCl), Donor-2 (10mM HEPES + 68.5mM NaCl), Donor-3 (5mM HEPES + 34.2mM NaCl), Donor-4 (137mM NaCl), Donor-5 (68.5mM NaCl), Donor-6 (34.2mM NaCl).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean Cumulative amount µg/cm²</th>
<th>Flux µg/ (cm².h)</th>
<th>Flux/I µg/ (mA.h)</th>
<th>Permeability coefficient cm/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEMQRR 1.13mM</td>
<td>9.7±4.3, 2.4</td>
<td>2.4±0.9, 0.5</td>
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<td>(2.5±1.0, 0.05) × 10⁻³</td>
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<td>pH 7.0 (Passive)</td>
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<tr>
<td>EEMQRR 1.13mM</td>
<td>34.0±17.9, 4.6</td>
<td>15.7±7.4, 2.3</td>
<td>43.7±20.7, 9.2</td>
<td>(1.7±0.7, 0.2) × 10⁻²</td>
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<tr>
<td>pH 7.0 (A-Ionto)</td>
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<tr>
<td>EEMQRR 1.13mM</td>
<td>5.8±3.1, 1.8</td>
<td>2.5±1.4, 1.3</td>
<td></td>
<td>(2.7±1.6, 0.09) × 10⁻³</td>
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<tr>
<td>pH 5.0 (Passive)</td>
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<tr>
<td>EEMQRR 1.13mM</td>
<td>11.3±9.0, 3.4</td>
<td>4.6±3.5, 1.3</td>
<td>12.7±9.9, 3.7</td>
<td>(4.8±3.8, 1.4) × 10⁻³</td>
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<td>pH 5.0 (A-Ionto)</td>
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<td>9.7±4.4, 1.8</td>
<td>4.4±4.3, 2.5</td>
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<td>(4.6±4.6, 2.6) × 10⁻⁴</td>
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<td>EEMQRR 11.3mM</td>
<td>91.5±21.1, 9.4</td>
<td>41.0±13.7, 6.1</td>
<td>113.6±38.0, 17.0</td>
<td>(4.3±1.4, 0.6) × 10⁻³</td>
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<tr>
<td>EEMQRR 11.3mM</td>
<td>3.1±1.2, 0.4</td>
<td>1.1±0.8, 0.3</td>
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<td>(1.2±0.8, 0.3) × 10⁻³</td>
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<td>pH 7.0 (C-Ionto)</td>
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<td>EEMQRR 1.13mM</td>
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<td>pH 5.5-6.0 in Donor-2</td>
<td>pH 5.5-6.0 in Donor-3</td>
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<td>EEMQRR 1.13mM</td>
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<tr>
<td></td>
<td>15.6±14.7, 2.3</td>
<td>13.9±6.2, 2.7</td>
<td>22.5±14.8, 1.3</td>
<td>0.9±0.2, 0.1</td>
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<td>8.9±5.1, 2.2</td>
<td>11.3±4.9, 2.1</td>
<td>12.8±3.3, 1.4</td>
<td>0.4±0.2, 0.1</td>
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<td>24.6±14.0, 6.3</td>
<td>31.5±13.5, 6.0</td>
<td>35.6±9.0, 4.0</td>
<td>1.3±0.6, 0.3</td>
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<td></td>
<td>(9.3±5.3, 2.4) × 10⁻³</td>
<td>(1.2±0.5, 0.2) × 10⁻²</td>
<td>(1.4±0.3, 0.1) × 10⁻²</td>
<td>(5.0±2.2, 1.3) × 10⁻⁴</td>
</tr>
<tr>
<td>pH 5.5-6.0 in Donor-5</td>
<td>EEMQRR 1.13mM</td>
<td>pH 5.5-6.0 in Donor-6</td>
<td>EEMQRR 1.13mM</td>
<td>EEMQRR 1.13mM</td>
</tr>
<tr>
<td></td>
<td>4.3±5.6, 3.2</td>
<td>1.5±2.0, 1.2</td>
<td>0.8±0.0, 0.0</td>
<td>0.3±0.0, 0.0</td>
</tr>
<tr>
<td></td>
<td>(1.6±2.1, 1.2) × 10⁻³</td>
<td>(1.6±2.1, 1.2) × 10⁻³</td>
<td>(3.7±0.0, 0.0) × 10⁻⁴</td>
<td>(3.7±0.0, 0.0) × 10⁻⁴</td>
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</tbody>
</table>
Based on the results presented above we concluded that, constant current (0.38 mA/cm²) a nodal iontophoresis enhanced human skin permeation of acetylhexapeptide-3 and that the enhancement was concentration and pH dependent. Reduction in pH of the peptide solution decreased iontophoretic delivery of the hexapeptide revealing the possible mechanism of iontophoretic enhancement to be electromigration. The lowering of pH of the peptide solution is expected to make the peptide more cationic and therefore aid in a nodal iontophoretic delivery. In comparison to the anodal iontophoretic permeability of the hexapeptide at 1.13 mM, a 10-fold increase in peptide concentration, increased the flux of anodal iontophoretic permeation by 4-fold was demonstrated. The results from the study on co-ions and counterions revealed a linear trend in the permeation of hexapeptide, with the linear reduction in concentration of co-ions and counterions present 20, 10, 5 mM HEPES with 137, 68.5, 34.2 mM NaCl respectively. Significantly lower amounts permeated when the peptide donor solution was prepared in NaCl (137, 68.5, 34.2 mM) alone. The 10-fold reduction in cathodal iontophoretic delivery of the hexapeptide further supports that the predominant mechanism of iontophoretic enhancement is electromigration and the direction of movement of charge carriers is from anode to cathode. There has been no reported published data on the iontophoretic delivery of acetylhexapeptide-3 alone and other reports posted by the cosmetic companies where acetylhexapeptide-3 is used in combination with several other cosmetic peptides like hexapeptide-5 and -8 and palmityl peptapeptide for anti-wrinkle creams. There are a number of patent applications for electrical devices to aid the delivery to such cosmetic peptides (51, 55, 85).
5.5.4.5 IONTOPHORETIC TRANSPORT OF TRiptorelin acetate ACROSS HUMAN EPIDERMIS

The iontophoretic delivery of triptorelin was carried out in three series of experiments.

![Graph showing cumulative amount of permeation](image)

**Figure 5.28:** Cumulative amount of triptorelin acetate (µg/cm²) permeating human epidermis to the receptor compartment under constant current Anodal iontophoresis (●) versus passive diffusion (○), (mean ± SEM; n=3 and 3 cells respectively)

The first set of permeation studies investigated the influence of anodal iontophoresis at 0.38 mA/cm² constant current applied for 0-2h on epidermal permeation of the decapeptide in comparison to passive diffusion. The plot of cumulative amount of the decapeptide, 0.9 mM prepared in water at pH 7.4 that permeated human epidermis with iontophoresis versus passive diffusion is presented in Figure 5.28. The transdermal flux and permeability coefficient calculated over 0-2h were 12.1±3.6 µg/(cm².h) and (1.2±0.3) ×10⁻² cm/h respectively as compared to 0.42±0.00 µg/(cm².h) and (4.4±0.0) ×10⁻⁴ cm/h by passive diffusion. The corresponding applied current normalised flux for iontophoretic triptorelin acetate was 33.7±17.4 µg/(mA.h). The results from this study demonstrated that anodal iontophoresis significantly increased
the epidermal permeation of this decapeptide as compared to no or low passive permeation.

Figure 5.29: Cumulative amount (µg/cm²) of triptorelin acetate (0.9 mM) without co-ions and counterions (■) and in the presence of co-ions and counterions (□) permeating human epidermis to the receptor compartment under constant current anodal iontophoresis, (mean ± SEM; n=3 and 3 cells respectively)

The second series of experiments was conducted to determine the influence of co-ions and counterions in the donor solution on the iontophoretic permeability of the decapeptide. The comparison plots of cumulative amount of iontophoretic permeation of the decapeptide in the presence of co-ions and counterions are presented in Figure 5.29. In the presence of 137 mM NaCl in the peptide donor solution containing 0.9 mM decapeptide, the anodal iontophoretic delivery of the decapeptide reduced by 2-fold. This small reduction in transdermal flux could be due to the difference in molar concentrations of the decapeptide to that of its counterions and co-ions (20mM HEPES and 137mM NaCl). The transdermal flux, applied current normalised transdermal flux and permeability coefficient of the decapeptide in the presence of co-ions and counterions was 6.5±2.4 µg/(cm².h), 18.0±11.7...
µg/(mA.h) and (6.8±2.6) ×10⁻³ cm/h respectively. The results from this study again agree with the literature that in the presence of co-ions and counterions there is a reduction in iontophoretic delivery of the primary charge carrying ion.

The third set of experiments was carried out to determine the concentration dependence on the iontophoretic delivery of the decapeptide. The plot of cumulative amount of decapeptide permeating human epidermis from two different peptide concentrations (0.9mM and 9mM) is presented in figure 5.26. The iontophoretic flux and applied current normalised flux were 21.5±6.5 µg/(cm².h) and 59.6±36.3 µg/(mA.h) respectively. The corresponding permeability coefficient for iontophoretic decapeptide was (2.3±0.6) ×10⁻³ cm/h. A 10-fold increase in the peptide concentration increased the rate of iontophoretic permeation by 2-fold. We suggest that this could be due to the size of the molecule. When the molecular size increases it is difficult to see a substantial increase in flux as large molecules are less mobile (86). Therefore, even if there is more drug molecule or ionised species available,

Figure 5.30: Cumulative amount (µg/cm²) of triptorelin acetate, 0.9 mM (■) and 9mM (■) solutions permeating human epidermis to the receptor compartment under the constant current anodal iontophoresis, (mean ± SEM; n=3 and 5 cells respectively)
their iontophoretic transport depends on the extent of ionic mobility (Eq-5.6) and molar fraction of the mobile ion.

All experiments with triptorelin acetate clearly demonstrated that iontophoresis enhances delivery of this decapeptide: the permeation parameters for the decapeptide are presented in Table 5.9. In all the conditions tested, low or negligible amounts of passive diffusion were seen.

### Table 5.9: Permeation parameters of decapeptide (Triptorelin acetate) anodal iontophoresis and passive diffusion, (mean ± SD, SEM)

<table>
<thead>
<tr>
<th>Decapeptide concentration and treatment</th>
<th>Mean cumulative amount µg/cm²</th>
<th>Flux µg/(cm².h)</th>
<th>Flux/I µg/(mA.h)</th>
<th>Permeability coefficient cm/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9mM (Pass)</td>
<td>1.6±0.0, 0.0</td>
<td>0.4±0.0, 0.0</td>
<td>(4.4±0.0, 0.0)</td>
<td>× 10⁻⁴</td>
</tr>
<tr>
<td>0.9mM (Ionto)</td>
<td>25.8±13.2, 7.6</td>
<td>12.1±6.2, 3.6</td>
<td>33.7±17.4, 10.0</td>
<td>(1.2±0.6, 0.3) × 10⁻²</td>
</tr>
<tr>
<td>0.9mM in co-ions and counterions (Ionto)</td>
<td>14.4±8.1, 4.7</td>
<td>6.5±4.2, 2.5</td>
<td>18.0±11.7, 6.7</td>
<td>(6.8±4.4, 2.6) × 10⁻³</td>
</tr>
<tr>
<td>9mM (Ionto)</td>
<td>48.0±28.0, 14.0</td>
<td>21.5±13.1, 6.5</td>
<td>59.6±36.3, 18.1</td>
<td>(2.3±1.4, 0.6) × 10⁻³</td>
</tr>
</tbody>
</table>

There have been very few studies published on the iontophoretic delivery of triptorelin acetate. Nicol et al. presented chemical and physical skin permeation enhancement of triptorelin (63), utilising various combinations of solvent mixtures such as ethanol and particularly 4% lauric acid as the chemical penetration enhancer, and 0.3 mA constant current iontophoresis for 1h. Although the skin penetration enhancement quoted in the paper is significant, it is difficult to directly compare our results to the results presented in this article due to the difference in solvents used. The authors quoted a flux that was calculated post iontophoresis of 4.09 µg/(cm².h) between 12-48h from triptorelin acetate prepared in 0.1M acetate buffer with 3%
w/v mannitol. The amounts of triptorelin permeation at 48h post 1h iontophoresis was 183±100 µg/cm² across full thickness human skin. An important aspect of this study was the post iontophoretic permeation of the decapptide as the current application was able to open the pores or create channels that lasted so long as 48h. They also reported that with the minimum input rate of 0.78 µg/h of triptorelin, the required flux of permeation was 0.0378 µg/ (cm².h) from a permeation area of 20cm² (63). The flux achieved in our study with 0.9mM triptorelin within 0-2h from 0.95 cm² cross sectional area was 12.1±3.6 µg/(cm².h). Schuetz et al published iontophoretic delivery of triptorelin acetate across porcine ear epidermis in vitro and compared different current densities (61). The authors elucidated the mechanism of iontophoretic transport with delivery of acetaminophen as electroosmotic marker. Again due to the difference in skin type, peptide donor solutions and current density, a direct comparison is difficult. However our results are comparable to the aforementioned study. The authors report the permeation data of triptorelin (3mM in 20mM Tris/Trizma buffer) with iontophoretic current of 0.3 mA/cm² which was applied for 0-8h. The amount of triptorelin that permeated the skin over 8h was 60±30 µg/cm². We report permeation of triptorelin (9mM in purified water) with iontophoretic current of 0.38 mA/cm² applied over 2h to be 48±14 µg/cm². A 3-fold increase in molar concentration revealed an appreciable difference in amounts; however the amounts reported in the paper were after 8h of iontophoresis. The authors have also presented a prediction of triptorelin delivery rate of 22 µg/(cm².h) at 7h with 0.5 mA/cm² which they concluded was sufficient to deliver therapeutic levels of triptorelin from a patch area of < 2 cm².
5.6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The peptides and the peptide-like molecules were chosen to provide a series with increasing molecular weight. Iontophoretic flux is dependent on the physicochemical properties of the molecule to be transported by iontophoresis. There have been various reports on inverse relationship of molecular weight to that iontophoretic flux and permeability coefficient (77, 88-92). We attempted to fit the results from our studies into one of the equations derived by Roberts et al. in the ionic mobility-pore model (89) to correlate the increase in molecular weight of the peptides with their iontophoretic permeability coefficient.

Applying the above equation the molecular weight dependency on the iontophoretic permeability coefficient could be expressed. The same has been reported with experimental evidence using small molecular weight local anaesthetics. The ionic mobility pore model identified ionic mobility and molecular size to be the major predictors of iontophoretic permeability. Ionic mobility of a molecule depends on its pKₐ, molecular weight and conductivity. In order to fit our results into this model, important parameters such as ionic mobility and charge of the compound were required and these are not available at this stage. Whilst this is disappointing there was considerable information generated in these experiments that are useful for further understanding the parameters that effect iontophoretic transport of these peptides.

A simple linear regression analysis was fit using the logarithmic permeability coefficient or flux as the dependent variables, and the molecular weight as the independent variable. The results (Figure 5.31) show that there was no apparent association between either of these variables (flux or permeability coefficient) and molecular weight. When the data from 5-ALA were excluded on the basis of 5-ALA being a peptide prototype and not a peptide, the results were still insignificant (Table 5.9). Further on the basis of the inverse trend seen in data obtained from dipeptide, hexapeptide and decapeptide a linear regression line yielded a negative slope of 0.02
Such a relationship has been reported in the literature, with Green et al reporting a negative slope of 0.0017 for cathodal iontophoresis of a series of amino acids (77, 93). Similarly DelTerzo et al reported a negative slope of 0.0062 for alkanolic acids (94) and Phipps et al reported a negative slope of 0.0032 for inorganic cationic drugs (15). Based on the free volume model by Yoshida et al a mean slope of 0.0032 was interpreted to give an average free volume equivalent to the molecular volume of an ionised solute with molecular weight of 135 (20, 95). However the calculated negative slope observed in the current study was not statistically significant to substantiate the correlation in the molecular weight of the selected peptides and their iontophoretic permeability coefficient, moreover such correlation was attempted in the absence of the tetrapeptide data. Although the molecular weight of the tetrapeptide falls in the range selected to run the regression analysis, but the results from its iontophoretic permeation clearly do not fit the trend. The reasons for such behaviour could be the diversity in the structure of the peptides and thus differences in charge, ionic mobility and conductivity (variable due to different pH adjustments) of the peptides. Further according to the literature, the electroosmotic flux is said to be less dependent of the molecular size due to the fact that they are movement of ions along the solvent flux (19, 27). Peptides are predominantly neutral or zwitterionic at physiological pH and hence without the knowledge of the primary mechanism of iontophoretic transport (electromigration or electroosmosis), correlation such as the one attempted in this study is rendered difficult.
Figure 5.31: Iontophoretic permeability coefficient (cm/h) for the compounds investigated, 5-ALA (○), Ala-Trp (♦), Ala-Ala-Pro-Val (●), acetylhexapeptide-3 (■) and triptorelin acetate (▲), versus their molecular weight 131.1, 275.9, 355.4, 888.9 and 1311.5 g/mol respectively (mean ± SEM)

Figure 5.32: Iontophoretic permeability (cm/h) for Ala-Trp (♦), acetylhexapeptide-3 (■) and triptorelin acetate (▲), versus their molecular weight of 275.9, 888.9 and 1311.5 g/mol respectively (mean ± SEM)
Table 5.10 Consolidated permeation results of all compounds investigated, transdermal flux µg/ (cm².h) and permeability coefficient (cm/h), (mean ± SEM) and p values of regression analysis

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Molecular weight</th>
<th>Flux µg/ (cm².h)</th>
<th>permeability coefficient (cm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-ALA</td>
<td>131.1</td>
<td>12.5±1.0</td>
<td>(130±11)×10⁻³</td>
</tr>
<tr>
<td>Ala-Trp</td>
<td>275.9</td>
<td>51.9±11.3</td>
<td>(5.5±1.2)×10⁻³</td>
</tr>
<tr>
<td>Ala-Ala-Pro-Val</td>
<td>355.4</td>
<td>175.5±7.1</td>
<td>(1.8±0.07)×10⁻²</td>
</tr>
<tr>
<td>Acetylhexapeptide-3</td>
<td>888.9</td>
<td>41.0±6.1</td>
<td>(4.3±0.6)×10⁻³</td>
</tr>
<tr>
<td>Triptorelin acetate</td>
<td>1311.5</td>
<td>21.5±6.5</td>
<td>(2.3±0.6)×10⁻³</td>
</tr>
</tbody>
</table>

Regression Analysis

<table>
<thead>
<tr>
<th></th>
<th>Flux µg/ (cm².h)</th>
<th>permeability coefficient (cm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All compounds</td>
<td>-0.037 (p=0.65)</td>
<td>-4×10⁻³ (p=0.65)</td>
</tr>
<tr>
<td>Excluding 5-ALA</td>
<td>-0.090 (p=0.37)</td>
<td>-9×10⁻³ (p=0.37)</td>
</tr>
</tbody>
</table>

The results obtained from individual peptide permeation studies comply with the generally accepted iontophoretic permeation theories as described in the literature:

The pH of the donor solution plays an important role in determining the ionised form of the molecule. In addition the pH of the donor solution containing the electrode also influences the permselectivity of the skin (96). Changes in the pH of the solution are particularly important for peptides and proteins as it greatly affects their chemical stability. Greater skin permeability at pH below the isoelectric point than at or above the isoelectric point has been reported for insulin (97-98). Based on skin’s isoelectric point (pI) of the skin being 4-5, it is desirable that peptides with pI either below 4 or above 7.3 are best suited for iontophoretic delivery (99). In addition the pH of the formulation is said to impact the transport number of the ionic species. At
extremely low or high pH, increased concentrations of hydroxonium ion (H$_3$O$^+$) and hydroxide ions can develop. These ions have high electrical mobility. At high concentrations these ions can significantly lower iontophoretic delivery of large molecular weight drug molecules which have low mobility (86).

The significance of pH of the vehicle containing the drug molecule has been documented for various solutes, including lidocaine across human epidermis (76, 100), 5-ALA across dermatomed pig skin (71), a series of amino acids across hairless mouse skin (77). Greater delivery of the tripeptide thyrotropin-releasing hormone (TRH) was achieved in its unionised form at pH 8 by electroosmosis than at pH 4 where it is 99% cationic; this was because under acidic conditions of the skin reverses its permselectivity from cationic to anionic and electroosmosis occurs from cathode to anode (101).

In summary the influence of pH of the donor solution containing the peptides was investigated to achieve efficient iontophoretic delivery of the ionised form. With respect to 5-ALA the change in pH indicated stability, and its partial cationic form at pH 5.5. Similarly for all the peptides investigated the role of pH adjustment proved significant in achieving maximum transdermal iontophoretic delivery. For the dipeptide, lowering of the pH from 7.4 to 5.5 increased the epidermal iontophoretic delivery by 40-fold (Figure 5.17 and Table 5.5). For the tetrapeptide lowering the pH from 7.4 to 5.5 did not influence the epidermal iontophoretic permeation. However when the pH was lowered to 3.0, the iontophoretic permeation of the tetrapeptide dropped dramatically (Figure 5.20 and Table 5.6) due to change in permselectivity of the skin. For the hexapeptide, lowering the pH from 7.0 to 5.0 reduced the epidermal iontophoretic permeation by 5-fold (Figure 5.24 and Table 5.7) suggesting the hexapeptide to be sufficiently cationic at pH 7.0.

The ion of opposite charge to the charge of ionic molecule to be transported is called a counterion and that of the same charge as co-ion. Addition of a background electrolyte, buffer or substance that could increase the ionic strength of the formulation increases the competition between the charge carriers and generally decreases the iontophoretic drug flux. The electrodiffusion model developed by Kasting and Keister based on single ion transport suggests that, in the absence of competing cations, iontophoretic flux is independent of concentration and dependent only on the ratio of mobilities of the cation and the main counterion (usually Cl$^-$)
arriving from beneath the skin (102). Although highest iontophoretic flux has been reported with the single ion situation, in practice multiple ions as cations and counterions exist. Hence knowledge of the transport number of a drug molecule becomes essential. Transport numbers are complex functions of the concentration and mobility of all the ions present in a system. They are always less than unity due to the presence of relatively small and mobile endogenous ions such as sodium and chloride ions that have transport numbers of $t_{Na^+} = 0.6$ and $t_{Cl^-} = 0.4$ (35). Bellantone et al demonstrated a reduction in benzoate flux by more than half in the presence of equimolar amounts of sodium chloride (103). Cázares-Delgadillo et al presented a similar observation with cytochrome C -A, a 12.4 k Da protein. The iontophoretic delivery of the macromolecule reduced 3.9-fold when the sodium ion concentration increased to 170mM (104).

In summary, for the peptides investigated in the current study, reduction in the iontophoretic flux of the corresponding peptide was observed in the presence of counterions and co-ions. For Ala-Trp the presence of sodium chloride, 137mM in the donor solution decreased the Ala-Trp delivery by 20-fold (Figure 5.18, Table 5.5). For the tetrapeptide, APV presence of 137mM sodium chloride reduced the iontophoretic flux of the tetrapeptide by 2-fold from a 28 mM donor concentration and 35-fold from a 2.8 mM donor concentration (Figure 5.21, Table 5.6). For the hexapeptide the presence of co-ions and counterions revealed a linear trend in the permeation of hexapeptide with the linear reduction in concentration of co-ions and counterions present 20, 10, 5 M HEPES with 137, 68.5, 34.2 M NaCl respectively. Significantly lower amounts of the hexapeptide permeated when the peptide donor solution was prepared in NaCl (137, 68.5, 34.2 mM) alone (Figure 5.25, Table 5.7). For the decapeptide the presence of 137mM NaCl in the peptide donor solution, 0.9 mM decapeptide, the iontophoretic delivery reduced by 2-fold (Figure 5.29, Table 5.8).

The parameters, concentration and presence of counterions and co-ions in iontophoretic drug delivery are interconnected. The concentration of ionised species is said to influence its electromigration component of the iontophoretic flux, only when it is applied along with counterions and co-ions (buffers, electrolytes and preservatives in the formulation - multiple co-ion and independent of concentration in
a single ion situation (35). According to the literature more than concentration it is the molar fraction of the drug molecule that plays a major role on iontophoretic delivery as the later gives information about its concentration relative to that of the competing ions (18, 105). The effect of concentration on the iontophoretic delivery has been well documented with various studies on small and higher molecular weight compounds to name a few, butyrate (94), diclofenac (106), arginine-vasopressin (107), that showed proportional increase in iontophoretic flux with increase in donor concentration. For benzoate (103) and LHRH (58) a modest linear relation was observed with increase in donor concentration. The iontophoretic flux of methylphenidate was found to increase with increase in concentrations up to 100 mM; however at this concentration the increase in flux was less evident. The authors suggested reasons for such behaviour to be due to charge saturation in the aqueous conducting pathways (108); a similar trend was observed for lidocaine (109). In summary all the peptides investigated in the study for effect of concentration on iontophoretic delivery revealed increase permeation with increase in donor concentration. However the effect was not pronounced. For Ala-Trp and hexapeptide a 4-fold increase in iontophoretic delivery was observed with a 10-fold increase in dipeptide and hexapeptide concentration where as for triptorelin only 2-fold increase in permeation was observed.

Cathodal iontophoresis was conducted to determine the predominant charge carrier in the peptides, since the peptides were zwitterionic. For AAPV the flux and the cumulative amount that permeated by cathodal iontophoresis was similar to anodal iontophoresis. This suggested that at the concentration selected for the study, approximately equal quantities of cations and anions were available as charge carriers. Further if the iontophoretic permeation was by electroosmosis, the direction of current would change the permeation of the molecule. Iontophoretic permeation of AAPV with an electroosmotic marker would clarify this. On the other hand for the hexapeptide a 10-fold reduction in cathodal iontophoresis suggested predominant charge on the hexapeptide to be cationic and iontophoretic permeation was by electromigration.
5.7 CONCLUSIONS

Iontophoresis enhanced the epidermal permeation of 5-ALA, Alα-Trp, Alα-Ala-Pro-Val, a cetyl hexapeptide-3 and triptorelin acetate. Various parameters such as pH, concentration of the peptide and presence of co-ions and counterions influenced effective iontophoretic delivery of these peptides and therefore provide considerable scope for optimisation of the application. Based on the key findings from these investigations, there is scope for effective therapeutic and cosmetic applications of these peptides when applied with iontophoresis.
REFERENCES


65. Fasano WJ, M anning LA, Green J W. Rapid i ntegrity a ssessment of r at a nd human e pidermal m embranes f or inv itro d ermal regulatory testing: c orrelation o f electrical resistance with tritiated water permeability. Toxicology In vitro. 2002;16:731-40.


6 CHAPTER 6

General Discussion and Future Directions
Successful transdermal delivery relies on achieving a suitable balance between effective delivery and safety to the skin. Between 1979 and 2007, the US FDA approved 19 transdermal patch delivery systems using both chemical and physical enhancers for a number of indications (1). In the future, an increased number of small molecular weight compounds are likely to be added to the transdermal market with the aid of new efficient chemical penetration enhancers. The transdermal delivery of biodrugs such as proteins, peptides and vaccines that has been a topic of extensive research in the recent past years is promising with more targeted delivery devices. Physical skin penetration enhancement techniques offer effective, safe and rapid delivery of drugs at the preferred site. On their own and in combination with chemical penetration enhancers, physical penetration enhancers have achieved success in delivering drugs and biodrugs across the skin. Some examples of the clinical impacts of physical skin penetration enhancement techniques include patient-controlled electronic iontophoresis dosing unit in pain management (2), local anaesthesia using cavitation sonophoresis (3) and successful clinical trials utilising microneedles for vaccine delivery. This project work focused on evaluating both novel and well-established physical skin penetration enhancement techniques to deliver drugs, therapeutic and cosmetic peptides across human skin. Table 6.1 presents the various techniques investigated in this project and the list of drugs that showed enhanced skin permeation using these techniques.
Table 6.1: List of physical skin penetration enhancement techniques and drugs investigated in the current project work.

<table>
<thead>
<tr>
<th>TECHNIQUES</th>
<th>COMPOUND NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>PULSED ELECTROMAGNETIC ENERGY</td>
<td>Naltrexone hydrochloride</td>
</tr>
<tr>
<td>(PEMF)-DERMAPORTATION</td>
<td>(NTX)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>MAGNETIC FILM ARRAY TECHNOLOGY</td>
<td>Urea</td>
</tr>
<tr>
<td>(ETP)</td>
<td></td>
</tr>
<tr>
<td>LOW FREQUENCY ULTRASOUND (SONOPHORESIS)</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td></td>
<td>(5-ALA)</td>
</tr>
<tr>
<td>SMALL CURRENT (IONTOPHORESIS)</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td></td>
<td>(5-ALA)</td>
</tr>
<tr>
<td></td>
<td>Dipeptide</td>
</tr>
<tr>
<td></td>
<td>(\text{L-Ala-L-Trp})</td>
</tr>
<tr>
<td></td>
<td>Tetrapeptide</td>
</tr>
<tr>
<td></td>
<td>(\text{L-Ala-L-Ala-L-Pro-L-Val})</td>
</tr>
<tr>
<td></td>
<td>Acetyl-hexapeptide-3</td>
</tr>
<tr>
<td></td>
<td>Ac-Glu-Glu-Met-Gln-Arg-Arg-NH\text{2}</td>
</tr>
<tr>
<td></td>
<td>Triptorelin acetate</td>
</tr>
<tr>
<td></td>
<td>(p\text{-Glu-His-Trp-Ser-Tyr-[D-Trp]-Leu-Arg-Pro-Gly-NH}\text{2})</td>
</tr>
</tbody>
</table>
6.1 SUMMARY

Using a novel skin penetration enhancement technique called Dermaporation that utilises pulsed electromagnetic energy (PEMF), the epidermal permeation of naltrexone hydrochloride was investigated. Chapter 2 presents epidermal penetration of naltrexone hydrochloride across human skin, \textit{in vitro} and gold nanoparticles, \textit{ex vivo}. Dermaporation significantly enhanced the epidermal permeation of naltrexone. With no enhancement in permeation of naltrexone across an artificial membrane, we concluded that the mechanism of enhancement by dermaporation is due to the interactions between epidermal structures and the PEMF energy. Confirmation was provided by the observation of penetration of gold nanoparticles across human skin \textit{ex vivo} as visualised using multiphoton tomography fluorescent lifetime imaging microscopy. The results demonstrated enhanced stratum corneum penetration of 10 nm particles and suggested the channels through which the nanoparticles move must be larger than the 10 nm diameter. Dermaporation is a promising new skin penetration enhancement technique; further investigations into optimisation are underway. The data in chapter 2 was published in 2009: Krishnan G, Edwards J, Chen Y, Benson HAE. Enhanced skin permeation of naltrexone by pulsed electromagnetic fields in human skin \textit{in vitro}. \textit{Journal of Pharmaceutical Sciences}. 2009; 99(6):2724-31.

Chapter 3 continues the investigation of magnetic energy. In this case enhanced skin permeation of urea was generated using a novel non-powered magnetic film array technology termed ETP. A 4-fold increase in skin penetration of urea \textit{in vitro} and increased hydration \textit{in vivo} was provided by magnetic delivery. Using optical resonance tomography the physical changes in the skin exposed to ETP induced urea penetration, \textit{in vivo} was visualised as a measure of epidermal thickness. The study revealed a 16% and 11% increase in epidermal thickness at 30 min and 60 min with urea gel applied with the magnetic film (ETP008) when compared to 3% and 6% increase at 30 min and 60 min with urea gel occlusion. The creation of hydrophilic diffusion channels within the skin by the magnetic field concurs with our previous finding that pores or channels are created within the skin in the presence of magnetic energy. Apart from being a hydrotrope, urea is a penetration enhancer in itself, therefore an active magnetic film array technology that increases urea penetration could be beneficial in treating hyperkeratotic skin conditions. Unpowered magnetic
film array technology offers the benefits of being formulated as a patch system. This study was published in 2010: Benson HAE, Krishnan G, Edwards J, Liew YM, Wallace VP. Enhanced skin permeation and hydration by magnetic field array: preliminary in vitro and in vivo assessment. *Journal of Pharmacy and Pharmacology* 2010; 62:696-701.

Low-frequency ultrasound or sonophoresis is a well-established skin penetration enhancement technique. Chapter 4 presents enhanced skin penetration of 5-aminolevulinic acid (5-ALA) *in vitro* and curcumin *in vivo*. Multiphoton tomography fluorescent lifetime imaging microscopy (MPT-FLIM) was employed to determine the biological effects of low-frequency ultrasound for the first time. Two sources of low-frequency ultrasound generators were employed in this study. Using curcumin as a dye, its permeation due to sonophoresis generated from both the devices was visualised by MPT-FLIM. The results revealed greater permeation of curcumin with 55 kHz sonophoresis with permeation reaching deeper layers than when applied by 21 kHz sonophoresis where permeation was confined to the superficial skin layers. The device that generated 55 kHz frequency sonophoresis is a clinically used ultrasound unit and the one that generated 21 kHz frequency is used in cosmetic facial therapy. MPT-FLIM analysis showed no damage to the epidermal cells treated with low-frequency sonophoresis. The results further substantiated a perturbation due to sonophoresis with increased metabolic rate in the epidermal cells. 5-ALA is a photosensitizing pro-drug used in photodynamic therapy (PDT) with poor skin permeation. Our results demonstrated that pre-treatment with low-frequency sonophoresis could offer effective PDT with enhanced 5-ALA penetration at the target site. Fluorescence lifetime imaging is a fairly new technique and whilst further work needs to be undertaken to optimise its use in permeation studies where it provides the opportunity to track and quantify the permeation of substances in the skin in real-time.

In Chapter 5 we present enhanced epidermal penetration of a peptide prototype and series of model therapeutic and cosmetic peptides using small current iontophoresis. Iontophoresis is a nother well established physical skin penetration enhancement technique. Various key parameters such as pH of the donor solution, presence of background electrolyte as co-ions and counterions in the donor solution, concentration of the peptide and direction of current that can optimise effective
Iontophoretic delivery. Iontophoretic current of 0.38 mA/cm² significantly enhanced the epidermal permeation of 5-ALA and the mechanism of iontophoretic delivery was found to be partially by electromigration and partially by electroosmosis at pH 5.5. Similarly, iontophoresis enhanced the epidermal permeation of a model dipeptide, Ala-Trp. A reduction in pH increased its ionised form yielding increased permeation at pH 5.5 than at pH 7.4. For the tetrapeptide iontophoresis significantly increased epidermal permeation. The presence of counterions and co-ions reduced its permeation and this effect was found to be concentration dependent. The epidermal permeation of the cosmetic peptide, acetyl hexapeptide was significantly increased by iontophoresis. There was an inverse relationship between the concentration of counterions and co-ions and the permeation of hexapeptide. The epidermal permeation of triptorelin was significantly enhanced by iontophoresis and therapeutic levels of the decapeptide were achieved within 2 h of iontophoresis application. We attempted to fit the results obtained from the permeation studies of these peptides in a mathematical model derived to show the molecular weight dependency on iontophoretic permeability. However, this was unsuccessful. We concluded that to investigate this relationship knowledge of the charge, ionic mobility or transport number of these peptides is required and these evaluations should be continued. Part this work has been published: Krishnan G, Roberts MS, Grice J, Anissimov YG, Benson HAE. Enhanced transdermal delivery of 5-aminolevulinic acid and a dipeptide by iontophoresis. Peptide Science. (in press).

Overall the various physical skin penetration enhancement techniques investigated in this project work successfully enhanced the permeation of drug molecules and various types of peptides. Further development of these technologies is warranted.

6.2 FUTURE DIRECTIONS

Dermaporation and ETP are new technologies and therefore further work needs to be undertaken to optimize the magnetic energy based technology. More mechanistic studies are required to substantiate the permeation pathway generated by these technologies. Further in vivo assessment of permeation needs to be carried out to quantify the levels of drug permeation enhancement by these technologies and their potential to generate therapeutically relevant drug levels. In addition, there needs to be research to investigate the structural characteristics that are best suited to the
influence of the magnetic field. In summary, understanding the interactions between the magnetic energy and lipid structure of the stratum corneum, and with the chemical structure of drug molecules needs to be clarified. Since ETP is an unpowered technology, it has the potential to be formulated as a transdermal patch system, and thus could be a useful, simple and inexpensive transdermal delivery technology.

Understanding the molecular weight dependency on iontophoretic permeability of peptides is warranted. To facilitate successful modelling, full characterisation of the peptides, and in particular determining the predominant charge on these peptides is essential. This can be carried out by capillary zone electrophoresis. Based on the charge of the peptide, its ionic mobility and the corresponding transport number of the peptide can be determined using appropriate equations. Characterisation of the relationship between amino acid sequence and iontophoretic delivery enhancement pattern needs to be established. This could answer the discrepancies in permeation pattern observed for some of the peptides investigated in the current research. Using an appropriate electroosmotic marker, the mechanism of iontophoretic permeation should also be determined. Ultimately the statistical significance of any model needs more data points than there was time to generate in the current research, therefore in future studies more peptides within the range and beyond the range of molecular weight would help draw better conclusions.

MPT-FLIM analysis has the scope to be an efficient quantitative non-invasive visualisation tool in transdermal permeation studies. We used MPM-FLIM analysis for the visualising gold nanoparticle permeation in ex vivo skin under the influence of Dermaporation experiments and also on in vivo human skin for visualising curcumin permeation under sonophoresis. The group at University of Queensland led by Dr Tarl Prow has continued to develop the effectiveness and extend the application of this technique in transdermal delivery. Recently the addition of different optical probes and filters has facilitated real-time imaging of these fluorescent materials at deeper skin layers and also made their quantification possible. Clear images of the permeation pathway, before and after intervention, are now achievable. Further along with the permeation pathways, pharmacokinetic studies of drug molecules with fluorophores can also be carried out as they cross the different layers of skin. The
further development and application of this important visualisation tool will further contribute to our understanding of a broad range of enhancement technologies.
6.3 REFERENCES


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