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To Or Through The Skin: A Rational Design Of Transdermal Drug Delivery

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TO OR THROUGH THE SKIN: A RATIONAL DESIGN

OF TRANSDERMAL DRUG DELIVERY

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DOCTORAL PROGRAM IN BIOMEDICAL ENGINEERING

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DEDICATION

To my mother

who used to sit with me when I was a child to watch Sesame Street.

I only sat there and watched the show because I wanted to be near you.

This simple act inadvertently taught me the awesome power of an education

and started me on the path

TO OR THROUGH THE SKIN: A RATIONAL DESIGN OF TRANSDERMAL DRUG DELIVERY

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To my lab mates Huda, Jade, and Chris; An anagram of the word "*dissertation"* is "*satirist"*. You have all taught me that this is not a coincidence.

To my family, who have always believed and encouraged me to be the best I can be. I could not ask for more. I am so lucky. My grandmother, Linda; you constantly remind me that I am my fathers' son, and that he has always been with me; I needed that reminder on many occasions. I think he would have liked that his son would carry his last name with the preceding title of Doctor.

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There are so many people who I have not mentioned individually here who have shaped who I am, and in-turn have made me a better person. I have only named a few who immediately come to mind, but there are countless more.

Robert Goddard once wrote "It is difficult to say what is impossible - for the dream of yesterday is the hope of today and the reality of tomorrow"[1]. This frame of mind is not to be taken lightly. When combined with his unbridled tenacity, his technology propelled mankind to the moon. His naive dreams became our reality.

As a member of the millennial generation, my inspirations have not been founded in 19th century scientists, but rather of those based in the 23rd. Fiction, specifically the television show "Star Trek" opened my eyes to an optimistic future, where a small rectangular handheld device known as a "tricorder" could be used to communicate anywhere, translate, and even diagnose disease. Sadly, the smartphone was already a well-established tool of my youth, so I had to look for ulterior messages within the subtext of Gene Roddenberry's world. I found my inspiration in Bones.

Leonard "Bones" McCoy was the ships doctor. Though, he did not practice medicine all that often. His character would usually shine a laser or apply a salve that would almost instantaneously heal the wounds of those in need. The power to heal was not based on years of practice to improve mending abilities, rather Bones himself was simply a man with incredible power; the power flowed through his hands from the tools he utilized. Technology was his only limit. Through this realization, I inadvertently discovered my tricorder.

A doctor is limited by the technology of his generation. Even the most well-meaning physicians in history were restricted in their practice by their rudimentary understanding of concepts we now find to be the foundations of medicine. Examples like antibiotics, blood pressure, germ theory, or even something as simple as sanitary technique, were so beyond the realm of understanding, even as early as 150 years ago, that death rates when compared to today's standards would be considered abhorrent. It is the responsibility of modern researchers to ensure that our practices today are viewed in the future with the same distaste as we see within our ancestors. This can only be accomplished through modest and gradual advances in technology which will further our understanding of the natural world. My personal contribution to this progress may be

considered somewhat ambitious, focusing primarily on the largest organ of the human body, the skin.

To answer these questions is by no means a small task; it is an endeavor that has consumed many researchers, myself included. However, incremental progress, inspiration through the dream world of science fiction, and the relentless optimism of those seeking a better tomorrow has led to several discoveries that offer those with ambition and yearning, the keys to overcoming our current limitations, and opens a window with a view of a brighter future with better treatments and possibilities just over the horizon of our current moment.

"If you know the enemy and know yourself, your victory will not be in doubt; if you know Heaven and know Earth, you may make your victory complete" -Sun Tzu

Most importantly of all, thank you to our God, the spiritual creator with many names. Thank you for the gift of life; I do my best to not waste something so precious.

ABSTRACT:

The skin is the largest organ in the body and plays many crucial roles through diverse functions ranging from protection, temperature regulation, energy storage via fat deposits, and is used to communicate with our surroundings. Various methods have been attempted to deliver drugs to target areas of the skin in effective dosages by a variety of means with limited success. Recently, advances in transdermal drug delivery have offered avenues previously unattainable through conventional means. Some of these new delivery methods have been reviewed, explored and offer promising results with the potential to carve a new avenue in the way therapeutic substances may be consumed.

In one study, a novel miconazole-ufosome nano carrier was characterized, the formulation was optimized in approximately a <300 nm diameter lipid carrier, and at the same time achieved an entrapment efficiency of 62%. In-vitro permeation studies displayed significant targeted transdermal drug delivery to the site of infection in higher amounts than conventional marketed creams, and antifungal studies showed that the current formulation greatly enhanced the efficacy of antifungal activity when compared to standalone miconazole while displaying low toxicity and high biocompatibility.

In another study, we have developed a combination of choline and octenoic acid ionic liquid with an experimental anticancer BCI-2 inhibitor, ABT-263 (navitoclax) to combat melanoma by delivering the drug directly to the site of infection. This formula was characterized and optimized for loading capacity, size, and stability. Cell internalization was achieved at higher levels due to the penetrative capabilities of the ionic liquid, while toxicity remained low. Skin permeation studies displayed significantly higher penetration into the target site, displaying capabilities previously unattainable for non-invasive transdermal drug delivery.

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CHAPTER 1:

A REVIEW OF CURRENT SPECIFIC DISEASES AND THERAPIES

Introduction

The skin is the most easily accessible organ in the body, however due to the strong diffusive properties of the skin, access to this avenue for drug delivery offers a plethora of challenges that are all too often circumvented through painful or minimally effective pathways [1] This limited scope of options has been persistent since the dawn of our more current forms of modern medicine. Recently however, several novel approaches have been published with assorted levels of success [2]. Often, due to toxic drug effects, targeted drug delivery must be implemented to ensure proper amounts of therapy is delivered to a specific area, while also minimizing contact with areas not affected by the disease. Hepatoxic materials must not only avoid direct contact with liver cells, but also must avoid systemic circulation [3]. Hemotoxic substances must avoid contact with blood altogether, severely limiting delivery options [4].

This skin serves a variety of purposes that includes temperature regulation, energy storage via fat deposits, and preventing crossing the toxic substances across the skin. Most importantly, skin acts as a barrier, protecting vital organs and processes from exterior threats, and is absolutely necessary to maintain a healthy internal homeostatic environment.

The outermost layer of the skin is composed of a thick layer of protein known as keratin. This building block is joined by a moist layer of fat which is interwoven between protein deposits. This layer is known as the stratum corneum, also called the “horny layer” or in shorthand, the SC layer. Below this layer is the lucidum, granulosum, spinosum, basale, and the dermis. Each deeper

layer considered easier to penetrate than the previous due to the looser configuration of the cells [5]. This proprietary structure delineates the SC as the main culprit in terms of penetration. The lifespan of a human keratin cell ranges depending on age from 14-90 days, being slowly pushed out over time, offering some recompense in terms of vulnerability.

This understanding naturally leads one to ask the question: What is to happen to the body when its protector is the system that is damaged? Aside from minor lacerations, burns, or intrusions, the answer is often death. The skin being the largest organ of the human body makes it a prime target for attempted infiltration. The unforgiving nature of nature itself, has honed the organ into one that offers much resilience, adaptivity, and strength when presented with adversity.

However, skin is not invincible. Like all organs, the passage of time through the slow breakdown of DNA through methylation and telomere shortening eventually takes a serious toll on the barrier. Protection from harmful radiation becomes less effective, elasticity is hindered and therefore sections no longer bounce back into shape as easily [6]. Tumors fester, and the malignancy of cancer welcomes death through uncontrolled growth. Other genetic betrayal can manifest in this organ as well, through the continuous creation of scars from scleroderma, which can be classified as anything from a minor inconvenience, to a death sentence [7]. External threats such as fungus, can penetrate, feed, and attack vulnerable areas [8].

Often these areas are quite difficult to treat, mainly due to the diffusive properties of the skin, which only allow. The skin has the ability to protect itself from external threats, even if the invader is not necessarily dangerous. Depravity is not a prerequisite for exclusion, so even the most desirable medicines and treatments are refused admittance through the barrier. This indiscriminate behavior has been the culprit for healers since the creation of the first salve. How does one get to or through the skin? How does one create treatments that target something that

nature designed specifically to be impenetrable? How can we save the barrier that has a primary function of protecting us from the exact type of physical invader we hope to emulate?

In this dissertation, an amalgamation of various delivery methods has been comprehensively gathered to determine the state of targeted drug delivery within the current scope of understanding. Several challenging diseases, such as melanoma, skin cancer, and scleroderma, have been lain out as examples of the criteria necessary to structure new targeted drug delivery systems specifically residing in skin layers. These layers often require the delivery of high molecular weight drugs which may have undesirable effects in off-target areas, while concurrently requiring a high dosage for maximum efficacy [9]. These diseases emphasize many of the challenges and realistic expectations that will drive therapies out of the laboratory, and into the market.

1.1 Skin disease and their location within the various layers

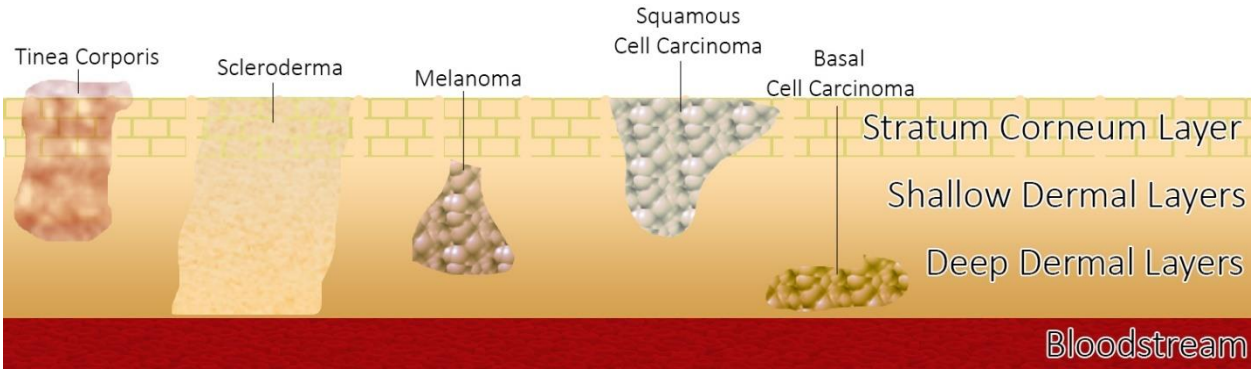

Figure 1.1: Skin diseases and their potential origin locations within the various layers.

Basal Cell Carcinoma

Basal Cell Carcinoma (BSC) is one of the most common types of cancers. The cancer begins in the basal cells, a cell within the lowest layers of the epidermis, which is responsible for the production of new skin cells [10]. Most cases of BSC are contracted by long term exposure to UV radiation or sun exposed parts of the body. People with fair skin are more than likely to contract it more than other races. Most cases of basal cell carcinoma occur in older adults beyond the age of 45, but BSC can also be seen in younger people who are exposed to abnormal amounts of radiation from the sun, such as seasonal farmers, truck drivers, and others that spend copious amounts of time in direct sunlight [11]. BSC is mostly found on the head and neck, however other body parts such as genitals can be infected as well [12]. More than 3 million cases are reported in the United States annually. The primary therapies used to treat BSC is by the use of targeted radiation, copious application of topical drugs, and surgical excision [13].

Squamous Cell Carcinoma

Squamous Cell Carcinoma (SCC) is also a common form of skin cancer [14]. This cancer develops in the squamous cells in the middle and outer layers of the skin. Squamous cells can be found all throughout the body, so the disease can occur anywhere. The cancer is not usually life threatening but it can be aggressive if left untreated. Most cases of SCC results from high levels of UV light from tanning beds or prolonged time in the sunlight [15]. All races are able to contract Squamous Cell Carcinoma, Caucasians are more likely to contract the disease. More than 1 million cases per year are diagnosed with 15,000 annual deaths. Treatment of SCC is almost identical to BCC [16].

Melanoma

Malignant melanoma, commonly referred to simply as melanoma, is relatively the most serious type of skin cancer when compared to BSC and SCC. This cancer develops in the cells that produce melanin. Melanoma can form outside of conventional skin areas, with reports of melanoma originating in the eyes, under the nails, in the mouth, and rarely in the digestive tract or other internal organs. Melanoma can be diagnosed in areas of the skin that are not exposed to the sun, such as spaces between the toes and the genitals [17]. As for the other causes of skin cancers, exposure to UV light and sunlight can be the cause for the cancer [18]. The risk of melanoma in people under 40 is increasing, especially in women [19]. Melanomas are harder to recognize on people with darker skin. It is still a common factor that Caucasian people have a higher risk of contracting the cancer [20]. More than 200,000 US cases are reported per year. Melanoma can be treated depending on the stage of the cancer. In treating stage 1, the melanoma is removed (excision) and the sample is sent to a lab for histological analysis. In other cases, the melanoma can be treated by the use of anti-tumor medication such as imiquimod or Mohs surgery, which is essentially an exploratory excision process that leads to large scarring [21]. The choice of treatment method is largely dependent on the infected area. Treating stage 1 melanoma starts with the excision of the infected area, moving on to a sentinel lymph node biopsy [22]. If the SLNB comes back negative, there is no further treatment. If the SLNB is positive, removal of the lymph nodes will take place or monitoring of the lymph nodes and surrounding areas for growth will be suggested. Additional treatment can be provided with targeted chemotherapeutics [23]. Treating stage 2 melanoma approaches the same steps as treating stage 1, but with more aggressive steps to ensure no further spread of the cancer [24]. In stage 3 of melanoma, the cancer has already infected the lymph nodes, the removal of the tumor and the lymph nodes will happen as well as in the other

stages. Targeted therapeutic agents and/or radiation can be used to prevent the cancer from recurring [25]. Depending on the location of the cancer, there are different treatment options. If tumors are found in the lymph nodes or just under the skin, they can be removed or injections of T-VEC vaccine [26] Basile Calmette-Guerin vaccine, or interleukin can be directly injected into the melanoma, as well as radiation [27]. For melanomas on the arms and legs, an option could be infusing the limp with chemotherapy [28]. When treating stage 4 melanoma, which is also known as metastases stage, the cancer has already spread through the body and lymph nodes, the tumors are sometimes removed depending on the placement, if they are cancerous, and the quantity of individual tumors. Immunotherapeutic drugs (checkpoint inhibitors) are used in those, whose cancer does not have BRAF gene changes. The use of the immunotherapeutic drugs allows for the tumor to shrink but there are serious side effects [29]. If the cancer cells have BRAF cell changes, then immunotherapy drugs such as pembrolizumab can be used to shrink the tumor or have a greater chance of shrinking them. Chemotherapy is commonly used in patients that have stage 4; the cancer shrinks but there is a possibility that the tumors can continue to grow that increases risk of spreading the cancer to other organs including liver, and kidney.

Scleroderma

There are two different types of scleroderma: localized and systemic. Localized scleroderma only affects the skin, causing the skin to become thick and hard. Systemic scleroderma is a more serious type of the disease that can affect skin, muscles, joints, blood vessels, lungs, heart, and other organs. Scleroderma is a rare disease, but 75,000 to 100,000 people in the United States are currently affected [30]. Twins and those with a family history of scleroderma or autoimmune disease are more likely to be afflicted [31]. There is no actual known cause for

scleroderma, but genetic factors and certain chemical exposure may play a factor in the development of the disease.

No two cases are alike, so the identification of the stage, type, and which organs are involved all play a factor in deciding proper treatment [32]. Although no cases are alike, the main features are the similar; inflammation, autoimmunity, vascular disease, and tissue fibrosis are all indicators of the disease [33]. Inflammation medications are used to treat the symptoms of the specific case in order to alleviate pain, and as a direct form of pain management. These anti-inflammatory medication treatments can treat the various forms of arthritis, myositis, and other serositis based problems [34]. Immunosuppressive therapy is the most popular approach for symptom alleviation. The most promising drugs are mycophenolate and cyclophosphamide in treating aggressive cases of scleroderma [35]. Drug therapy of vascular disease allows for the drugs to treat the medium and small arteries that the disease infects [36]. Treatment of the vascular disease is crucial in order to control the disease and to prevent organ damage. Anti-fibrotic agents are used to reduce collagen production or reduce tissue collagen [37]. Colchicine, PABA, dimethyl sulfoxide, and D-penicillamine are the common drugs used to treat excess collagen being produced due to scleroderma [38, 39]. Experts believe that these drugs do not have enough potency to take effect or benefit the patient. The search for new drugs to treat the fibrotic reaction is one of the most active areas of research for scleroderma. Navitoclax, a chemotherapeutic agent used to treat melanoma, is one of these potential promising agents. However, the high molecular weight and hemotoxic qualities of the drug make conventional delivery methods unsustainable in effective quantities.

Tinea Corporis

Tinea Corporis, commonly known as ringworm, is a rash contracted by a fungal infection. This dermatophyte feeds directly on the keratin layer of skin. The contagious fungal infection is spread by a parasite that lives on the outer layer of the skin. Ringworm can be spread from direct contact from humans, animals, objects, and the soil. people who live in warm climates, work with animals or participate in contact sports (such as wrestling or martial arts) are more likely to contract the diseases than others living a more secluded lifestyle. This common disease affects more than 3 million US cases per year, but globally many mild cases are not reported. Ringworm can usually be treated with non-prescription antifungal creams, lotions, or powders. These azoles are used in order to treat fungal infections without having to see a physician, however patient compliance is very low. Most antifungal medications are over the counter and easy to access. The typical application period is multiple times a day for 4 or more weeks. This leads to a lack of patient compliance, and eventually consistent recurrences of the infection which leads to more medical issues, and/or scarring of the affected area.

Athletes foot is a common fungal infection that usually starts between the toes. People whose feet become sweaty from tightfitting shoes are more likely to contract the infection. The infection is caused by the same type of fungus that causes ringworm. Men are at higher risk of contracting athletes' foot than women. Frequently wearing sweaty socks or tightfitting shoes allows for the fungus to grow due to damp conditions. Poor hygiene and sharing infected linens can cause the fungus to spread from person to person. more than 3 million US cases are reported annually.

Although there are countless skin infections and diseases, the aforementioned ailments tend to offer a diverse insight into the various forms of treatment necessary to adequately produce

proper treatment regiments, with respect to time, area, infection, and levels of seriousness associated with various diseases. With these targets in mind, one must realize that a large toolbox of potential treatments is vital for the proper implementation of disease control; ones that may target a wide variety of areas, and allow proper penetration, as well as accurate and precise targeted drug delivery.

To the skin

Various strategies have been attempted for targeted drug delivery to the skin with mixed levels of success, but with each offering limitations to counter certain obstacles. We will examine each of these potential drug delivery methods, as well as their advantages and disadvantages. Each historically, has their fair share of trials and errors.

Transdermal patch

This form of topical treatment allows for specific areas of the skin to be treated. There are four different types of transdermal patch delivery systems; membrane permeation-controlled systems, matrix diffusion-controlled systems, adhesive dispersion-type systems, and Microreservoir types Transdermal delivery allows for the medication to move through the skin, usually by ensuring sustained contact through synthetic patches held in place on the skin by glue. This uncomfortable and inconvenient form of delivery is necessary to ensure permeation of the SC layer. These patches release a specific dose of medication through the skin, usually controlling drug release through a hydrogel. The advantages to using this type of system include enabling the

avoidance of gastrointestinal absorption, consistent dosage rather than peaks in medication levels commonly associated with other forms of delivery, and the capacity to immediately terminate drug dosage (and potentially effects) via immediate removal of the patch. Disadvantages include potential irritation of the skin, a lack of universal consistency to skin types when considering adherence, discomfort in certain areas of application, high cost per dose, and the inability to administer high amounts of the drug into the bloodstream due to the size of the patch and the low amount of drug that can be present on each individual patch. Importantly, the major limitation to transdermal patch is that it welcomes a very high potential chance for leakage [40], concluding that the administration of treatment may not be sufficient; as well as the fact that individuals may be allergic to the adhesive material. These patches release a specific dose of medication through the skin, usually controlling drug release through a hydrogel.

Membrane permeable control system

A membrane permeable control-transdermal drug delivery system (TDDS) of isosorbide dinitrate (ISDN), the model drug is prepared and mixed with polyvinyl alcohol aqueous gel. The membrane consists of ethylene vinyl copolymer membrane and acrylic adhesive [55]. These membranes have some advantages: (i) Prolonged action owing to sustained drug release [56]. (ii) Similar drug delivery both in vivo and in vitro model [57]. (iii) Prevention of high plasma concentration in case of skin damage [58]. Advantages of this system are only for high permeable drugs, but these functions have limited application. This form of delivery is desirable for a wide variety of drug applications. Function achieved is independent of skin condition and application site. In this application, skin permeation rate of the drug should be high enough to allow systematic pharmacological application. High skin penetration can be achieved by the use of penetration

enhancer such as ethanol, medium chain ester, or Azone [59]. However high levels of penetration through the use of this method may cause inflammation. If the skin barrier function is remarkably decrease due to inflammation or placement over a scar, drug delivery rate will be still equal to the permeation rate through the controlling membrane and the plasma concentration should be maintain below the toxic concentration [55], however this may manipulate the dosage. Another transdermal patch application has been created entirely out of collagen. One major advantage of this system is that collagen membrane is very skin friendly and significantly reduces skin damage. However, collagen membranes could not be used as such for an application without stabilizing it by crosslinking [60]. With traditional crosslinking agents like glutaraldehyde, the potential to cause some adverse side effect is raised. To overcome such limitations, a polycationic biopolymer chitosan form stable composite with collagen was created [61]. In this study, authors propose collagen-chitosan membrane for transdermal drug delivery using the lipophilic drug nifedipine, and a hydrophilic drug, propranolol hydrochloride (prop-HC1) [60].

Matrix diffusion control system

Verapamil hydrochloride is a calcium ion influx inhibitor. It is widely used in the treatment of angina, hypertension, and supraventricular tachyarrhythmias [62]. It is suitable for transdermal delivery owing to its low molecular weight (491.07) and low melting point (144˚ c). Transdermal patches of verapamil hydrochloride were prepared using four different polymers (individual and combination): Eudragit RL100 (ERL100), Eudragit RS100 (ERS100), hydroxypropyl methylcellulose 15 cps (HPMC), and ethyl cellulose (EC), of varying degrees of hydrophilicity and hydrophobicity. Authors use both water-soluble polymers like hydroxypropyl methylcellulose (HPMC) and water insoluble polymers, ERL 100, ERS 100, and ethylcellulose (EC), were

selected as carriers into which the drug was incorporated [63]. In another study, authors prepared, designed, and evaluated matrix diffusion controlled transdermal patches of verapamil hydrochloride. Dexibuprofen is chemically (2S)-2-[4-(2-ethylpropyl) phenyl] propanoic acid and used as an NSAID for management of pain and inflammation associated osteoarthritis. In this study, matrix transdermal patches prepared with dexibuprofen exhibited better drug permeation [64]. In another study authors demonstrate a matrix diffusion patch containing rosin: Eudragit RL PM in the ratio 6:4 with 5%w/v of camphor achieved the desired objectives of transdermal drug delivery systems, which according to the study were overcoming of first pass metabolism effects, extended release, and reduced frequency of administration [65].

Micro reservoir type system

The drug reservoir is made of a homogenous dispersion of drug particles suspended in an unleachable viscous liquid medium (e.g. Silicon fluids) to form a paste like suspension, gel, or a clear solution of drug in a releasable solvent (e. G. Ethanol) [66]. The drug reservoir formed is sandwiched between a rate controlling membrane and backing laminate. The rate controlling membrane can be nonporous so that the drug is released by diffusing directly through the material, or the material may contain fluid filled micropores in which case the drug may additionally diffuse through the fluid, thus filling the pores [67]. In the case of nonporous membrane, the rate of passage of drug molecules depends on the solubility of the drug in the membrane as well as the thickness. The drug reservoir is formed by suspending the drug solids in an aqueous solution of water miscible drug solubilizer e.g. Polyethylene glycol. The drug suspension is homogenously dispersed by a high shear mechanical force in lipophilic polymer, forming thousands of unleachable microscopic drug reservoirs (micro reservoirs) [62]. This system is exemplified by the

development of a nitrodisc. The micro reservoir type transdermal system is shown in the figure 1.2 below:

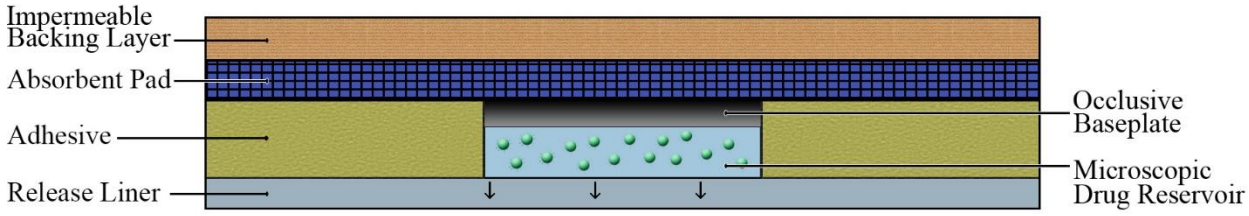

Figure 1.2: Design of micro-reservoir transdermal patch

Adhesive dispersion type system

The drug and other selected excipients, if any, are directly incorporated into the organic solvent-based pressure sensitive adhesive solution, mixed, cast as a thin film, and dried to evaporate the solvents leaving a dried adhesive matrix film containing the drug and excipients [69]. This drug in an adhesive matrix is sandwiched between release liner and backing layer. The drug-in-adhesive patch may be single or multi-layered. The multi-layered system is different from single layer in that it adds another layer of drug-in-adhesive, usually separated by a membrane [70]. This serves to adhere the components of the patch together along with adhering the patch to the skin. The adhesive must possess enough adhesion property so that the TDDS should remain in place for an extended period of time. Pressure sensitive adhesives are commonly used for transdermal patches to adhere to the skin. Commonly used adhesives are silicone [69], polyisobutylenes, and poly acrylate-based adhesives [71].

The mechanism of the transdermal patch has been stated by Prausnitz and Langer (vii) that these patch-based transdermal drug delivery systems *have yet to achieve its potential* as an alternative to oral and hypodermic injection. With that stated, much work is still expected to take place in this field to improve upon these techniques for delivery.

Electrophoresis

Electrophoresis is a method used to separate DNA fragments as well as other macromolecules (RNA) according to the size of their particles. A current is run through a gel that contains the molecules that are needed to be separated. The molecules will travel through the gel at different speeds and directions which allows for them to be separated from each other. The size of the molecule depends on how the gel will react based on speed and direction. In order for the molecules to be placed, the gel must be placed in a gel box. The box is hooked up to a positive and negative electrode which allows for a current to take place. Once turned on, the current flows through the wells, the gel is running. The gel running towards the positive electrode shows the fragments of the molecules, as well as the lengths.

This same type of technology can be utilized on the skin by placing electrodes on the outer layer of skin. This electric effect may allow small molecules to travel through the skin layer through the pores, or by accelerating movement through the lipo-sections of the keratin layer. This process may be effective with low molecular weight drugs, however these size limits are far from its detractions. This type of electrical shock may permanently alter the skin, causing hair loss, inflammation, and destruction of the skin pores.

Ultrasound

Ultrasound therapy has been a beneficial use for therapeutic purposes since the 1950s. Ultrasound therapies were originally used for the treatment of tendinitis and bursitis. As utilization became more advanced, the use of ultrasound therapy became beneficial for various alternative ventures such as uterine fibroid ablation, cataract removal, surgical tissue cutting, hemostasis, transdermal drug delivery, and in the acceleration of bone fracture healing. Therapeutic ultrasound therapy uses short bursts to deliver beneficial energy to the tissues. Ultrasound heat induced therapy uses the absorption of ultrasonic energy in the tissues. Temperature elevations are usually kept at low to limit the temporal average intensities as well as exposure to the heat. Applications of heat induced ultrasonic therapy use long durations of heat with unfocused beams or higher intensity focused bursts. Unfocused heating commonly used in physical therapy is used to treat highly absorbable tissues such as the bones or tendons. The use of the unfocused heating is to produce healing without injury.

Therapeutic ultrasound uses an electric signal and a handheld transducer. The transducer is applied with a coupling gel and moved into a circular motion over the injured area. The transducer is commonly used to treat bursitis or tendonitis to accelerate healing. The coupling gel can also be beneficial to the area of treatment, allowing for the transport of the compound into the skin. Commonly used drugs such as lidocaine or cortisol have been used during ultrasonic therapy. Although there is a chance of burns from the ultrasound therapy, it is still considered to be a benefit for patients as well as being effective. Ultrasound therapy has been founded to be proficient because of its capability to focus energy several millimeters away from the transducer plane. Before using ultrasound therapy, it is important to accurately determine the location of the treatment. As a transdermal delivery system, ultrasound works in a similar manner as

electrophoresis, and has similar benefits. Ultrasound heating can cause irreversible tissues changes based on the heating, coagulative necrosis, and tissue vaporization.

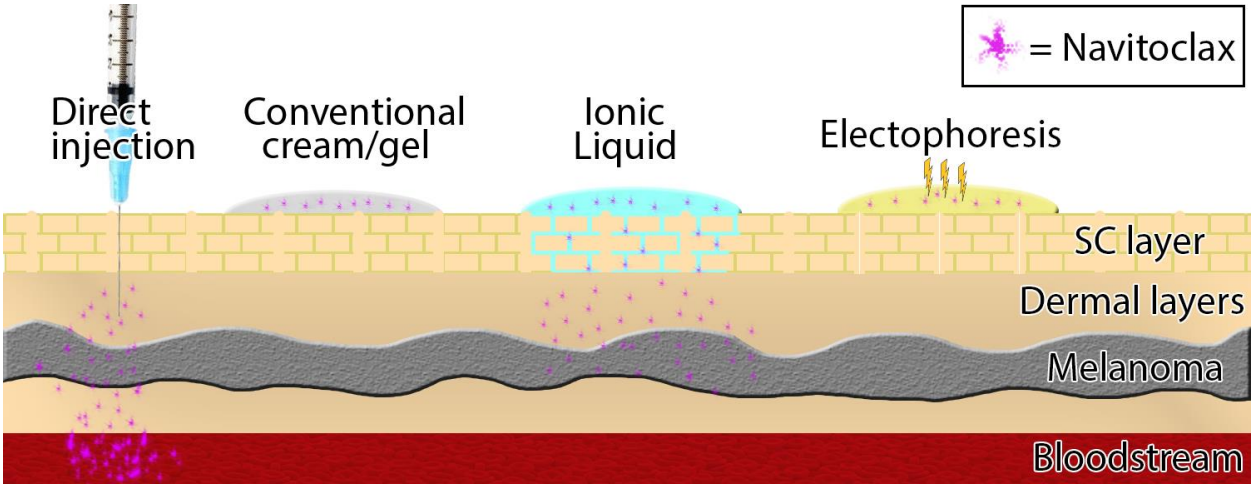

Figure 1.3: Transdermal delivery approach offers one of the most convenient ways of drug delivery to or through the skin. The proposed ionic liquid mediated transdermal delivery is very unique in terms of biosafety and the ability to transport to the dermis of the layer of the skin.

Light based therapies

Photoactivated approaches to transdermal therapy have attracted interest among several research communities. Light based drug delivery has the ability to enable precise control in a highly spatial and temporal manner. The spiral and temporal manner allows for a controlled release of drug molecules. Several photo responsive systems use ultraviolet (UV) or visible light (400-700 nm) to undergo multiple reaction pathways, such as photocleavage and photoisomerization[73]. Although UV light is used mostly in light activated cancer therapies, UV light (<400 nm) has poor tissue penetration and high phototoxicity. The potential issues of using UV light has brought upon the idea of near infrared (NIR) light (700-1000nm) which has been proven to have distinct advantages amongst cancer therapies. All of the NIR advantages, such as the deep penetration and

low cell damage are important for cancer diagnosis and therapies[73]. This type of activation is useful in drug delivery systems because the manual targeting of drug activation may allow more precise control of drug delivery underneath the skin after insertion into systemic circulation via injection or oral introduction. This method could potentially significantly lower

UV light

The commonly used light sources in photoinduced chemotherapy mainly include light energy at UV or short wavelength range.[52]. There are different kinds of approaches that can be taken with using UV or short wavelength light energy; photoactivation through photocaged approach and photoisomerization. The first strategy towards the photoactivated therapy is the photolysis of photocaged molecules, in which a beam of light will be utilized to activate a photocleavable functional group that blocks the activities of therapeutic molecules.[52]. Using photoactivation through the photocaged method achieves the use of a controlled delivery release system of molecules in a living system. Several photocaged systems are based on chemical groups such a o-nitro benzyl, pyridylmethyl ester, and coumaroyl ester to regulate biomolecule activities such as real time monitoring cell trafficking and controlled release of therapeutic molecules. Such chemically photocaged approaches have also been documented for rational design of photoactivable bioluminescent probes for the remote control of optical imaging in living subjects.[52]. Using UV light controlled photocaged strategy has been promising in the usage of bioimaging but can also be promising for the controlled delivery of specific anticancer regents including doxorubicin, paclitaxel, and calprotectin.

Photoisomerization is a reversible strategy that uses the chemical groups to link with the carrier system. Using UV exposure, photosensitive moieties could undergo reversible *cis-trans* light switches and result in the conformational exchanges between inactive and active states and thus controlled the drug release from the carriers[52]. Photoisomerization molecules include azobenzene, spiropyran, and dithienylethene have been studied to be used in conjugation with UV light triggered drug delivery and imaging.

Visible light:

UV light is the most popular strategy for photo-induced therapeutics[74].UV light has had initial success in its field but also has widely accepted disadvantages such as poor ability of tissue penetration, and often have biological damage. In terms that UV light has these disadvantages, longer wavelength exposure at visible range can be used in order to cause less damage to the tissues. Visible light may serve as an alternative option to more damaging wavelengths and has been proposed for light activated drug delivery. Photo responsive organic molecules including vitamin B12 derivatives, ruthenium complexes, and dithiocarbonates have been reported to be of use for visible light- triggered release. Several functionalized nanostructures used in drug delivery are activated by visible light[76].

Near infrared light

Lights with short wavelengths may bare enough energy to induce disruption of chemical structures by either breaking a covalent bod of causing a *cis-trans* isomerization but despite their promising photoactivation applications, there are still some cons; UV light has poor capability of

tissue penetration, high autofluorescence background, and potential phototoxicity. The development of a more reliable alternative that has a high depth tissue penetration and more precise of photoactivation reaction as well as minimum cellular damage is needed. NIR (700-1000nm) shows unique advantages toward deep tissue penetration, low fluorescence background and limited photodamage, the exact opposite of UV light therapy.

Photoactivated drug delivery and bioimaging using NIR light

Photoactivated delivery strategies could enhance therapeutic quality by preventing nontargeted effect through the spatial and temporal precision of light irradiation. For short wavelength they can bear enough energy to induce disruption of chemical structures but despite their promising photoactivation applications, they are shown to have limited tissue penetrating capability, high autofluorescence, and potential phototoxicity. NIR enables a high tissue penetration and more precise control of photoactivation reaction as well as minimal cell damage. NIR provides advantages toward deep tissue penetration, low fluorescence background and limited photo damage. One common use of NIR is to utilize photosensitive nanoplatforms, converting NIR light irradiation into UV and spatiotemporally triggering the release of payload reagents into living systems. Photothermal effect may cause the temperature to increase caused by the light transduced heat to trigger the drug release from nanostructures to tumor sites.

NIR triggered to therapeutic agents UCNPs can serve as phototherapeutic platforms for the controlled delivery of drugs using NIR. NIR regions could absorb photoenergy and convert them to thermal energy. The heat generated by the photoenergy will increase temperatures in the surroundings which can induce the rapid release of the drug molecules. Gold related nanomaterials

have the capability to absorb NIR light and can produce photothermal conversion. Gold based nanoparticles can be modified with thermosensitive polymers (liposomes, mesoporous silica shells, and red blood cells have the ability to absorb NIR light and produce photothermal conversion.

Light mediated therapeutic strategies have recently emerged as a promising and effective tool to precisely treat cancer as effectively as possible. Using light mediated therapeutic strategies allows for a patient to receive exceptional care without the high costs commonly associated with some transdermal therapies. When it comes to cancers, precise surgery and accurate medical diagnosis is necessary, but with light-based drug delivery medical professionals can do so with minimal invasiveness. NIR has displayed several notable advantages relating to precision and trauma, such as deep penetration, low autofluorescence in living cells and tissues, and minimimal photodamage[73]. Due to the minimized photodamage, the light therapy also has the advantage to be used more often. Deep penetration allows for the light to go into the skin deeper and have a more precise control than UV or visible light. The cells can absorb light, too much autofluorescence can cause the image to be hard to read but with using NIR, low autofluorescence allows for the cell to be easily read.

Although light mediated therapies have achieved initial success, the conventional strategies use short wavelengths which can have some undesired drawbacks. UV and visible light both suffer from light scattering, limited tissue penetration depth, and potential phototoxicity to off-target damage[75]. UV light cannot travel long distances into the skin in order to treat the cancer effectively. When using light mediated therapies, the cells can absorb the light which can cause the cell to absorb too much light or involve the autofluorescence to scatter outside of the cell. High autofluorescence can cause the image to be hard to read or locate the cell that is being treated.

Phototoxicity or photosensitivity; a chemically induced skin irrational from the light can be seen when using UV or visible light. UV or visible light are not the only types of light mediated therapies that can have negative side effects but NIR as well. NIR also has some clinical drawbacks; low water solubility, troublesome synthetic procedures, and limited accumulation abilities in targeted areas.

Lipid Nanoencapsulation

Another recent advancement has been the prospect of nanoencapsulation of drugs in lipid-based vesicles for stratum corneum penetration. This strategy has shown promising results in the delivery of azoles, which are antifungal agents used to treat *tinea*-based diseases such as jock itch, athletes' foot, and ringworm. The drugs are encapsulated in a vesicle containing cholesterol, sodium oleate, and the antifungal agent. This is accomplished through a combination of mechanical forces, including intense sonication, and a thorough washing of the product to ensure safe removal of the more toxic materials such as chloroform and methanol. Upon completion of this process, the nanovesicles can be added to a variety of creams or gels for simple application

Efficacy of these nanovesicles has been most promising, delivering 2-3x more of the drug to the target layer and due to the lipid encapsulation, allows the drug to reside in the infected zone for an extended period of time. This additional time and concentration allow for fewer applications, driving down the typical 50-60 necessary doses to potentially far less, which would significantly improve patient compliance and concurrently lower the amount of recurring infections.

The only major disadvantage for this type of application is the molecular size limit that comes naturally with this type of application. The stratum corneum skin layer consists of hard keratin

surrounded by a patchwork “brick and mortar” type of configuration where lipids encompass the mortar. This mortar will accept other lipids for transport through the layer but must adhere to a low molecular weight limit.

Ionic liquid

Recent studies involving new avenues have shown promising results involving the use of ionic liquids for transdermal drug delivery [50]. Macromolecule permeability is increased when ionic liquids are present. The liquid extracts high amounts of lipids from the SC layer of the skin and allows for very large molecules, some exceeding 160kda, to permeate the skin. This new avenue offers promising new alternatives for transdermal drug delivery, one that does not require nano-level breakdown or encapsulation of drugs, simplifying and expediting what would otherwise be an endeavor with a meager payoff.

Comparing to IL/drug methods, patients may no longer need to be wary of having to face particles pressuring their way into the vessel, that will obstruct from a mixture of high pressure plus nanoparticles crystal formation. Ultimately, the aim for IL/drug method to replication or mimic these methods can reshape the future of medicinal application where patients no longer need to fear treatment that is necessary for their pain, cancer treatment, or addiction.

Presenting the flexibility of IL/drug treatment. The ideal mechanism of IL is very simply, as previously stated macromolecule permeability is increased when ionic liquid is present. To support this claim in one study [50], the researchers concluded that the IL induces lipid extraction, followed by skin lipids replaced with IL that allows water to mediate faster through skin pores. Therefore, from the SC layer of the skin; molecules exceeding 160kDa may permeate through the skin.

Further experiments in the near future could open IL potential in controlling how deep a particle is permitted to travel. As previously mentioned, IL can penetrate through the SC layer, this potentially can open the discussion if IL can be controlled to deliver drug treatments to the venous system as well.

IL has convenient applications in vitro, in vivo, and fluorescently-labeled dextrans to study permeability for both experiment and theoretical [[50]]. With that said, TDD can successfully deliver only a few hundred Daltons in molecular mass, versus as previously mentioned IL potentially exceeding 160kDa. As drug therapy passes through the IL, their molecular structure will not be compromised[[47]], due to controlling stability. With these known advantages, high-molecular weight hydrophobic drugs such as navitoclax and other hemotoxic substances now have a delivery method that can circumvent systemic circulation [**Figure 1.3**]. Some studies have reported slight skin inflammation at the site of application, but no long-term damage has been observed.

1.2 Through the skin: Ways we deliver therapy to the bloodstream

Intravenous injection

Intravenous injection is one of the most popular forms of transdermal drug delivery, which involves inserting a hollow and sharp metal rod directly into a vein through the skin. The drug is injected directly into the vein, and dosage is controlled via pressure, as well as the gauge of the rod, which allows for precise drug concentration in the plasma. Advantages such as immediate access to the circulatory system, easy routes for patients that cannot tolerate oral medications, and instant drug action via systemic circulation, make IV injections one of the most popular forms of

drug delivery in hospital settings. Despite the advances, there are major disadvantages in regard to this form of delivery; dispersing the medication too quickly can cause speed shock, and once infused, the side effects cannot be reversed. This type of danger offers little recompense for mistakes and can easily lead to serious injury that may ultimately lead to extensive tissue damage, high pressure injuries, and tearing an aorta or vein. Extravasation can occur when the medication leaks into the surrounding tissue due to excess pressure. There can also be the negative side effects of physical, chemical, and therapeutic incompatibly with systemic drug circulation. Many of these factors can inevitably lead to death. Due to the high possibility of infection, these types of delivery systems are rarely seen outside of hospital settings, and patient compliance in home settings is low due to the pain and required precision associated with injections. Though it accomplished its goal of delivering treatment to patient, by a professionally trained individual is required to understand how the needle must be administered upon a patient's skin, with the bolus into the subcutis [49]

Intra-arterial injection

Under certain circumstances with analgesic, antibiotic, antifungal, and anticancer drugs in an effort to deliver a high concentration of the drug to a particular tissue, the administration of Intra-arterial injection is a viable option. This method requires the drug treatment to enter the cerebrospinal fluid via injection; then from the subarachnoid space of the spinal cord it will bypass the blood-brain barrier, allowing drugs with poor penetration easy access to the central nervous system. Advantages to using this method include good absorption, a more rapid onset than oral and rectal routes, and offers a long duration of action. This particular type of injection requires an experienced professional for proper administration. One study [72]presented intra-arterial drug

injection for treating lethal brain diseases. The idea of IA can only be effective when three criteria is met; (i) when there is low regional blood flow, (ii) high regional extraction, and (iii) high systemic clearance [72]. Some disadvantages to using this method include the somewhat common unpredictable absorption, and the painful injections may cause serious swelling or bruising that may lead to further complications. Previously it has been reported that an accident involving intra-arterial drug injection caused a sporadic blood vessel deformation[44]. Conclusions drawn from the authors determined that IA drug injection may cause serious vessel injury, deforming vessels from the drug's particles being large enough to disrupt these vessels post-injection.

Table 1.1: Overview of Transdermal Drug Delivery Systems.

Form of Delivery	Type of Disease	Pros	Cons
Ultrasound	• Bursitis • Tendonitis • Muscle strains • Sprains • Joint contracture	• Reduce swelling, inflammation, and promote bone healing • Accelerate bone healing time • Promote tissue healing • Noninvasive	• Cannot be used over cancerous areas • Cannot be used on children, near the eyes, around the heart, in people with vascular problems

Topical	• Ring worm • Athletes foot • Psoriasis • Acne • Eczema	• Fewer risks of gastrointestinal difficulties • Easy to administer	• Irritation • May not adhere to all skin types
IV Injection	• Cancers • Heart failure • Crohns disease • Immune deficiencies • Rheumatoid arthritis • Sclerosis	• Direct access to the circulatory system • Instant drug action • High drug concentrations • Instant drug termination	• Errors with IV mixing • Adsorption • Speed shock • Chemical phlebitis • Extravasation of agent
Electrophoresis	• Seizure control • Transdermal • Electrically modulated	• Non-toxic • Various buffers can be used, easily made and sets quickly,	• High percentage gels may not set evenly, • low percentage gels may break, • does not have a uniform pore size • permanent damage

UV light	• Eczema • Psoriasis • Vitiligo • Lymphoma	• Possess higher energy • Activates therapeutic agents • Noninvasive • Precise control	• Ageing of the skin • Skin darkening • Increased risk to other types of cancers • Limited tissue penetration • Potential photodamage
Visible light	• Cancers • Acne • Seasonal disorders • Neurodermitis • Eczema	• Controllable operation • Precision • Lower expenditure • Noninvasive	• Potential photodamage • Limited light penetration depth
NIR light	• Osteoarthritis • Tendinitis • Cancers	• Minimized photodamage • Deep penetration • Low autofluorescence • Noninvasive	• Low water solubility • Limited accumulation properties

Lipid Nanoencapsulation	• Antifungal agents	• Higher levels of drug concentration	• 500Da molecular weight limit. • Must be encapsulated in lipids
Ionic Liquid	• Antifungal agents • Anticancer therapy	• 160+kDa molecules	• Slight inflammation

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CHAPTER 2

NANO-VESICLE BASED ANTI-FUNGAL FORMULATION SHOWS HIGHER STABILITY, SKIN DIFFUSION, BIOSAFETY AND ANTI-FUNGAL EFFICACY *IN VITRO*

Abstract

Opportunistic fungal infections are responsible for over 1.5 million deaths per year. This has created a need for highly effective antifungal medication to be as potent as possible. In this study, we improved the efficacy of a common (over-the-counter) OTC antifungal skin medication, miconazole, by encapsulating nano-molecules of the drug in cholesterol/sodium oleate nano-vesicles. These nano-vesicles were characterized to optimize their size, zeta potential, polydispersity index and encapsulation efficiency. Furthermore, these nano-vesicle were compared to a conventional miconazole-based commercially available cream to determine potential improvement via permeation through the stratum corneum, cytotoxicity, and antifungal capabilities. Our results found that the vesicle size was within the nano range (~300 nm), with moderate polydispersity and stability. When compared with the commercially available cream, Actavis, as well as free miconazole, the miconazole nano-vesicle formulation displayed enhanced fugal inhibition by a factor of 3 or more when compared to free miconazole. Furthermore, with smaller NP sizes, higher percentages of miconazole may be delivered, further enhancing the efficacy of miconazole's antifungal capability. Cytotoxicity studies conducted with human dermal fibroblast cell confirms its biosafety and biocompatibility as cell survival rate was observed 2 folds higher in nano-vesicle formulation than free miconazole. This formulation has the potential to treat

fungal infections through increasing the retention time in the skin, improving the treatment approach, and by enhancing the efficacy by use of the nanovesicles.

1. Introduction

Globally, 300 million people are inflicted with a serious fungal infection annually[1]. Of those infected, 25 million will face the possibility of losing their sight[1]. Fungal infections are the cause of over 1.5 million deaths per year[1]. Most of these deaths are caused by opportunistic infections acting on already immunocompromised individuals, such as those diagnosed with HIV/AIDS, influenza, cancer, COPD, asthma, tuberculosis, or those currently taking immunosuppressants for a variety of medical reasons[2]. Essentially, these fungal infections prey on societies most vulnerable, and therefore, a high degree of significance must be placed to ensure that current therapies are achieving maximum efficacy.

Tinea Corporis, a dermatophyte commonly known as ringworm, is one of these opportunistic infections that plagues many individuals, especially the immunocompromised[3].This type of disease subsides deep within the skin, using these layers as a barrier to protect itself from external threats, while it manifests and spreads beneath the stratum corneum, wreaking havoc on the body and if left untreated in an immunocompromised adult may eventually lead to serious illness or death[4]. Tinea Corporis is a pathogenic fungus that is often found on all areas of mammalian skin, and can be seen on the surface layers, often creating a distinctive ring pattern, and is especially prominent on those with weakened immune systems, such as those infected with HIV/AIDS [5].

Superficial skin infections are often difficult to treat and require the use of systemically dispersed drugs to properly attack the site of infection[6]. This is primarily due to the makeup of the

skin, which consists of a protective outer layer of keratin and lipids known as the Stratum Corneum (SC) layer. This layer is the primary defense against foreign invaders and allows very few molecules to pass through into the deeper layers of the skin[7]. Due to its protective nature, passing foreign material through this layer is quite challenging, and requires specific size and outer makeup to pass to the lower layers of skin[8].

Miconazole is an imidazole antifungal agent commonly used to treat various fungal infections of the skin and vagina[9]. It is available in tablet, cream, ointment, and suppository form, and may also be delivered intravenously in some circumstances. This drug is currently listed on the World Health Organization's list of essential medicines: those which are considered to be the most effective and safe medicines necessary to meet the major and minor needs of a health system[10]. This drug is also extremely affordable, being one of the most cost-effective antifungal agents available over the counter, and available in virtually every country worldwide[11]. Miconazole is not considered toxic due to its high LD50 and is especially attractive due to its low EC50[11]. However, miconazole is water-insoluble, making it difficult to deliver to the target area in large quantities beneath the skin[12].

This blaring issue involving miconazole has led to most creams and conventional treatments to be limited to 1-2% drug with an abundance of lipid fillers to transport the drug to the target site[13]. On average, for a typical ringworm infection, miconazole cream must be applied twice daily, for 4 weeks to eliminate the infection[9]. This means that a conventional treatment requires almost 60 applications over the course of a month. Due to this issue, patient compliance is low and leads to recurring infections that may last years in an otherwise healthy person. Continuous manifestation may lead to scarring at the site of infection and can both spread and infect new hosts during this time.

This study attempts to remedy this plethora of issues by securing adequate transport of miconazole to the site of infection **[Figure 2.1]**, while still involving a familiar route of skin entry through conventional transdermal skin application. Lipid encapsulation has been shown to enhance delivery while also increasing efficacy of antifungal drugs[14]. Previous studies involving the use of a combination of cholesterol and sodium oleate with proper ratios have been successful in transdermal drug delivery, while maintaining respectable drug encapsulation efficiency[15] Understanding the proper ratios of miconazole, cholesterol, and sodium oleate, as well as measuring vesicle size, polydispersity index, zeta potential, and encapsulation efficiency are paramount to understanding overall drug efficacy, and were intimately studied to ensure the best combination to battle infection would be properly utilized for further study.

Additionally, proper measurement through the skin layers was of prime importance[16], especially when compared to Actavis creams, and across a spectrum of *in-vitro* human skin types to ensure result consistency[17]. *In-vitro* cytotoxicity and efficacy against dermal cell line and potential fungi, respectively[18] was also required to showcase the efficacy of lipid-enhanced miconazole nanoparticles when compared to free miconazole.

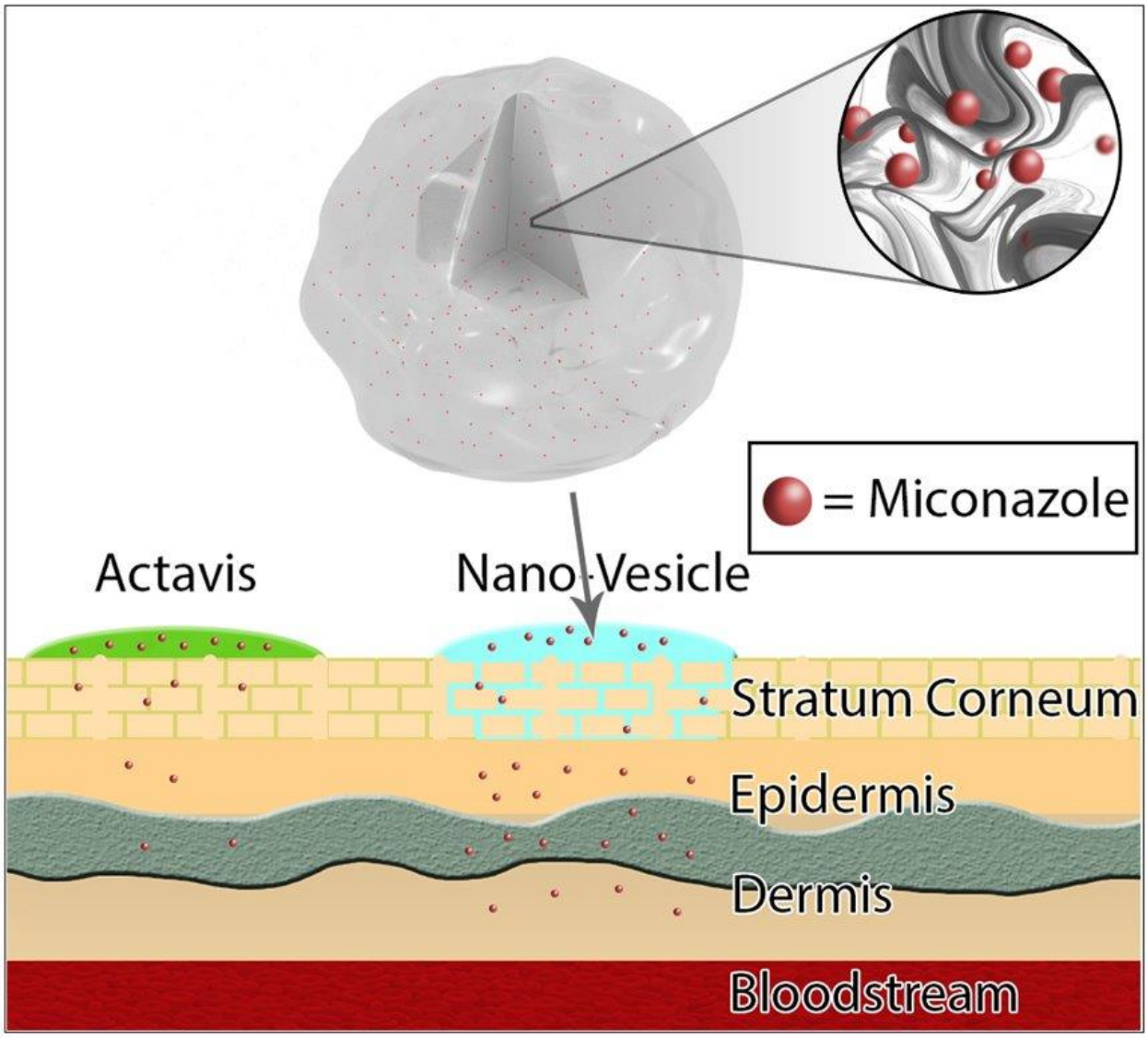

Figure 2.1. Miconazole loaded nano-vesicles offer a higher delivery of drug content through the epidermis to the target site of fungal infection. The high lipid content of the nano-vesicles allows the drug to be carried through the stratum corneum and offers a previously unattainable avenue of drug delivery in high content for lipophilic nano-sized drugs.

2. Materials and Methods

2.1. Materials

Miconazole was purchased from MP Biomedicals (Solon, OH, USA). Actavis (miconazole 2%) was purchased from a local pharmacy store in El Paso, TX, USA. Cholesterol was purchased from Alfa Aesar (Ward Hill, MA, USA). Cadaver skin for permeation and skin diffusion studies was obtained from Zen-Bio Inc., (Research Triangle Park, NC, USA). Deionized water (resistivity of 18.2 MΩ) used for all experiments was obtained from in-house Milli-Q® IQ 7000 Ultrapure Water System (EMD Millipore, Bedford, MA, USA). Sodium oleate was purchased from TCI America (Portland, OR, USA). Methanol (ACS grade), PBS (pH 7.4), and chloroform were procured from Fisher Chemicals (Fair Lawn, NJ, USA).

Table 2.1: Display of the various ratios of miconazole, cholesterol, and sodium oleate used in each formulation. Miconazole was used as the determinant anchor to maintain consistency with testing, and to offer a support when comparing with Actavis brand marketed cream

Formulations	Miconazole (mg)	Cholesterol (mg)	Sodium Oleate (mg)	Miconazole loading efficiency (%)	Size distribution(nm)	Zeta potential (mV)
1:0.5:1	100	50	100	43.9±7.8	252±62	76±5
1:1:0.5	100	100	50	59.7±3.3	337±58	85±3
1:1:1	100	100	100	3.1±0.5	383±103	88±5
1:2:1	100	200	100	3.1±0.6	269±57	93±4
1:1:2	100	100	200	3.3±0.6	308±79	74±7
1:2:2	100	200	200	3.4±0.5	372±135	64±7

2.2. Preparation of Nano-vesicles

Miconazole loaded nano-vesicles were prepared by using a thin-film hydration method previously used in a similar azole study[15]. Miconazole, cholesterol, and sodium oleate were dissolved in 10 mL of methanol and chloroform in a 1:1 ratio solution. This solution was placed in a 15 mL conical tube and left in an ultrasonic bath until completely dissolved, approximately

10 min. The solution was then moved to a 50 mL beaker and left to stir at 100 rpm overnight to ensure complete evaporation of the solvents. The resulting thin film was rehydrated using 5 mL of PBS (pH 7.4) for 2 hr. Residual amounts of methanol and chloroform may be unnecessarily toxic to off-target cells, and therefore these solvents had to be completely removed. The nano-vesicles were washed by placing the solution in an ultracentrifuge and were spun at 15,000 rpm for 1 hr. The supernatant was replaced with PBS, vortexed until dispersed, and the nano-vesicles were then washed again using the same process for a second time. Finally, the formed vesicular dispersion was sonicated for 6 min to de-clump the mass of lipids, and to ensure the nano-vesicles retained similar sizes. Various ratios of miconazole, sodium oleate, and cholesterol [**Table 1**] were prepared to determine ideal amounts to maximize entrapment efficiency[19]. All samples were produced at least in triplicate. All experiments were also performed at least in triplicate.

2.3. Determination of Vesicle Size, Polydispersity Index, and Zeta Potential

Vesicle size and polydispersity index were determined by using dynamic light scattering (DLS) and zeta potential was determined by electrophoretic mobility[20]. 100 µL of nano-vesicles were diluted in 10 mL of de-ionized water and measurements were taken using a Malvern Zetasizer (Nano series ZS90, Malvern, Worchestershire, UK) at 25°C.

2.4. Determination of Entrapment Efficiency

Entrapment efficiency (EE) of the formulations was determined using an ultracentrifugation method[21] using a Optima XPN-90 Ultracentrifuge (Beckman Coulter Life Sciences, Indianapolis, IN, USA) at 15,000 rpm for 4 hr at 4°C. After centrifugation, the supernatant was discarded to

remove any unentrapped miconazole. 10 mL of methanol was added to the lipid precipitate, and then bath sonicated for 1 hr, and left for 12 hr in a shaking water bath (25°C at 100 rpm) to completely extract the entrapped miconazole. The resultant solution was centrifuged at 15000 rpm for 1 hr at 4°C to separate the methanol lipid layer. The supernatant was then diluted, and the concentration of miconazole was determined using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and encapsulation efficiency was determined by using the following formula:

Entrapment Efficiency = (Miconazole remaining in vesicles) x 100/ (Initial miconazole)

2.5. Determination of Viscosity

Viscosity testing was determined by using a fungilab Viscolead One (Fungilab, Hauppuage NY, USA) following manufacturers instructions for a 200 mL sample. The sample was placed in a cold-water bath and then tested at 5 °C, 24 °C, and 37 °C. These temperatures were chosen to determine if there would be significant changes in the formulation viscosity when at temperatures relating to cold storage, ambient temperature, and the average temperature of human skin.

2.6. *In Vitro* Permeation Study

Skin preparation studies were performed using skin harvested from two human sources provided by the ZenBio corporation (ZenBio, East Triangle Park, NC, USA) listed below:

Table 2.2: Skin samples used for diffusion studies. Samples were taken from the same location (abdomen) and with similar scarring (severe), however the age, gender, and ethnicity were

different between the samples. This was done purposely to determine if the intake of miconazole vesicles would act differently when alternative factors were introduced, and to compare these potential differences with those of a marketed cream (Actavis).

Ethnicity	**Gender**	**Age**	**BMI**	**Stretch marks**	**Location**
Caucasian	Male	24	29.9	Severe	Abdomen
African/Black	Female	43	26.9	Severe	Abdomen

Various skin sources were used to determine any potential discrepancies in permeation due to sex or ethnicity, although few were expected[22]. Skin samples were stored in -20 °C until needed, in which they were thawed for 24 hr in 4 °C and then left to equilibrate at 24 °C for 20 min, and then lightly shaved to remove any hair, if any. The skin was then soaked in 150 mL of PBS (pH 7.4) for 20 min for rehydration. The skin was then cut into squares approximately 0.7-inch x 0.7 inch to fit into the ILC07 automated flow-through system (Permegear Inc. Hellertown, PA, USA). The skin was then placed individually in one of the 7 donor/receptor compartments.

These receptor compartments were locked in place with a small lower chamber that pumped PBS (pH 7.4) directly underneath the skin at 37 °C at a flow rate of 4 mL/hr to simulate typical flow conditions of the human body[23]. The pump used was a multi-channel peristaltic pump IPC (Ismatec, Zurich, Switzerland) and the heating source was a Julabo BC4 circulating water bath (Julabo Pumps, Seelbach, Germany). The liquid that flowed through the chambers was collected in 20 mL scintillation vials, and changed at various time points (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 and 24 hr) to determine how much drug penetrated the final skin layer and could potentially enter the bloodstream. This process is presented in **Figure 2.4A**.

2.7. Separation of the SC Layer

In order to quantify the amount of drug left in the skin, the Stratum Corneum layer of skin was removed from the inner layers using a tape strip and scrape method[24]. The skin was carefully detached from the cells and placed on a flat metal surface. The skin was gently dabbed to remove excess formulation, and a piece of scotch tape was pressed against the skin to ensure maximum adherence. This strip was removed with light force and discarded. An additional piece of tape was placed on the skin, and the SC was then forcefully ripped from the other layers quickly in a single pull. This process was repeated 10 times. Additionally, a surgical scalpel was used to carefully scrape away and collect any leftover SC left after the tape stripping. The tape strips, along with the SC, were collected in conical tubes and dissolved in 10 mL of methanol.

The remaining dermal layers were cut with surgical scissors into the smallest pieces mechanically possible and placed into their own respective conical tubes. Each tube was then filled with 10 mL of methanol and sonicated for 30 min. The tubes were left overnight to soak at 4 °C. Finally, the solution was centrifuged at 15000 rpm for 30 min at 4 °C and the supernatant was collected for drug quantification by UV-Vis spectrophotometer.

2.8. Fungal Assay

The fungal organisms tested in this study was *Cryptococcus neoformans*. The fungus and fungal study were both provided and conducted in Dr. Luis Martinez' laboratory of the department of Biology, University of Texas at El Paso. To evaluate the efficacy of miconazole-ufozome nano-vesicles, *Cryptococcus neoformans*[33], a powerful and somewhat resilient fungus, was cultured on a sabouraud dextrose agar plated petri dish[34]. To duplicate results, a total 8 plates with 4 treatments was prepared. Using a Bunsen burner, the loop/needle were sterilized and with the assistance of

the loop. After the fungus was successfully plated, the 4 treatments were plated with the fungus with PBS, 1% miconazole drug, 1% miconazole NP and 2 % miconazole nano-vesicle respectively. The application was placed in 4 different spots in each petri dish with different treatments. A drop of the formulation (about 0.01 mL) was then placed in the cavity slide and was incubated for 25 ± 2 °C in a moist chamber to maintain proper humidity. Two replicates were maintained for each treatment including the control. Each petri dish was examined for 48 hr by observing the amount of dead space between the drug and fungal colony. The total area of the petri dish was measured using ImageJ software by NIH and using CAD software[35]. The ImageJ calculation has been tabulated below in **Table 2.3**.

No of Reading	1X PBS	1% Miconazole drug	1% Miconazole NP	2% Miconazole NP
1st Reading on Area (mm^2)	19.64	19.33	15.634	11.886
2nd Reading on Area (mm^2)	19.64	19.3	15.45	11.59
Percentage of Fungal Survival	100	98.82232	79.10906298	59.34459805
Average	19.64	19.315	15.542	11.738
SEM	0.00	0.015	0.092	0.148

2.9. *In vitro* Cytotoxicity/Biosafety

Human dermal fibroblast (HDF) cells were purchased from ATCC, USA. HDF cells were routinely cultured in DMEM supplement with 10% FBS and 0.1 % antibiotics (100 U/mL penicillin, 10 µg/mL streptomycin) in a humidified incubator with 5% carbon dioxide at 37 °C.

When cell confluence reached 80%, they were incubated $5X10^3$ cell/well in a 96-well plate for 24 hr. 200 µL of medium was added in each well. 5 different treatments were prepared with medium only (without cells), PBS, 1% miconazole- nano-vesicle nanoparticles (MUNP), 2% MUNP and 1% miconazole drug. Cells were prepared according to different time points starting from 1, 2, 4, 8 and 12 hr respectively. Vybrant® MTT cell proliferation assay kit of Invitrogen of Thermo Scientific was used according to required specifications. MTT cytotoxicity assay quick protocol[25] was also used according to the manufacturer's specified instructions. In brief, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414) of 12 mM MTT stock solution by adding 1 mL of sterile PBS to one 5 mg vial of MTT and dimethyl sulfoxide (DMSO) was used in this protocol. First, the medium was removed and replaced with 100 µL of fresh culture medium. Then 10 µL of the 12 mM MTT stock solution was added to each well with 100 µL and incubated at 37˚C for 4 hr. After labeling the cells with MTT, as described above, all but 25 µL of medium was removed from the wells. Then, 50 µL of DMSO was added to each well and mixed thoroughly with a pipette. After incubation for 10 min at 37 °C, absorbance was read at 540 nm using UV spectrophotometry Synergy 4 (BioTek, U.S.). Each sample was tested in triplicate. The untreated cells that were incubated with PBS were used as negative control.

3. Results and Discussion

3.1. Preparation and Characterization

Various ratios of miconazole, cholesterol, and sodium oleate were evaluated to determine ideal size, polydispersity, zeta potential, and encapsulation efficiency. The most ideal formulation would be used for further study.

Miconazole sizes were found to be all within the range of less than 400 nm [**Figure 2.2**]., which according to previous studies, would prove to be ideal for passing through the stratum corneum to deliver miconazole to the deeper layers within the skin[26]. Standard deviation remained relatively consistent, allowing any of the formulation ratios to be considered for additional evaluation. These results were to be expected due to the identical preparation methods and would not be the main determinant for drug delivery. Similar findings were observed in previous studies with clotrimazole and diclofenac[27].

Similar to the size evaluation, the measurement of the electrokinetic zeta potential was proved to be equally acceptable across all ranges, displaying excellent stability. This is a key indicator of colloidal dispersions, and offers a promising view of the formulation stability[28].

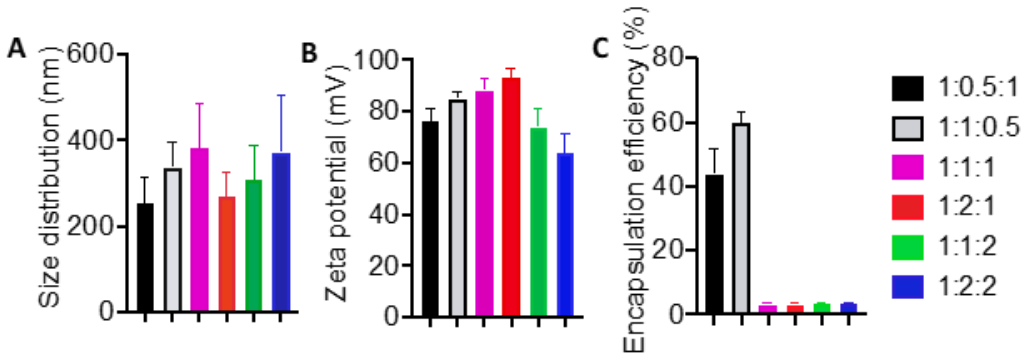

Figure 2.2. Size, zeta potential, and encapsulation efficiency amongst the various ratios of miconazole:cholesterol:sodium oleate. (A) Size distribution and (B) zeta potential remained relatively similar, however high variation between vesicles eliminated consideration for 1:1:1 and 1:2:2 due to an inordinate amount of vesicles stretching beyond the nano-range. (C) Encapsulation efficiency amongst the various ratios eliminated several formulations from consideration due to poor performance in relation to drug delivery, while two ratios (1:0.5:1 and 1:1:0.5) conversely displayed exceptional loading potential. All data are presented as mean ± SE (n = 3).

The encapsulation efficiency is a prime indicator that the formulations had a key difference between each-other [**Figure 2.2**], and eliminated the concept of allowing certain ratios to be considered for further study as drug carriers[29]. Four of the formulations displayed abysmal encapsulation efficiency, all approximately at 3%, making them relatively inadequate for any state of efficient drug delivery. Two of the formulations (1:1:0.5 and 1:0.5:1) displayed high rates of encapsulation, however the 1:1:0.5 formulation offered the highest encapsulation efficiency at 59.71% with a lower standard deviation than its counterpart. Similar studies involving ufosomes have displayed similar results involving certain ratios with significantly improved EE[[15]]. Due to these advantages, the 1:1:0.5 formulation of miconazole:cholesterol:sodium oleate was determined to be the best ratio combination to use as a transdermal drug delivery vehicle for miconazole, and was used for all further studies beyond this point[30].

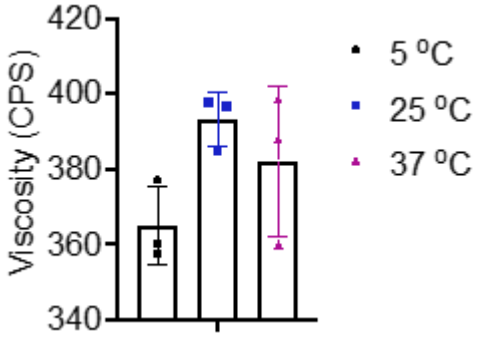

Figure 2.3. Viscosity testing was performed at various temperatures to determine optimal temperature during administration, as well as to see if any major change occurred at temperatures similar to contact with human skin. Viscosity remained relatively constant throughout the spectrum, maintaining between 355-405 CPS. All data are presented as mean ± SE (n = 3).

Viscosity studies provided insight into the changes at various temperatures [**Figure 2.3**]. Viscosity remained within 355-405 CPS, in-between all temperature changes, and performed as expected with very little difference in viscosity. The various materials used in the formulation may have impacted the formulation slightly with the temperature changes, but all maintained a very liquid state throughout. With the abundance of PBS present in the formulation, the application would be expected to display little to no difference regardless of conventional temperatures while in cold storage, or after being left at room temperature.

3.2. *In vitro* diffusion

In vitro diffusion studies supported the notion that large amounts of miconazole stayed within the dermal layers after 24 hr [**Figure 2.4. B1, B2**], significantly more than when compared to the Actavis market cream[31]. This supports the notion that nano-vesicles are more effective in delivering this insoluble drug to the target site than conventional delivery methods currently being marketed[27]. These higher quantities can offer significantly more therapy than their marketed counterparts. Higher amounts of miconazole were found in the younger Caucasian male. Younger skin tends to display higher levels of elasticity and retains significantly more moisture than aging skin. This may be the reason why a significantly higher amount of miconazole was able to penetrate and reside in the younger skin when compared to the older skin [**Figure 2.4. B1, B2**].

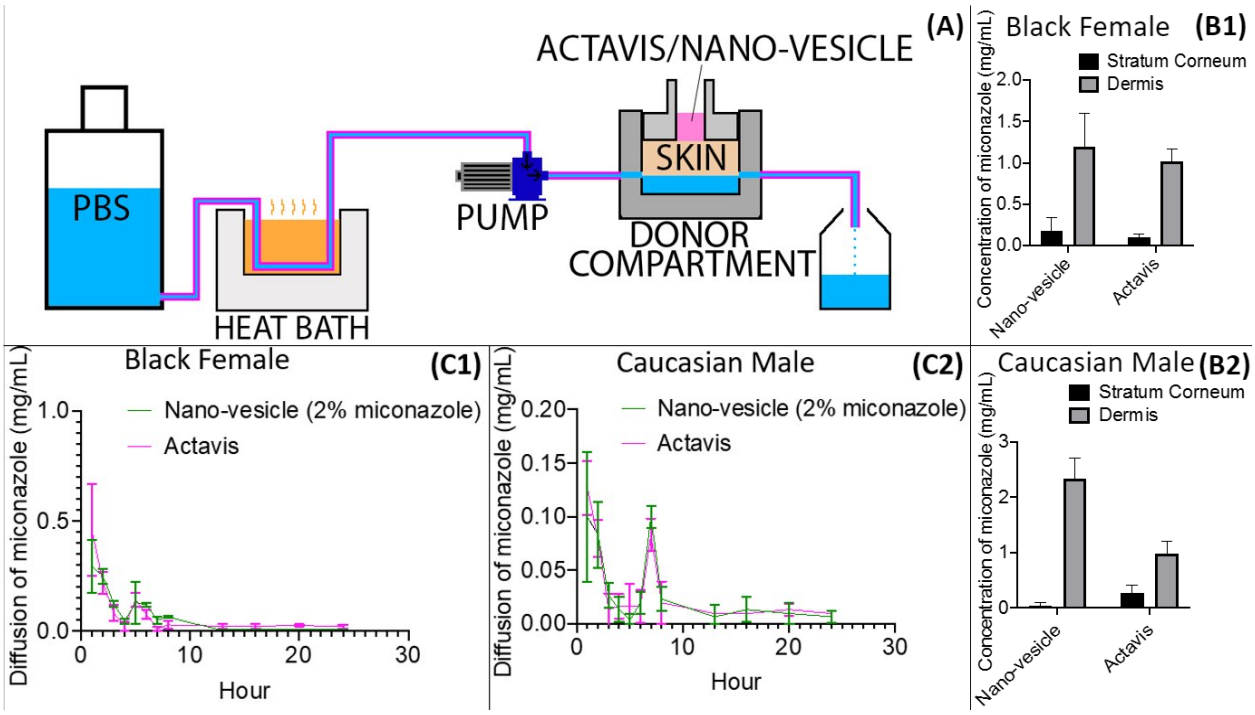

Figure 2.4. (A) Diagram of the Permegear Franz-diffusion cell system. PBS is warmed to 37°C before being pumped into the donor compartment, underneath the skin, and finally resides in the scintillation vial, which was changed hourly. (B1, B2) UV-Vis comparing the amount of miconazole located within the Stratum Corneum and lower dermal layers after 24 hrs in the Permegear diffusion system. (C1, C2) Hourly readings of the PBS collected scintillation vials, identifying the amount of drug that may pass completely through the skin and into the bloodstream within 24 hrs. All data are presented as mean ± SE (n = 6).

Analysis of the PBS collected hourly showed very little miconazole passed beyond the skin [**Figure 2.4. C1, C2**], indicating that most of the drug achieved prolonged presence in the target site. Like other studies, this again supports the notion that lipid-based nanoparticles greatly enhance the penetration of molecules through the skin while staying within the target site instead of being metabolized by the body. These promising results signify the potential to greatly increase

the current levels of drug delivery through the skin while avoiding systemic circulation and elimination[32].

3.3. Anti-fungal Study

In **table 2.3** the summarized effectiveness of miconazole-ufozome nano-vesicle has been displayed, showing both 1% and 2% miconazole containing nano-vesicle were able to kill higher fungal colonies compared with 1% miconazole-vesicles, as well as the drug itself. This clear visual experiment shows that miconazole nano-vesicles can inhibit fungal colonies at a rate of 20% and 40% (**Figure 2.5, 2.6**) with 1% and 2% miconazole nano-vesicle treatment, respectively. The maximum zone of inhibition was shown by 1% and 2% 15.542±0.092 mm^2 and 11.738±0.148 mm^2 respectively when compared with 1% drug and negative control with 19.315±0.015 mm^2 and 19.64±0.0 mm^2 (**Figure 2.5**). The projected efficiency of nano-vesicles to inhibit fungal colony germination compared with a negative control and 1% of standalone drug in terms of total area is also exhibited. The presence of a larger inhibition zone in the fungal colonies[36] clearly indicates a higher level of efficiency of nano-vesicles over direct drug application.

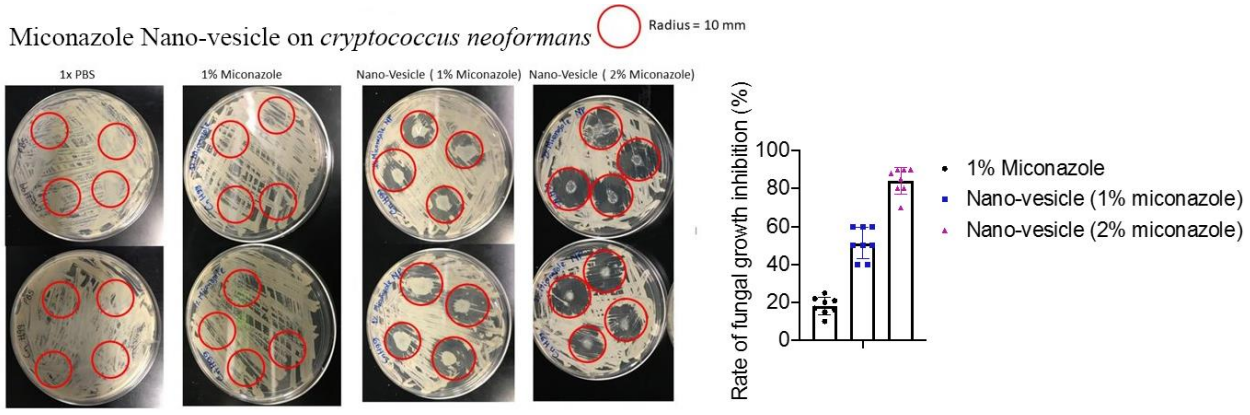

Figure 2.5. Fungal growth inhibition using free miconazole, as well as nano-vesicles at 1% and 2% miconazole. PBS was used as a control. (A) Significantly higher levels of inhibition were expressed using nano-vesicles as opposed to free miconazole, supporting the notion that nano-vesicles act as an inhibition enhancer. (B) Around 20% and 40% fungal colonies were inhibited with the 1% and 2% miconazole nano-vesicles treatment respectively. All data are presented as mean ± SE (n = 8).

3.4 Cytotoxicity Biosafety Study

As seen in **figure 2.6**, nano-vesicles loaded with 1% miconazole displayed higher cell viability than the free miconazole counterparts. However, when nano-vesicles loaded with 2% miconazole were introduced to the cells, significantly lower survival rates were observed. This may be due to the overabundance of miconazole being present, however the amount of drug present is still well below the expected LD50. Alternatively, this may be due to a lower drug loading capability of the miconazole due to the 2% formulation essentially being a concentrated combination of nano-vesicle and miconazole. Both of these hypotheses lend credence to the notion that further observation is required for the application of this transdermal drug delivery method[39].

This data also suggests that 1% miconazole nano-vesicles are much safer alternative to 1% free miconazole drug treatments[40].

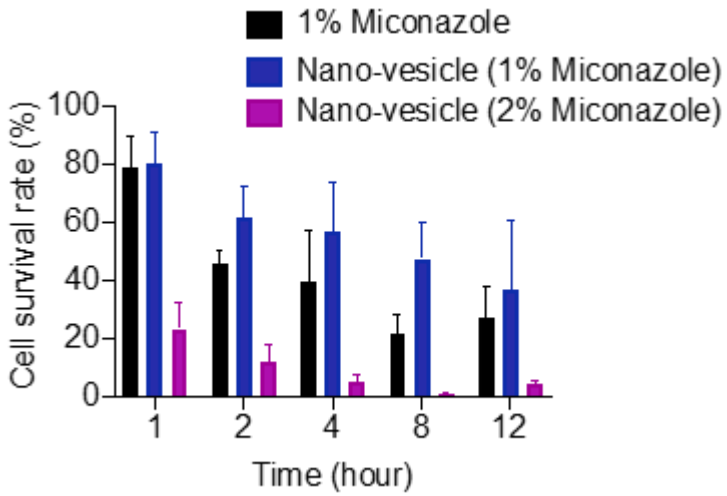

Figure 2.6. Cell survival rates at varying time points. 2% miconazole nano-vesicles displayed lower cell viability at all time points when compared with 1% miconazole nano-vesicle and 1% free miconazole. All data are presented as mean ± SE (n = 9).

Conclusions

Increased bioavailability of conventional over-the-counter (OTC) drugs can greatly enhance their efficacy simply and effectively. In this study, we characterized a novel miconazole nano-vesicle carrier, optimized the formulation (100 mg miconazole, 100 mg cholesterol, 50 mg sodium oleate) in approximately a 400 nm diameter lipid carrier, and at the same time achieved a high entrapment efficiency of 59.71%. *In vitro* permeation studies display significant targeted transdermal drug delivery to the site of infection in higher amounts than conventional marketed cream. Antifungal studies show that the current formulation greatly enhances the efficacy of antifungal activity when compared to standalone miconazole. These enhancements have the potential to revitalize the efficacy and conventional use of miconazole, even at the over the counter

market level, and therefore require further development to demonstrate and understand its efficacy in an animal model. This novel miconazole delivery system offers the potential to transform the current market, revolutionizing conventional medicines and offering a new wave of enhanced revitalized treatments using already understood conventional drugs and therapies.

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CHAPTER 3:

IONIC LIQUID MEDIATED BCL-2 INHIBITOR DELIVERY FOR TARGETING TREATMENT OF MELANOMA

Abstract

Melanoma affects over 200,000 Americans every year [1]. Current therapies to fight early stages of this disease involve invasive surgeries that lead to permanent scarring of the skin and may also include systemic circulation of toxic chemotherapeutics with devastating side-effects [2]. These averse and often unsuccessful treatments have left a dire need for new less-invasive drug delivery methods with a greater focus on the target site of infection [3]. Recently, several advancements in transdermal drug delivery have shown promising results with the ability to transfer large molecules through the stratum corneum and allow anchoring within these deeper skin layers [4].

In this study, we have developed a combination of choline and octenoic acid ionic liquid with an experimental anticancer BCI-2 inhibitor, ABT-263 (navitoclax) to combat melanoma by delivering the drug directly to the site of infection. This formula was characterized and optimized for loading capacity, size, and stability. Cell internalization was achieved at higher levels due to the penetrative capabilities of the ionic liquid, while toxicity remained low. Skin permeation studies displayed significantly higher penetration into the target site, displaying capabilities previously unattainable for non-invasive transdermal drug delivery.

Introduction

More people are diagnosed with skin cancer in the U.S. than all other cancers combined.[5] Melanoma, one of the most common skin cancers, originates in the dermal layers and will eventually metastasize deeper until reaching the lymph nodes and bloodstream.[1] Despite having numerous therapeutics to combat this type of cancer, they all suffer from off-targeting toxicity that comes in tandem with intravenous delivery.[6] These delivery methods also lack the proper therapeutic concentration that would allow for proper treatment while avoiding the devastating side-effects common with chemotherapy.[7] Intravenous and oral routes are utilized for conventional drug delivery; however, these routes are systemic, and usher a plethora of side-effects that include hair loss, cardiac malfunction, and troubled breathing that cannot be easily countered.[8] The high efficacy of these drugs is not limited to melanoma, and the indiscriminate destruction of healthy cells is problematic. This system-wide application of these drugs through intravenous can oftentimes be more devastating than therapeutic.[9]

Transdermal drug delivery (TDD) offers an alternative to IV/oral delivery, circumventing systemic circulation by homing in on the site of melanoma. This direct form of delivery may result lowering the dose to achieve effective concentration, and significantly reduce non-specific internalization mediated side-effects.[10] Due to these advantages, TDD may be considered as an effective approach to treat melanoma. Stratum corneum (SC), the outermost layer of the skin, is responsible for keeping the inner layers of the body separate from exterior threats. This layer prohibits the vast majority of solids and liquids from passing through by offering a physical barrier of keratin and lipids to block these foreign materials from entering the body.[11] To overcome this barrier, a variety of strategies have been tested, including ultrasound and electrical stimulation,

lipid encapsulation, and light therapies.[12,13] While these strategies have been met with limited success, they only allow specific, very small molecules to bypass the SC layer.[4]

In this study, Ionic liquids (IL)composed of choline bicarbonate and octenoic acid that retain a liquid state at room temperature have been developed. This allows for a variety of applications due to their poorly coordinated ions.[4] The potential to overcome the skin barrier by utilizing these ILs allows hydrophobic molecule diffusion through the SC layer of the skin. A recent report shows navitoclax, a BCI-2 inhibitor, is highly effective in killing melanoma as well as the stem cells that are responsible for chemotherapy resistance.[14,15] However, due to severe risk of thrombocytopenia and lack of skin penetration ability, navitoclax is limited exclusively to oral delivery, and this route of administration does not result in effective therapeutic concentration within the melanoma sites.[15] Therefore, the objective and goal of this study was to allow the expression of its most promising qualities while avoiding the serious detriments of its toxic effects. This can be achieved by avoiding the introduction of the drug into the patient's bloodstream. The implementation of Ionic liquids (IL's) for TDD due to their ability to function as a delivery vehicle through and to the skin for hydrophilic molecules will allow for safe administration of this potent drug. Using an IL, it is possible to anchor a large dose of navitoclax beyond the stratum corneum and deliver it into the progressively deeper layers of the skin without contacting the bloodstream.

Materials & Methods

Pig skin was purchased from a local butcher store in El Paso, TX, USA. Deionized water (resistivity of 18.2 MΩ) used for all experiments was obtained from in-lab Milli-Q® IQ 7000 Ultrapure Water System (EMD Millipore, Bedford, MA, USA). PBS (pH 7.4) was procured from

Fisher Chemicals (Fair Lawn, NJ, USA). Ionic Liquid was created in-lab and also donated from Samir Mitragotri Lab at Harvard University. A-375 (epithelial, malignant melanoma, Female, aged 54 years) cells were donated from the University of Texas at El Paso Border Biology Research Center, by Dr. Armando Varela of the biology department, and was cultured in vitro., and Passages 9-10 were used for these experiments.

Preparation of IL/Navitoclax

Choline and octenoic acid Ionic liquid was sonicated with various percentages of navitoclax to determine maximum stable loading capacity of drug in liquid form. Sonication was performed using a probe sonicator at 30% intensity with a 15 second on/off cycle for 90 seconds. This method determined that the highest loading capacity was 1% navitoclax. Higher amounts of drug would either immediately precipitate or not suspend at all. Sonication was attempted at higher intensities for longer periods of time with no avail. Therefore, 1% was determined to be the ideal loading capacity for this suspension.

Formula Characterization

Size and polydispersity index were determined by using dynamic light scattering (DLS) and zeta potential was determined by electrophoretic mobility. 100 µL of IL/navitoclax were diluted in 10 mL of de-ionized water. This formulation was lightly sonicated in a bath for 5 minutes to ensure consistent dispersion and measurements were taken using a Malvern Zetasizer (Nano series ZS90, Malvern, Worchestershire, UK) at 25°C.

Cell Internalization assay

A375 cells were cultured in DMEM with 10% FBS and 0.5% PS. When the cells reached 70-80% confluence, they were split and seeded in 96 well plates with approximately 1000 cells per well. A total of 15 wells were prepared for the three different treatment groups (5 wells each)**.** After 48 hrs of seeding, the treatment with cells began. Coumarin 6, a fluorescent agent with an almost identical molecular weight to navitoclax was used as a substitute for the drug to offer a visual representation for imaging purposes. The bright glow offers insight to the behaviors and penetrative capabilities of IL, and to clearly show if the IL is acting as a penetration enhancer at the cellular level.

Three different treatment groups were utilized, including a control group with medium only, Coumarin 6 treatment group, and lastly an ionic liquid w/ coumarin 6 treatment group. Coumarin was diluted in a 1mg/1 ml DI H_2O solution. Ionic liquid and coumarin 6 treatments were prepared at a 1:1 ratio. After 6 hrs of treatment, the cells were washed three times with 1X PBS and replaced with 190 mL of fresh medium in each well. Then, 10 µL of Hoechst was added to stain the live cell nucleus. After 2 hrs of staining treatment, the cells were again washed three times with 1X PBS and replaced with 200 µL of fresh medium in each well. An LSM 300 confocal microscope was then utilized to capture fluorescent images. The images were taken at 20X magnification at both green (488 nm for coumarin) and violet (410 nm for Hoechst) wavelengths.

LDH Toxicity assay

A375 cells were cultured in DMEM, 10% FBS, and 0.5% PBS. When the cells reached 70-80% confluence, they were split and seed in 96 well plates at around 3000 cells in each well. After

48 hrs of seeding, the cell treatment was initiated. Four different treatments were used, namely the control group with medium only, Navitoclax, Ionic liquid, and Ionic liquid with navitoclax. A Cyquant LDH cytotoxicity assay kit was then used as per manufacturers instruction. An optimal number of 3000 cells/well were plated in 100 μL of the medium in triplicate wells in a 96-well tissue culture plate. The LDH activity of A375 cells was measured in all four different treatments at 1, 2, 4 and, 6 hr time points. To the set of triplicate wells serving as the Maximum, spontaneous, and chemical LDH Activity Controls, 10 μL of 10x lysis buffer was added, 10 μL of sterile ultrapure water, and 10 μL of the vehicle containing chemical compound respectively. They were then mixed by gently tapping the plate. Then, 50 μL of each sample medium (Spontaneous LDH Activity, Maximum LDH Activity, and Chemical-treated LDH activity) was transferred to a 96-well flat-bottom plate in triplicate wells. 50 μL of Reaction Mixture was aliquoted to each sample well, then mixed thoroughly. The plate was then incubated at room temperature for 30 min while protected from light, and later 50 μL of Stop Solution was added to each sample well. Finally, absorbance was measured at 490 and 680 nm, respectively. To determine LDH activity, the 680 nm absorbance value (background) was subtracted from the 490 nm absorbance before the calculation of % cytotoxicity.

In Vitro Permeation Study

Skin preparation studies were performed using skin harvested from locally harvested porcine sources, originating on the belly of the animal. Skin samples were stored in -20 °C until needed, in which they were thawed for 24 hr in 4 °C and then left to equilibrate at 24 °C for 20 min, and then lightly shaved to remove any hair, if any. Several layers of muscle and fat tissues were attached to the skin. This was carefully removed using a scalpel, with much emphasis placed

on not disturbing or piercing the skin. Special consideration was taken to ensure the minimum amount of scar tissue was present on these samples to ensure a consistent flow amongst the various samples, and to improve homogenous results related to permeation over material selection.

The skin was then soaked in 150 mL of PBS (pH 7.4) for 20 min for rehydration. The skin was then cut into squares approximately 0.7-inch x 0.7 inch to fit into the ILC07 automated flow-through system (Permegear Inc. Hellertown, PA, USA). The skin was then placed individually in one of the 7 donor/receptor compartments.

These receptor compartments were locked in place with a small lower chamber that pumped PBS (pH 7.4) directly underneath the skin at 37 °C at a flow rate of 4 mL/hr to simulate typical flow conditions of the human body [23]. The pump used was a multi-channel peristaltic pump IPC (Ismatec, Zurich, Switzerland) and the heating source was a Julabo BC4 circulating water bath (Julabo Pumps, Seelbach, Germany). The liquid that flowed through the chambers was collected in 20 mL scintillation vials, and changed at various time points (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12 hr) to determine how much drug penetrated the final skin layer and could potentially enter the bloodstream.

Separation of the SC Layer

In order to quantify the amount of drug left in the skin, the Stratum Corneum layer of skin was removed from the inner layers using a tape strip and scrape method. The skin was carefully detached from the cells and placed on a flat metal surface. The skin was gently dabbed to remove excess formulation, and a piece of scotch tape was pressed against the skin to ensure maximum adherence. This strip was removed with light force and discarded. An additional piece of tape was

placed on the skin, and the SC was then forcefully ripped from the other layers quickly in a single pull. This process was repeated 10 times. Additionally, a surgical scalpel was used to carefully scrape away and collect any leftover SC left after the tape stripping. The tape strips, along with the SC, were collected in conical tubes and dissolved in 10 mL of ethanol.

The remaining dermal layers were cut with surgical scissors into the smallest pieces mechanically possible and placed into their own respective conical tubes. Each tube was then filled with 10 mL of ethanol and sonicated for 30 min. The tubes were left overnight to soak at 4 °C. Finally, the solution was centrifuged at 15000 rpm for 30 min at 4 °C and the supernatant was collected for drug quantification by a UV spectrophotometer.

Results and Discussion

Formula Preparation and Characterization

1% navitoclax formulation

		Standard deviation
Size	213.3 nm	±57.2
Polydispersity Index	.366 (0-1)	±.045
Zeta Potential	-5.11 mV	±.46

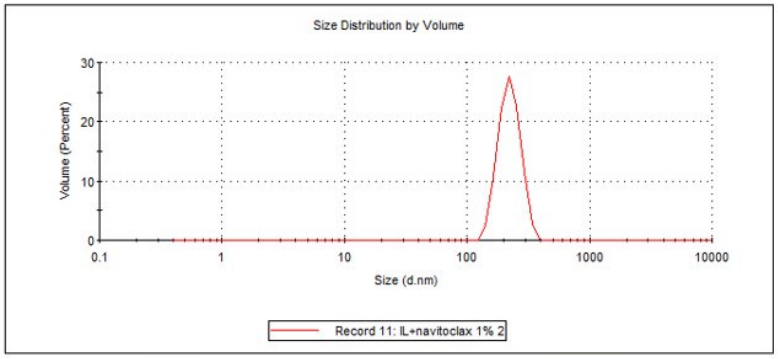

Figure 3.1. The IL/navitoclax formulation was evaluated to determine size, polydispersity, zeta potential, and maximum load.

The formulation displayed an average size of 213 nm, placing the formulation well within the size expected to traverse through the SC layer to deliver navitoclax to the deeper layers of the skin. Standard deviation remained within this boundary, and offered a consistent formulation which can offer more consistent dosage [**Figure 3.1**].

Similar to the size evaluation, the measurement of the electrokinetic zeta potential was proved to be equally displaying excellent stability. However, polydispersity index had some results

which initially displayed a low amount of dispersion, but if the formulation was left undisturbed for more than 10 hr, the index would rise sharply, as seen in sample 3 of **Figure 3.2**. This study was repeated to confirm results. Having a varied, time-dependent, polydispersity index indicates that like most suspensions, one would be expected to shake well before use. However, shaking in this case involves probe sonication. Therefore, in order to ensure consistency, the formulation would have to be created or at the very least sonicated the day of application to ensure a consistent and proper dosage of navitoclax to the target site. This precipitation seems to be unique to ionic liquid when compared with H_2O and may be due to the electrophoretic relationship between navitoclax and IL.

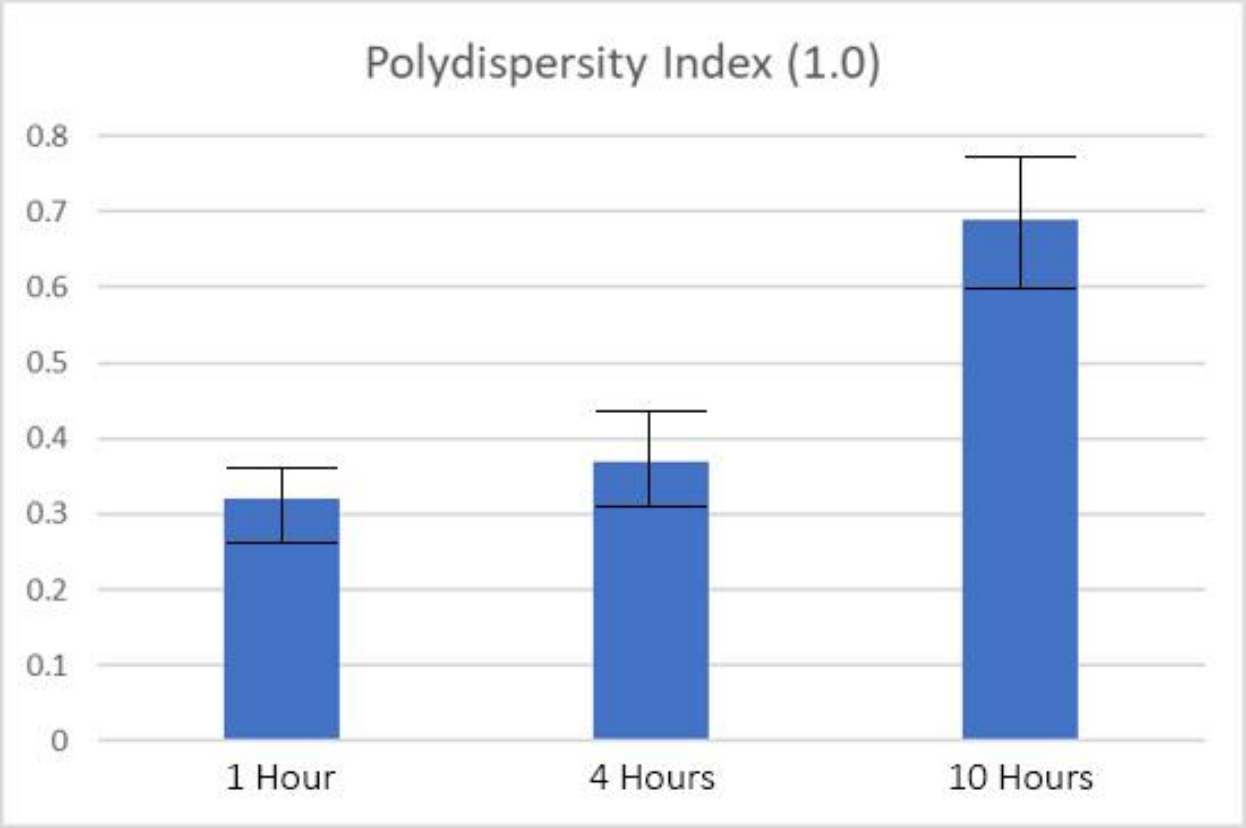

Figure 3.2. Polydispersity of navitoclax loaded IL varies at different time points within a 10-hour span. Increased time displayed higher diversity as well, displaying instability in the suspension.

Cell Internalization assay

Visual imaging of the coumarin 6 substitute was slightly modified using photo software to offer a clearer picture of cell internalization; the color of coumarin 6 was changed from orange/yellow to green to emphasize the integrative role associated with C6/IL [**Figure 3.3]**. Controls offered no green glow whatsoever, indicating negative presence of ulterior materials than may have offered colorimetric interference. Standalone C6 had some residual flakes post-washing. This may be due to the extracellular fluids holding the C6, or the adherence of the plate itself. This

may have allowed a small amount to stick, however very little C6 was able to penetrate the cells, which can be indicated by the lack of color mixing on those particular plates. However, the IL/C6 plates clearly display a vast distinction, producing a teal color mixture of blue and green within the cells, which is indicative of C6 entering almost all of the displayed cells. This supports the notion that IL acts as a penetration enhancer, allowing vastly more potential drug penetration at the cellular level.

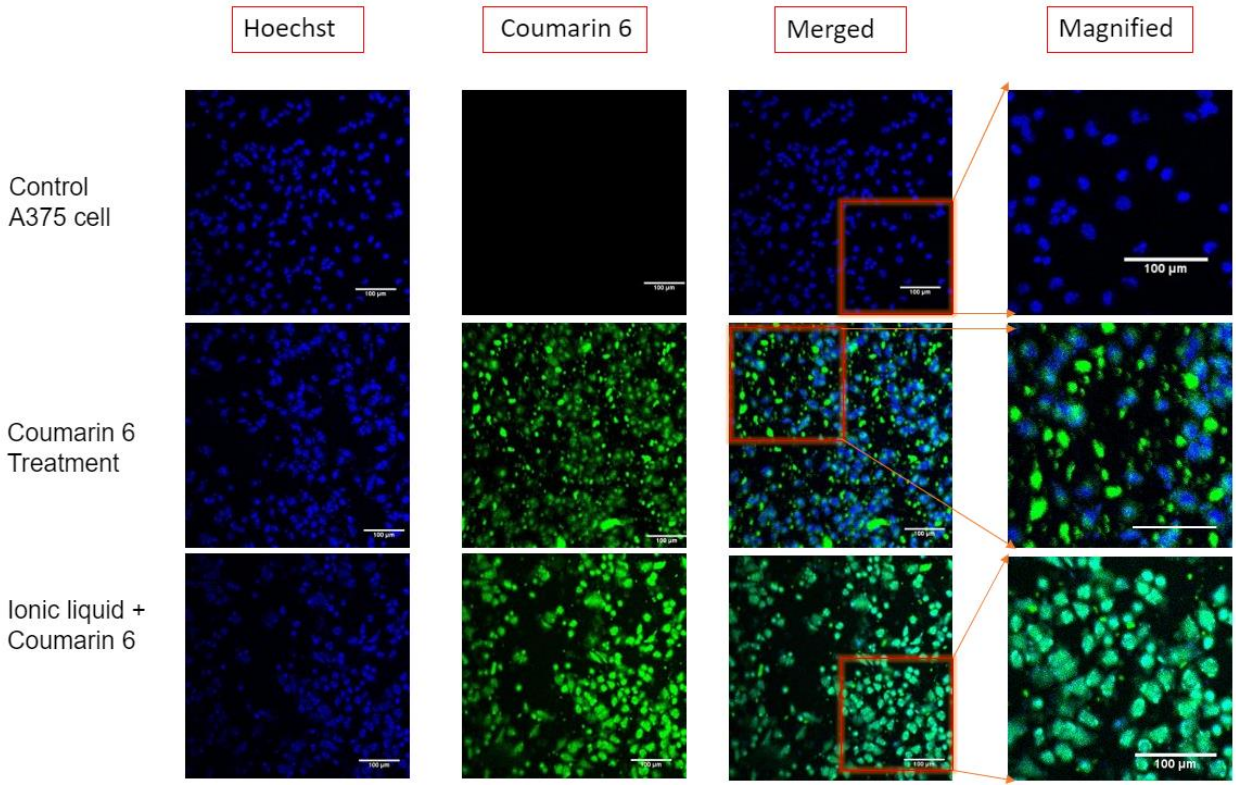

Figure 3.3. Potential of ionic liquid to enhance cellular uptake of a therapeutic molecule. The feasibility study was conducted with Coumarin 6, a hydrophobic small molecule with fluorescence properties, as a model molecule. The images show enhanced uptake of coumarin 6 by the A375 cell when co-incubated with ionic liquid compared to free coumarin 6, upon 4 hr of incubation.

LDH Toxicity assay

LDH offers insight into the cells themselves, fluorescing only when the lactate dehydrogenase successfully escapes the cell, and is a common indicator of cell death. Primary concerns with enhanced cell penetration involved the possibility that cell death would also increase due to this potential cellular damage involving the utilization of IL. Live cell percentages stayed consistent in wells containing only IL[**Figure 3.4]**. These wells increased their live cell % over the 6-hr period, indicating little to no toxicity for standalone IL. However, standalone navitoclax and the IL/navitoclax formulation both dropped at approximately the same level within the same time period, mimicking each other with similar cell death rates. Enhanced cell permeation did not act on an immediate time scale; however, it is important to take note that navitoclax is already highly effective as an inhibitor. From these results, one can derive that IL does not enhance immediate results and does not act as a toxic agent without navitoclax present. The LD50 is also extremely similar to that of standalone navitoclax, which is promising considering the aim of this study is more related to delivery than drug efficacy enhancement.

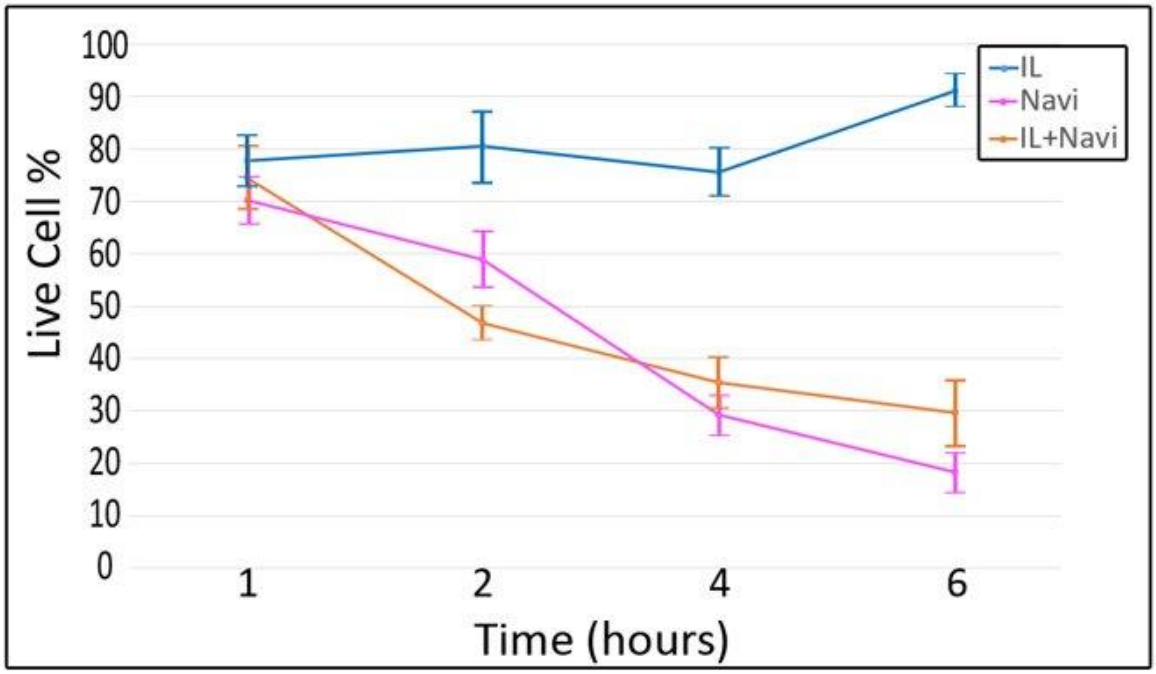

Figure 3.4. Cytotoxicity of ionic liquid, navitoclax and formulation was investigated with A375 cell upon incubation for various time points. Ionic liquid itself does not show any sign of toxicity whereas both free navitoclax and ionic liquid containing navitoclax show 70% cell death after 6hr of co-incubation. Data represent mean±SD, where n=6.

Ex Vitro Permeation Study

Ex Vitro permeation results displayed enhanced penetration in multiple layers of the skin, with minimal penetration entirely through the skin into the bloodstream. The SC layer had a high initial absorbance value in the PBS control when compared to the deeper epidermal layers, most likely due to residual material being left over after centrifugation[**Figure 3.5]**. However, IL/C6 clearly displayed significantly higher absorbance readings when compared to standalone C6. PLB and IL standalone both had similar values, emphasizing these individual controls similarities.

These increased absorbance values show that even in the outermost layer, absorbance was greatly enhanced. Although the SC was a secondary target, promising results of enhanced penetration bring value to overall therapy.

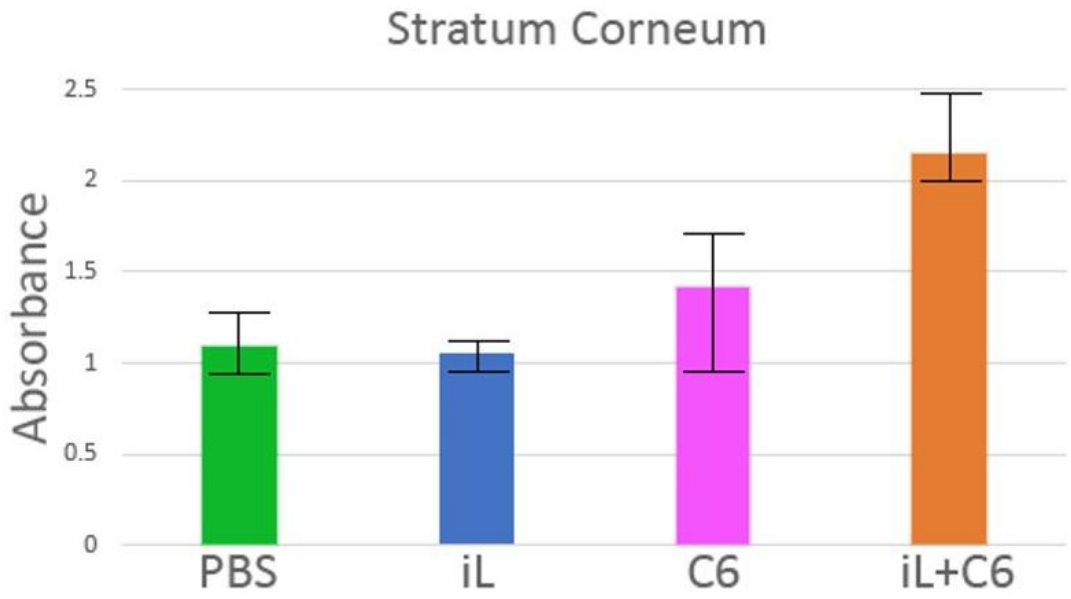

Figure 3.5. To investigate the diffusion ability of ionic liquid and potential as transdermal drug delivery carrier, we have performed a diffusion study with pig skin. The results show absorbance values of coumarin 6 within the stratum corneum is as much as 2 folds higher than PBS and free coumarin 6. Data represent mean±SD, where n=6.

Deeper into the epidermal layers, IL/C6 toppled absorbance values by a significant degree[**Figure 3.6**]. More importantly however, is the consistency when compared to standalone c6, which had a widely diverse range in both the epidermis and SC layers. This higher consistency lends credence to the notion that higher degrees of dose accuracy may be possible with this new delivery method.

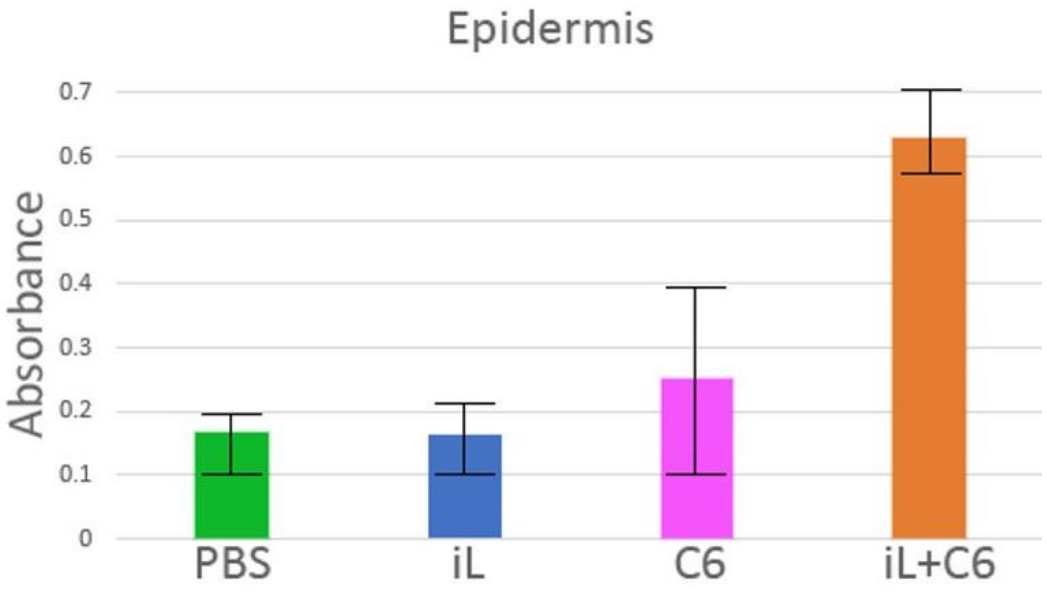

Figure 3.6. To investigate the diffusion ability of ionic liquid potential as transdermal drug delivery carrier, we have performed a diffusion study with pig skin. The results show that absorbance readings of coumarin 6 within the epidermis layer is as much as 3 folds higher than PBS and free coumarin 6. Data represent mean±SD, where n=6.

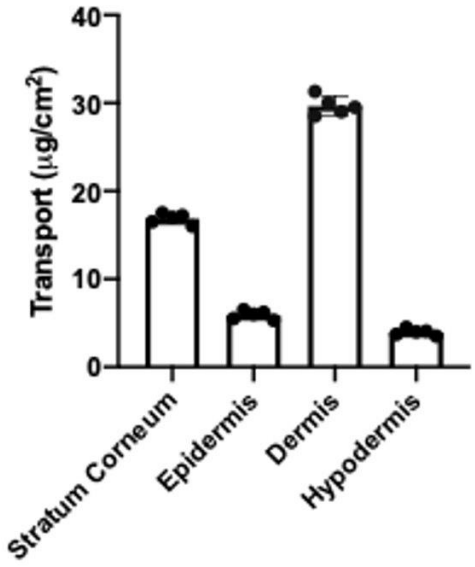

Figure 3.7. To investigated diffusion ability of ionic liquid and potential as transdermal drug delivery carrier, we have investigated diffusion study with pig skin. The results show that concentration of coumarin 6 within the dermis layer is 2 folds higher than Stratum Corneum and 5 folds higher than epidermis. Data represent mean±SD, where n=6.

Due to the recent COVID-19 outbreak, testing of the scintillon vials has not been possible due to lab restrictions. However, no visual observation of the C6 was present, indicating from the picture of the skin below [**Figure 3.8**] that if there were significant amounts of C6 that were able to traverse into the bloodstream, the distinctive color and glow would have been somewhat noticeable. Additionally, our recent collaboration with Harvard involved a similar study performed with IL/C6 and displayed similar results [**Figure 3.7**]. Sadly, the planned animal studies ended with the euthanasia of all mice meant for this study. One noteable limitation is that this unfortunate event left us with only coumarin 6 as a penetrative agent, which has a significantly lower molecular weight over navitoclax, and a lower hydrophobicity. According to previous studies[4], these

limitations should not be of consequence, but without proper testing, one cannot draw this conclusion with absolute certainty.

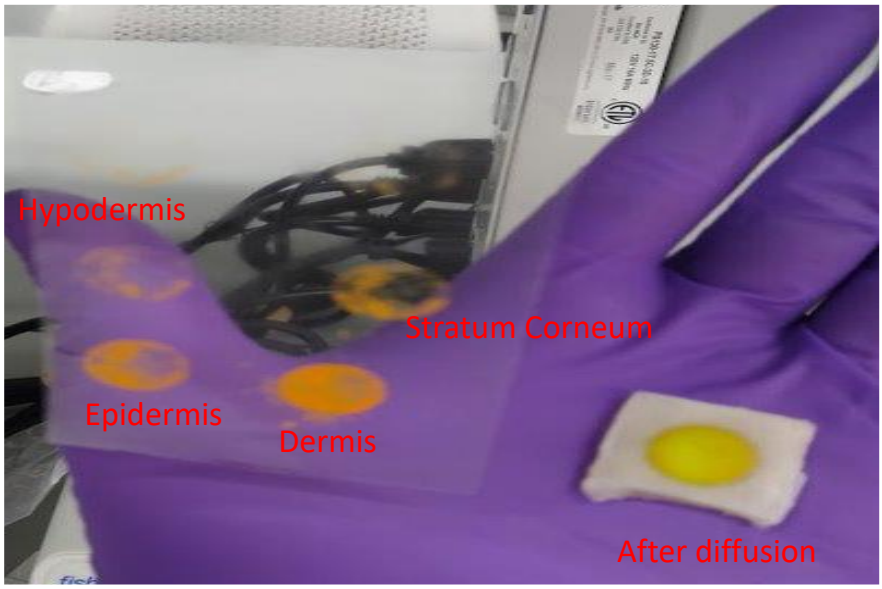

Figure 3.8. The photograph shows coumarin 6 in different layers of the skin after diffusion.

Conclusions

IL mediated transdermal transportation enhancement has shown promising results in the delivery of navitoclax or other similar molecules to the target site of epidermal layers, without traversing significantly into the bloodstream. This targeted drug delivery system has the possibility to offer stronger and more effective therapies to those who are afflicted with this debilitating and potentially deadly cancer. This type of application also offers the potential to increase patient compliance due to the non-invasive method and offers an avenue previously unattainable through conventional routes of drug administration. Looking back at the archaic methods of treatment involving an invasive and scarring surgery, the application of a simple ionic liquid, gel, or salve may be the potential lifesaving tool that will increase patient compliance and stop this disease from reaching the more deadly stages of cancerous growth, spread, and infection. The formulation can

be optimized with further studies in animal model and treatment feasibility studies in other skin related diseases such as scleroderma.

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DISSERTATION CONCLUSIONS

Overall, the culmination of these transdermal drug delivery systems presented in these studies has proved to be both an exciting and fruitful endeavor. Penetration of nano-scale molecules using an enhanced lipid-based carrier was successful in the transportation of one of the most widely used antifungal agents in history in a safe and more effective manner has essentially improved the outcomes of one of the most popular antifungal combatants used in conventional medical application. This ability to improve one of the most affordable treatments may have a significant impact in the safe, effective, and easy-to-use tools in a physicians arsenal. This improvement has the ability to save many lives if taken to the commercial market.

Contrarily, a rare, large-molecule, experimental, anti-melanoma drug has been shown to be able to traverse the SC layer of the skin in large amounts and attack the target site without the use of systemic circulation. This revolutionary way of penetrating the skin and cells may be a new avenue in fighting one of the most common cancers in the United States. This form of therapeutic relief may circumvent the plethora of side-effects associated with more popular forms of chemotherapy and may soon change the future of how certain cancers are dealt in the clinic. The association of an IV needle and cancer is now slightly closer to becoming an archaic evocation of scientific progress.

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Isaac Giovanni Deaguero holds a Bachelors of Science in Biology from the University of Texas at El Paso(2014). He is a Veteran of the Unites States Navy(2003-2008), where he served as a Fire Control Technician onboard two nuclear submarines and attained the rank of Petty Officer Second Class (E-5). During his graduate studies, he taught various courses including pharmaceutical compounding, global health for biomedical engineers, and biomedical device design & regulation.

Published Works

Hossain, R. F., **Deaguero, I. G.**, Boland, T., and Kaul, A. B. (2017). Biocompatible, large-format, inkjet printed heterostructure MoS2-graphene photodetectors on conformable substrates. *npj 2D Mater. Appl.* 28, 1–10.

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