TRANSDERMAL DELIVERY OF BIOMACROMOLECULES USING LIPID-LIKE NANOPARTICLES

A thesis submitted by

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ABSTRACT

Transdermal delivery is one of the methods for administering drugs through the skin both locally and systemically. The transdermal delivery of biomacromolecules, including proteins and nucleic acids, is challenging, owing to their large size and the penetration-resistant nature of the stratum corneum. Thus, an urgent need exists for the development of transdermal delivery methodologies. This research focuses on the use of cationic lipid-like nanoparticles (lipidoids) for the transdermal delivery of proteins, and establishes an in vitro model for the study. The lipidoids used were first combinatorially designed and synthesized; afterwards, they were employed for protein encapsulation in a vesicular system. This research has successfully identified active lipidoids capable of efficiently penetrating the skin; therefore, loading proteins into lipidoid nanoparticles will facilitate the transdermal delivery of proteins.

Chapter one introduces the research; the need and the goals of developing a novel method for enhancing the transdermal delivery of biomacromolecules. Chapter two gives a review of the literature by giving background information on transdermal delivery. Chapter three explains the materials and methods employed for the study. Chapter four describes the penetration study of this research. A skin penetration study demonstrated that lipidoids enhance penetration depth in a pig skin model, overcoming the barrier that the stratum corneum presents. Chapter five describes the permeation study which is for studying the penetration of protein from one layer of the skin to another. This study shows that the protein is able to travel through the epidermis to the dermis of the skin and also through the full-thickness skin samples. Chapter six provides the conclusion of the entire research and findings. It also discusses future directions on investigating the enhancement of transdermal delivery of biomacromolecules using lipidoids.

DEDICATION

I dedicate this thesis to my loving and outstanding father, Late Lucian Adetuyi Ige, for teaching me that knowledge and its application is power. I will continue to love and cherish you. May your gentle soul rests in perfect peace.

ACKNOWLEDGMENTS

I thank God Almighty for His grace, favor, mercy and blessings throughout my stay at Tufts University and especially for seeing me through the research.

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My deepest and special thanks goes to my mother, Mrs. Felicia Ige and my siblings, Victor Ige, Jude Ige and Regina Ige for their care, love, support, encouragement and best wishes. I also appreciate the effort and support of some of my friends, Bridget Harrison, Mauricio Dinarte, Benjamin Melançon and Micky Metts. I would like to extend my unflinching love to my precious daughter, Anne Bello, for her understanding, love and even kisses when I'm tired. I love you my baby.

Finally, thanks to my husband, Aaron Bello, for his support and willingness to manage our household activities when I was working on my research. I cannot forget those nights you spent with me at school just to support my research.

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CHAPTER ONE

INTRODUCTION

Recently, transdermal drug delivery has gained interest due to its obvious advantages such as the possibility of localized drug therapy, avoidance of first pass metabolism and the minimal side effects [Wagner et al., 2001]. This mode of drug delivery involves drug administration or application through the skin. The skin with its large contact area is a very useful and significant route to administer drugs locally and systemically. One major challenge of transdermal drug delivery is the protective function of the skin whereby it acts as a barrier to passage of drugs. The skin being the largest organ of the body, with more than 10% of the body mass and average surface area of $2m^2$, presents opportunities to interact intimately and dynamically with the surrounding environment. The skin provides a multifunctional interface between the body and its surrounding media, and hence is very vital to human survival in an aggressive environment. The functions of the skin are protective, homeostatic, and sensorial in nature. The first two are mainly a function of its barrier properties, allowing humans to protect themselves and adapt to changes in temperature, relative humidity and presence of dangerous substances such as chemicals, bacteria, allergens, radiation and others. Due these properties, the skin membrane is capable of limiting the molecular transport from and into the body. Transdermal drug delivery is possible only by overcoming this barrier function. The stratum corneum which is the outermost layer of the epidermis consists of 15 – 25 flattened, stacked, hexagonal, and cornified cells anchored in a mortar of highly organized intercellular lipids. This structure known as the brick and mortar model controls the rate of transdermal absorption of substances and poses a challenge to drug delivery into and through the skin [Contreras, 2007].

Several methods are being employed to overcome the challenges offered by stratum corneum. Some of these methods are:

- Electroporation in which short and high voltage pulses are used to reversibly disrupt cell membranes for gene transfection and other applications
- Biochemical enhancers which are highly specific bioactivity enabled peptide chemistry that can enable delivery via targeted routes through the skin
- Ultrasound to provide the driving force to move drugs into the skin by disrupting stratum corneum lipid structure and thereby increase permeability
- Iontophoresis in which a continuous low voltage current is used as an electrical driving force for transport across the skin
- Microneedles used for piercing the stratum corneum with very short needles
- Thermal ablation for heating the skin surface to generate micron-scale perforations in the stratum corneum
- Microdermabrasion which is akin to the use of sandpaper to alter and remove skin tissues
- Nanoparticles whose nano-size helps to improve drug transport into the skin and through biological membranes by encapsulating a variety of molecules.

The existing methods of enhancing transdermal delivery have disadvantages such as low delivery efficiency, cost and the need for additional equipment. The disadvantages call for a novel method for enhancing transdermal delivery. This research is undertaken to study the transdermal delivery of biomacromolecules using lipid-like nanoparticles (lipidoids). The research aims at the studying lipid-like nanoparticles for transdermal delivery of biomacromolecules including proteins and siRNA (short interfering Ribonucleic acid), and establishing the in vitro model for this study. This study focuses on the use of protein, a biomacromolecule. Several protein and

peptide-based drugs have been formulated for therapeutic and clinical applications due to the recent advances in the field of pharmaceutical biotechnology. The therapeutic outcome of a drug depends on the route of administration as well [Jitendra et al, 2011]. The nano-size (50 to 300 nm) and other novel properties of the lipid-like nanoparticles (lipidoids) help to inprove drug transportation into the skin; and through the biological membranes by encapsulating molecules into the nanoparticles. These lipidoids have been studied for intracellular delivery of cytotoxic protein for cancer treatment [Wang et al., 2014]. Walters reported that for efficient penetration of particles through the skin, the particle size must be less than 3 μm; and hence the hypothesis is that the lipidoids can be used for the transdermal delivery due to the possibility that lipidoids can be used for the formulation of protein nanoparticles. The research will involve two test systems; one is to study the permeation of the protein through the skin and the second test system is for studying the penetration of the protein into other layers of the skin.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 SKIN

Skin is comprised of specialized cells and structures for its function of protection, as it forms the barrier between the underlying organs and the external environment. It also plays its role in the regulation of the body temperature to maintain the functions of the body. Apart from that, it is also a sensory organ that plays an important role in providing immunity against different diseases [Brannon, 2014].

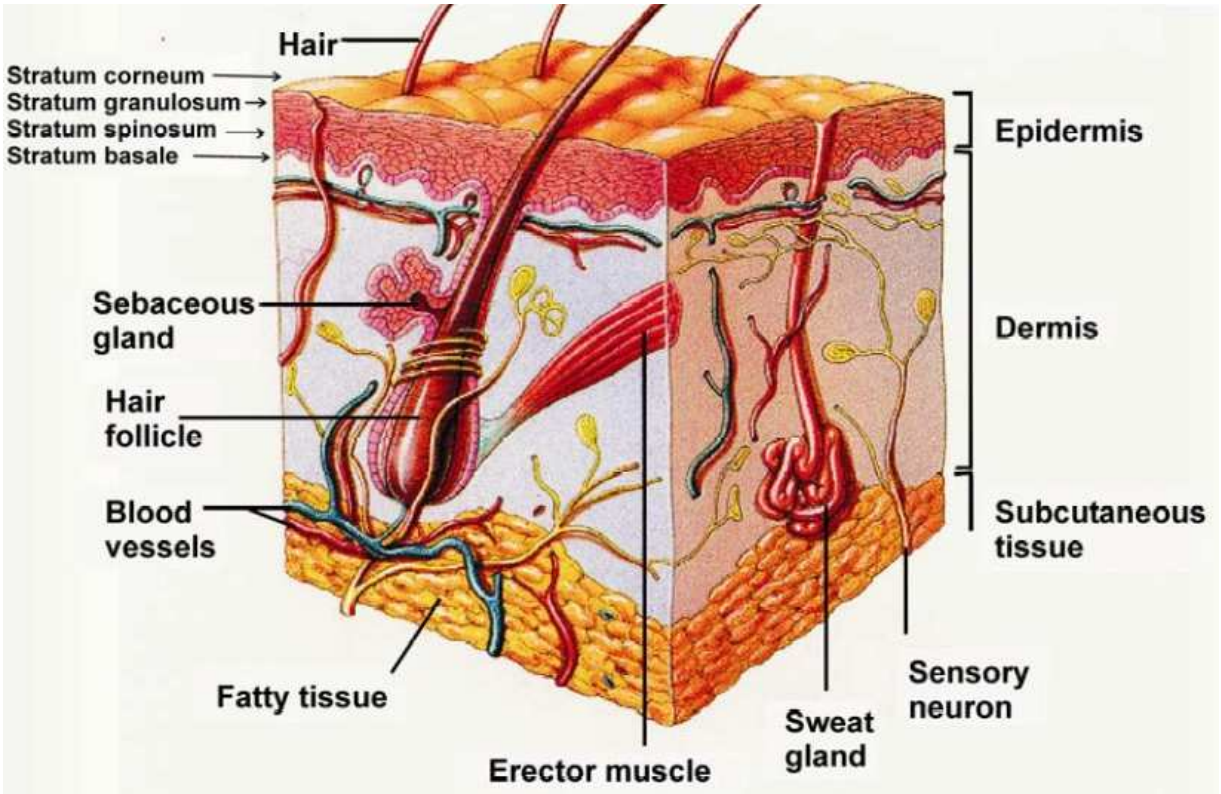


Figure 1: The structure of the skin [Contreras, 2007].

2.2 LAYERS OF THE SKIN

In order to understand the function of the skin, it is important to note here that it is divided into the epidermis, the dermis and the subcutaneous tissue [Brannon, 2014]:

2.2.1 Epidermis

The epidermis is the first and outermost layer of the skin, which measures around 0.05 mm (on the eyelids) to about 1.5 mm (on the soles and palms). The five layers of the epidermis are stratum basale, stratum spinosum, stratum granulosum, stratum licidum and stratum corneum [Brannon, 2014].

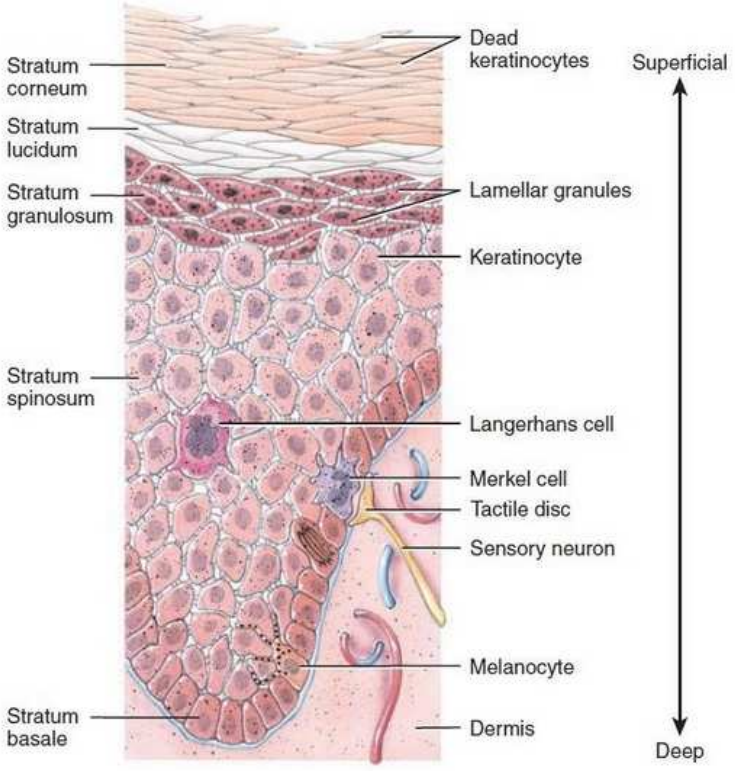


Figure 2: The layers of the epidermis.
[https://classconnection.s3.amazonaws.com/285/flashcards/2127285/jpg/epidermis-13E98A1132C76706014.jpg]

The outermost layer of the epidermis is the Stratum corneum and it measures about 10-20 micrometers in thickness; however this thickness varies according to different sites of the body. In a single cross-section this layer is made up by 15-25 hexagonaly shaped flat cells that are arranged in a proper manner by the cellular lipids. These flat cells are referred to as corneocytes or horny cells. The aforementioned structure of lipids and cells is said to be as mortar and brick model, which is considered to be important with respect to the regulation of the transdermal transport of substances [Contreras, 2007]. The stratum basale, the deepest layer, is composed of column-shaped cells. The cells of this layer divide and push these cells to the upper layers. As the cells move up, they become flat and part of the dead cell layer. Stratum corneum is composed of flat and dead cells that are replaced every 2 weeks [Brannon, 2014].

Epidermis contains three kinds of specialized cells; these are [Brannon, 2014]:

a) The melanocyte which is known to produce pigment (melanin).
b) The Langerhans' cell serves as the first line of defense for skin immune system.
c) The Merkel's cell's

2.2.2 Dermis

The dermis which is known to provide strength to the epidermis is composed of a fiber layer. The thickness of the membrane is supposed to range between 2-3 mm. Dermis comprised of a matrix of loosed connective tissue composed by collagen, a fibrous protein, embedded in a semi gel matrix that contains water, ions and mucopolysaccharides. The prime function of the matrix is to hold the cells and allow the oxygen and nutrients to diffuse to the epidermal cells. This layer is comprised of blood vessels and nervous network, as well as hair follicles, sebum and sweat (eccrine) glands. The papillary layer plays not only a nutritional function but is also known for

its role in maintaining temperature, pressure, and pain regulation [Contreras, 2007]. Blood vessels and nerves pass thorough this layer. Three types of tissues related to the layer are collagen, elastic tissue and reticular fibers. The two layers of the dermis are papillary and reticular layer. Thin arrangement of collagen fibers is contained in the upper, papillary layer. The lower, reticular layer is comprised of thicker collagen fibers that are settled equivalent to the skin surface [Brannon, 2014].

The dermis is comprised of numerous specialized cells and these cells are [Contreras, 2007] [Brannon, 2014]:

1. Fibroblasts, responsible for connective tissue synthesis.
2. Mast cells, involved in inflammatory and immune responses.
3. Melanocytes, involved in melanin production
4. Vater-Pacini and Meissner's corpuscles, these are specialized nerve cells that transmit the sensations of touch and pressure.

2.2.3 Subcutaneous Tissue

Subcutaneous tissue or hypodermis is the deepest layer of the skin [Escobar-Chávez et al., 2012]. The subcutaneous tissue is a layer of fat and connective tissue that houses larger blood vessels and nerves. The hypodermis acts as a shock absorber, a heat insulator, and a region of energy storage. The major role of hypodermis is to carry the vascular and neural systems for the skin. The layer performs the main task of regulation of the thin layer differ all over the body and from individual to individual [Brannon, 2014]. It also anchors the skin to the underlying muscles. In this layer, the cells present are [Escobar-Chávez et al., 2012]:

1. Fat cells are arranged in lobules and associated to the dermis through connecting elastin fibers and college.
2. Fibroblasts
3. Macrophages

2.3 SKIN FUNCTIONS

The functions of the skin are viewed as being vital for the survival of the human body and organs against the hostile external environment. Generally, the functions can be categorized into sensing, homeostasis, and protection. The significance of the homeostatic and protective functions makes it possible for the human beings to withstand the changing temperature of the environment, the presence of bacteria, radiation, fungi, and allergens as when as the changes in the water content of the body. Secondly, the skin is considered to be the most important organ for control of blood pressure, heat regulation, and composition. The stratus corneum helps regulate the water losses from internal components of the body. The epidermis, on the other hand, regulates pain, pressure, and temperature. The sebaceous gland and the hair follicle lubricate the skin with sebum, and the apocrine and eccrine glands play the role of cooling and protection. Heat insulation is specifically the function of the hypodermis, as it also stores energy and acts as a shock absorber. The neurovascular components of the skin are also carried by the hypodermis [Escobar-Chávez et al, 2012].

2.4 TRANSDERMAL DELIVERY

Transdermal drug delivery is used for administering drugs through the patient's skin and is an alternative for oral and hypodermic injection delivery [Sadrieh, 2009] [Prausnitz and Langer, 2008]. The first adhesive transdermal delivery system, which is a patch for drug delivery of scopolamine for motion sickness, was approved by the Food and Drug Administration (FDA) in USA in 1979. Since then several transdermal delivery systems have been designed, approved and used. Nicotine patches were approved in 1990 and around 1billion patches have been manufactured to date. Patches are being used for transdermal delivery of several drugs. Some of these are analgesics like fentanyl, contraceptives like ethinyloestradiol and norelgestromin, anesthetics like lidocaine, estradiol for female sex hormone, testosterone for hypogonadism and oestradiol and progesterone for hormone replacement [Prausnitz and Langer, 2008] [Margetts and Sawyer, 2007].

2.4.1 Advantages and Challenges of Transdermal Drug Delivery

In general, transdermal delivery is noninvasive and easy to use. Advantages of transdermal delivery when compared to oral drug delivery are [Sadrieh, 2009]:

- Bypass first-pass inactivation by the liver.
- Improved patient compliance.
- Easier for patients with difficulty in swallowing tablets or capsules.
- Controlled delivery through the skin can provide less fluctuation in circulating levels of drugs (avoid drug spike levels seen after oral delivery).
- Avoids irritation of the gastrointestinal (GI) mucosa.
- Termination of dosing by removal of transdermal drug delivery system (TDDS).

Advantages of transdermal delivery when compared to hypodermic injection delivery include [Wilson, 2011]:

- Eliminates the first pass effect where active drug molecules are rendered inactive or cause side effects.
- Provides steady plasma levels because the drug delivery rate of the patch is constant.
- Easy to use and noninvasive.

Challenges of transdermal drug delivery [Wilson, 2011] [Uchechi et al, 2014] are:

- Currently transdermal delivery is limited to a narrow range of molecules like lipophilic molecules which are small and can pass through the lipid bilayer "mortar" of the stratum corneum using traditional patch technology. Complex treatments involving larger molecules cannot be delivered using this method. Nicotine, estradiol, scopolamine, testosterone, fentanyl have molecular weight of 162.24 g/mol, 273.29 g/mol, 303.35 g/mol, 288.42 g/mol and 336.47 g/mol respectively. These molecules are organic, lipid soluble and can be delivered through the skin. These molecules are nonpolar, made of carbon and hydrogen atoms but still consist of polar atoms like oxygen and nitrogen. Molecules like fentanyl that have molecular weight over 300 g/mol; are categorized as large organic molecules, but smaller than a small protein like insulin.
- Only small drug quantities can be delivered and hence they have to be drugs which are potent even at low doses.
- Drugs that require high blood levels cannot be administered.
- Can cause sensitization or irritation which must be evaluated fairly early in the development process.

- Special equipment is required for manufacture. The formulation is more expensive than conventional dosage forms and therefore non-economical for most patients.
- The lag time for the drug to reach systemic circulation makes it ineffective for drugs requiring rapid action. It can be used only for low dosage and high permeable drugs with molecular weight less than 400, logP=2-3 and dosage less than 10 mg.

2.4.2 Transdermal Routes

Drug penetration happens through different transdermal routes. The drug diffuses through skin appendages like hair follicles and sweat glands which form shunt pathways through the intact epidermis, occupying only 0.1% of the total human skin [Escobar-Chávez et al., 2012].

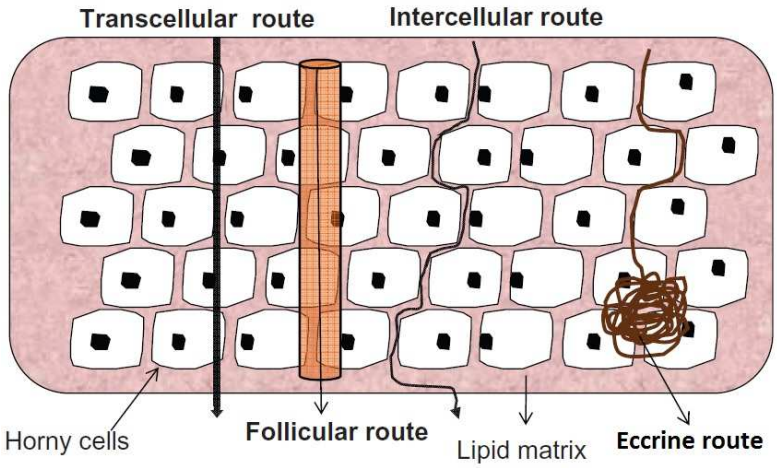


Figure 3: Transdermal routes [Escobar-Chávez et al., 2012].

a) The intercellular lipid route (between the corneocytes)

Non-planar spaces are formed between crystalline lipid lamellae and their adjacent cells' outer membrane, due to the presence of less ordered lipids and flexible hydrophobic chains. The transepidermal diffusion of lipidic and amphiphilic molecules happens through the non-planar

spaces [Xiang and Anderson, 1998] [Geinoz et al, 2004]. Hydrophilic molecules diffuse laterally along the surfaces of the few water filled inter-lamellar spaces. Polar molecules use the free space between a lamella and the outer membrane of a corneocyte for diffusion [Cevc, 1997].

b) The transcellular route (through the corneocytes)

Keratin present in the intracellular macromolecular matrix of stratum corneum gives it mechanical support [Cevc and Vierl, 2010]. The narrow aqueous transepidermal pathways have regions of poor cellular and intercellular lipid packing result in wrinkles on the skin surface and also offer lower resistance to the transport of hydrophilic molecules. This pathway lies between clusters of corneocytes in areas with no lateral overlap [Schätzlein and Cevc, 1998]. Hydrophilic conduits have openings ≥5 μm in skin appendages and ≤10 nm in narrow inter-corneocyte pores. Sweat ducts (≥50 μm), pilosebaceous units (5-70 μm), and sebaceous glands (5-15 μm) offer largest width and lowest resistance [Mitragotri, 2003]. Pore penetrant or opener can open hydrophilic conduits across the skin are which are 20-30 nm wide, through its thickness [Schätzlein and Cevc, 1998]. By electro-osmosis, the pores can be expanded to around of 22-48 nm [Aguilella et al, 1994]. The lipophilic cutaneous barrier's height is governed by molecular weight and distribution coefficient rather than molecular size [Johnson et al, 1997]. Its height decreases with increasing lipophilicity of permeant. However, permeant molecules heavier than 400-500 DA are too large for diffusion [Mitragotri, 2003] [Johnson et al, 1997] [Guy, 2003].

c) Follicular Penetration

Follicular penetration is a major focus area in the treatment of skin diseases. Though follicular orifices occupy only 0.1% of the total skin surface area, the hair follicles can be a way to trough the skin [Escobar-Chávez et al, 2012]. Use of follicular pathways for topical administration of polystyrene nanoparticles was studied ex vivo in porcine skin and in vivo in human skin. Surface

images showed polystyrene nanoparticles accumulating more in follicular openings, distribution increasing in a time-dependent manner, and follicular localization being favored by smaller particle size. There was similarity in the penetration between porcine and human skin membranes. Nanoparticles can be used as efficient drug carriers through the follicle or as follicle blockers to stop the penetration of topically applied substances [Uchechi, 2014].

2.4.3 Systems for Transdermal Delivery

The backbone of the transdermal drug delivery system is polymers. For transdermal delivery, the systems are fabricated as multilayered polymeric laminates. In this, a drug–polymer matrix or a drug reservoir is sandwiched between two polymeric layers. The systems of Transdermal drug delivery are categorized into three major types as: matrix, reservoir, and microreservoir [Kandavilli et al, 2002] [Sadrieh, 2009]. The outer impervious backing layer prevents the drug loss by the backing surface, while inner layer acts as a rate-controlling or an adhesive membrane. The layers delivery systems are backing layers, release liners, rate-controlling membranes, matrix formers and PSAs (Pressure-sensitive adhesives) [Kandavilli et al, 2002].

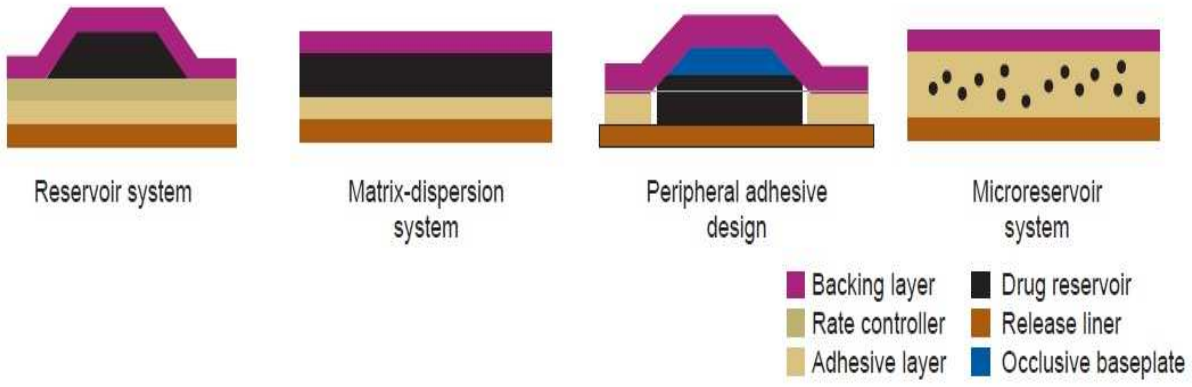


Figure 4: Transdermal delivery systems [Kandavilli et al, 2002].

2.5 METHODS OF TRANSDERMAL DELIVERY ENHANCEMENT

The skin acts as a barrier limiting drug delivery through the stratum corneum. Several physical and chemical methods have been studied to enhance drug delivery mechanism [Margetts and Sawyer, 2007]. An ideal enhancer should have the following characteristics [Prausnitz and Langer, 2008]:

(i) Disrupt the stratum corneum structure to increase skin permeability

(ii) Provide an added driving force for transport of drugs into the skin.

(iii) Ensure that the deeper, living tissues are not damaged

2.5.1 Non-cavitational Ultrasound

Therapists discovered that using ultrasonic heating probes for massaging anti-inflammatory agents into the skin increased efficacy, thereby recognizing the potential of ultrasound as a skin permeation enhancer. Ultrasound consists of an oscillating pressure wave at a frequency too high for human ear to detect. Ultrasound functions by disrupting stratum corneum lipid structure and thus increasing permeability, although there are speculations that the pressure gradients and oscillation created provides an added driving force to permeate drugs into the skin. Non-cavitational ultrasound is commonly used to enhance small, lipophilic compounds [Prausnitz and Langer, 2008].

2.5.2 Cavitational Ultrasound

This type of ultrasound can cause cavitation, which is the formation, oscillation and, sometimes, collapse of bubbles in an ultrasonic pressure field. Cavitation is only generated under certain specific conditions (like in the case of low-frequency ultrasound) which are different from that of ultrasonic heating or imaging devices. Cavitation bubbles concentrate the energy of ultrasound and thus cause targeted effects at the site of bubble activity [Wu and Nyborg, 2006] [Ogura et al, 2008]. This presents a lot of opportunities in transdermal drug delivery. Cavitation occurs within a coupling medium (e.g., a hydrogel) between the ultrasound transducer and skin, as it is difficult to create and oscillate bubbles within densely-packed tissue. In a cavitational ultrasound, the bubbles oscillate and collapse at the skin surface. This generates localized shock waves and liquid microjets directed at the stratum corneum, thereby disrupting its lipid structure and increasing skin permeability for several hours without damaging deeper tissues. It can increase skin permeability to both small and macromolecules as large as tens of kilodaltons [Prausnitz and Langer, 2008]. However, it does not create a driving force for transport. One main disadvantage of ultrasound was the large size of the device needed to send the sound waves [Maione et al., 2002].

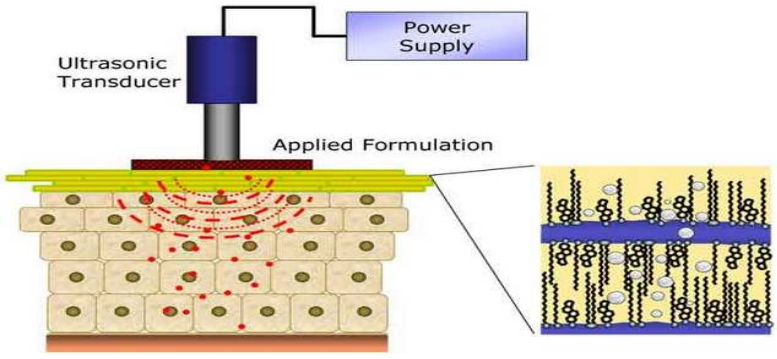


Figure 5: The use of ultrasound for transdermal delivery enhancement.
[http://www.skin-care-forum.basf.com/images/37_strategien-zur-verbesserung-der-hautpenetration/strategies-for-skin-penetration-enhancement_skinpenetration37_06_large.jpg?sfvrsn=2]

2.5.3 Electroporation

Electroporation is the use of short, high-voltage pulses to reversibly disrupt lipid bilayer structures in the skin and cell membranes for gene transfection and other applications [Denet et al, 2004][Li, 2008]. The electric field applied provides an electrophoretic driving force and diffusion happens through electropores which can last several hours. Thus order of magnitude of transdermal transport increases for small model drugs, peptides, vaccines and DNA. The order of magnitude of electrical resistance of stratum corneum is greater than that of deeper tissues Because of this; the electric field applied is initially concentrated in the stratum corneum. However, once the stratum corneum's lipid bilayers are subjected to electroporation its resistance drops rapidly, and the electric field distributes into the deeper tissues containing sensory and motor neurons, thereby causing pain and muscle stimulation [Pliquett and Weaver, 2007]. Due to complexities in device design electroporation has limited applications in transdermal delivery in humans [Prausnitz and Langer, 2008].

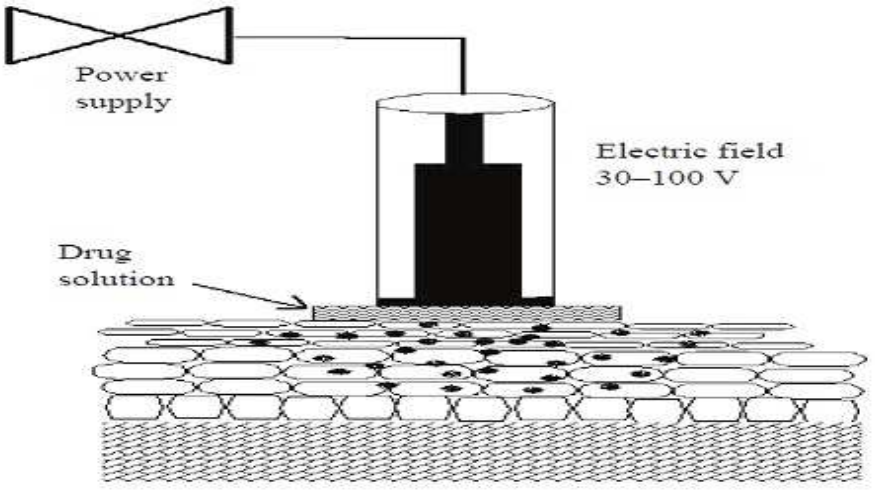


Figure 6: Transdermal enhancement using electroporation.
[http://i1082.photobucket.com/albums/j370/rajesh190888/pharmatutor-art-1465-10.png]

2.5.4 Microneedles

Microneedles help deliver drugs into the skin by selectively permeating it in a minimally invasive manner, without reaching the nerves in the underlying tissues. Solid microneedles can pierce the skin, without causing pain, to increase skin permeability for several small molecules, proteins and nanoparticles from an extended-release patch. Drug formulations are coated on or encapsulated within them for rapid or controlled release of peptides and vaccines in the skin [Prausnitz and Langer, 2008]. Microneedles can be 200-750 microns in length and are fabricated in batches called arrays that can contain 150-650 microneedles/cm^2. They are made of silicon, metal, sugar and plastics [Wilson, 2011].

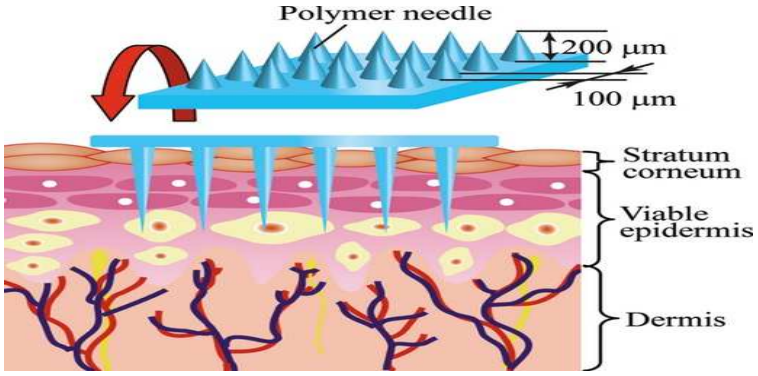


Figure 7: Microneedles for transdermal delivery.
[http://nanolithography.spiedigitallibrary.org/data/Journals/MOEMS/23521/011503_1_1.png]

2.5.5 Thermal Ablation

Thermal ablation generates micron-scale perforations in the stratum corneum by selectively heating the skin surface. Transiently heating of the skin's surface to extreme temperatures for microseconds to milliseconds localizes heat transfer painlessly and without damaging any viable tissues This involves rapidly vaporizing water in the stratum corneum, so that the resulting

volumetric expansion ablates micron-scale craters in the skin's surface [Prausnitz and Langer, 2008].

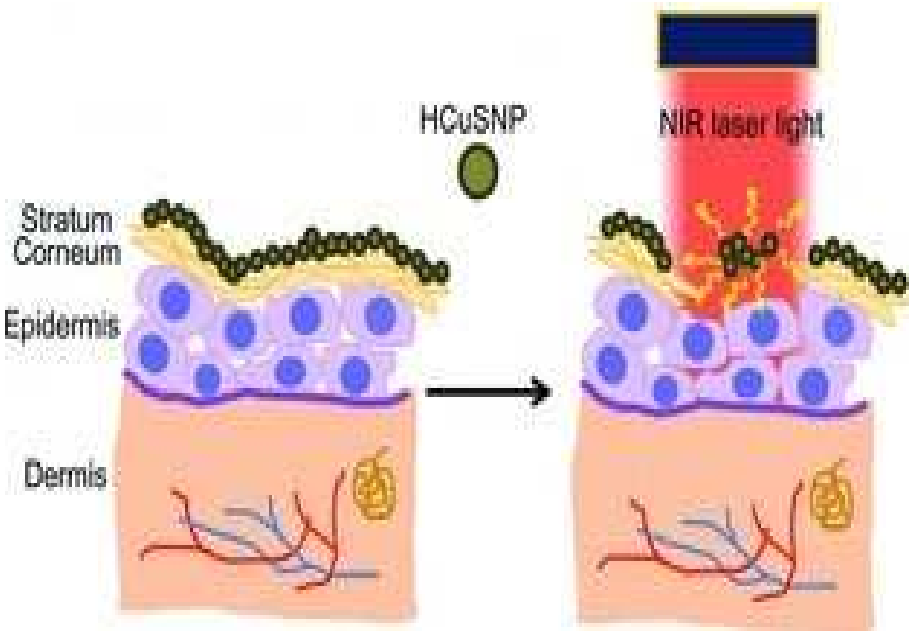


Figure 8: Photothermal ablation-enhanced transdermal delivery.
[http://onlinelibrary.wiley.com/store/10.1002/smll.201200783/asset/image_m/mcontent.jpg?v=1&s=da53847de1addc5487f9b0db7e70c45be1d63aff]

2.5.6 Microdermabrasion

Microdermabrasion is widely used for altering and removing skin tissue for cosmetic purposes. This abrasive mechanism, which is similar to sand blasting on a microscopic scale, increases skin permeability to drugs like lidocaine and 5-fluorouracil. This opens up opportunities for using it in transdermal drug delivery [Illel, 1997]. Skin abrasion using sandpaper facilitates vaccine delivery across the skin [Xiang and Anderson, 1998].

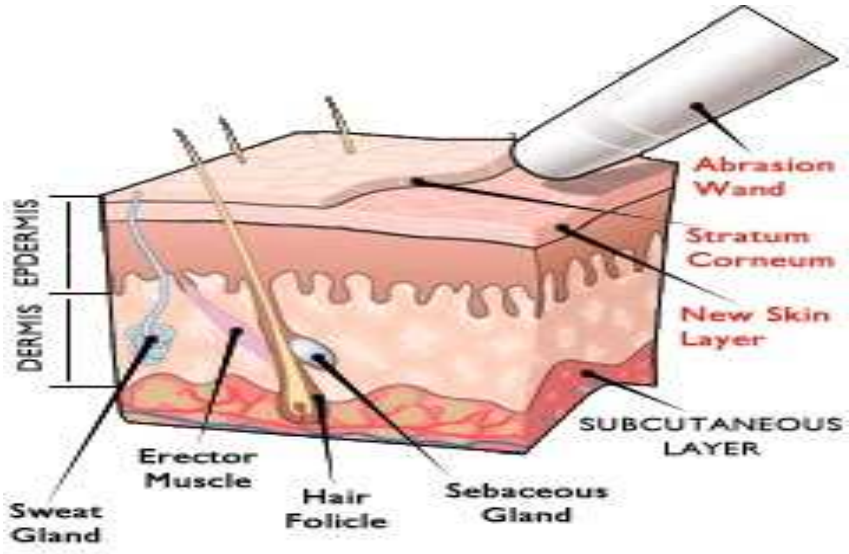


Figure 9: Microdermabrasion for enhancing transdermal delivery.
[http://ottawamedispa.ca/wp-content/uploads/2014/06/micro.gif]

2.5.7 Biochemical Enhancers

Peptides have been recently found to enhance skin permeability. In one method, screening a peptide library using phage display yielded an 11-amino acid synthetic peptide that enhanced transdermal delivery of insulin in diabetic rats by targeting a pathway via hair follicles [Chen et al, 2006]. A natural pore-forming peptide, magainin, can be used to increase skin permeability through stratum corneum lipid bilayer disruption without affecting deeper tissues [Kim et al, 2007]. The magainin was effective only when used with a surfactant chemical enhancer. The chemical enhancer increases skin permeability to the drug and magainin penetration into the stratum corneum. Covalently attaching cyclosporine to a polyarginine-heptamer cell-penetrating peptide, led to increased topical absorption that inhibited cutaneous inflammation [Rothbard, 2000]. These examples show that peptide chemistry causes a highly specific bioactivity which can enable delivery via targeted routes through the skin [Prausnitz and Langer, 2008].

2.5.8 Iontophoresis

Iontophoresis involves the continuous application of low-voltage current to provide an electrical driving force for transporting drugs across the stratum corneum by increasing skin permeability [Pikal, 2001]. Iontophoresis does not primarily change the skin barrier and hence is commonly used for transporting small charged molecules and a few macromolecules up to a few thousand Daltons. The rate of drug delivery varies with the electrical current and can be readily controlled by a microprocessor or the patient. So drug delivery can be turned on, off or modulated over time to enable complex delivery profiles and manage pain due to the method's inability to localize its effects to the stratum corneum [Prausnitz Mark and Langer, 2008].

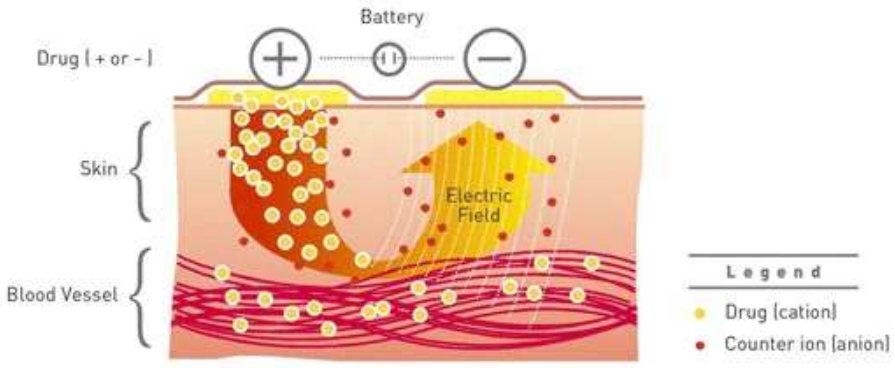


Figure 10: Transdermal delivery enhancement using Iontophoresis method.
[http://felixjtapia.org/blog/wp-content/uploads/2013/12/active-iontophoresis-1.gif]

2.5.9 Chemical Enhancers

Chemical enhancers disrupt the highly ordered intracellular lipid bilayers in stratum corneum. Known permeation enhancers include solvents (water, alcohols, pyrrolidones, dimethyl sulfoxide (DMSO), and l-dodecylazacycloheptan- 2-one also called Azone), urea, fatty acids such as oleic acid, sugar esters, and surfactants [Amsden and Goosen, 1995]. This is done by inserting

amphiphilic molecules into these bilayers to distort the molecular packing or by using solvents and surfactants to extract lipids and create lipid packing defects of nanometer dimensions. One disadvantage of chemical enhancers is that skin irritation increases with permeation enhancement. Chemical enhancers can also provide an added driving force for drug transportation by increasing drug partitioning into the skin and thereby increasing the concentration gradient driving diffusion [Prausnitz and Robert Langer, 2008]. Liposomes, dendrimers and microemulsions can also act as chemical enhancers that can increase skin permeability, drug solubilization in the formulation and drug partitioning into the skin [Kogan and Garti, 2006][Touitou and Godin, 2007].

2.6 NANOCARRIER SYSTEMS

Nanocarriers have several advantages like increased drug absorption, penetration, half-life, bioavailability and stability. They are too small that they can remain undetected by immune system and transport drugs to the target organ at lower doses with minimal side effects. Nanocarriers can be administrated in multiple ways one of which is the dermal route [Escobar-Chávez et al, 2012].

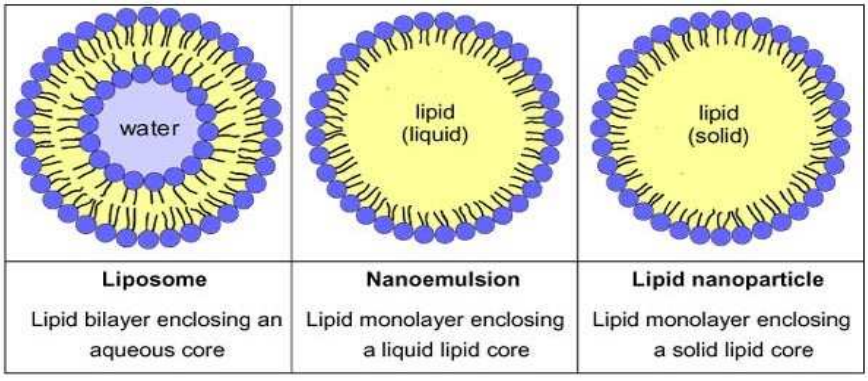


Figure 11: The structure of nanocarriers.
[http://www.intechopen.com/source/html/47025/media/image6.png]

2.6.1 Nanoemulsions

Nanoemulsions consist of isotropic dispersions of two non-miscible liquids. It is usually an oily liquid in an aqueous medium or aqueous liquid forming droplets in an oily medium or other oily liquid molecules of nanometric sizes (100 nm). The small size and use of surfactants keep it stable for a long duration. Nanoemulsions can use either hydrophobic or hydrophilic drugs [Sonneville-Aubrun et al, 2004]. They are non-toxic as well as non-irritant and hence used on skin or mucous membranes for cosmetic applications via parenteral and non-parenteral administration [Escobar-Chávez et al, 2012].

2.6.2 Microemulsions

Microemulsions are droplet sized dispersion of 10 to 100 nm size with low coalescing tendency. They form spontaneously with appropriate amounts of lipophilic and hydrophilic ingredients, and a surfactant and co-surfactant. Their physicochemical properties include transparency, optical isotropy, low viscosity and thermodynamic stability. These are used in both transdermal and dermal drug deliveries, although they have limited applications in transdermal delivery due to very low viscosity [Uchechi, 2014].

2.6.3 Vesicular Systems

a) Liposomes

Liposomes are hollow bilayer lipid structures made of cholesterol and phospholipids. They can transport hydrophilic drugs inside the core and hydrophobic drugs between the bilayers, in a stable manner depending on the polymer added to the surface [Justo and Moraes, 2011] [Bangham, 1993]. Liposomes are non-toxic and remain inside the bloodstream for a long time,

and hence are excellent for drug delivery. They can be surface-charged as neutral, negative or positive, depending on the functional groups and pH medium. They can be of different types: small (25 nm to 100nm), medium (100 nm and 500nm), large and giant unilamellar vesicles, oligolamellar vesicles, large multilamellar vesicles and multivesicular vesicles (500 nm to microns) [Justo and Moraes, 2011].

b) Niosomes

Niosomes are unilamellar or multilamellar vesicles capable of entrapping hydrophilic and hydrophobic solutes. They are used for non-ionic surfactant vesicular drug delivery systems in which the medication is encapsulated in a vesicle. They are stable, biodegradable, biocompatible, non-immunogenic, have lower costs than liposomes and do not have the variable purity issues of phospholipids [Uchegbu, 1995].

c) Transfersomes

Transfersomes are specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. In morphology, they are smilar to liposomes, but functionally, they are highly deformable as they are metastable with very flexible vesicles membrane. This enables them to penetrate pores much smaller than their own size [Jain et al, 2003]. Incorporating an appropriate edge activator like surfactants into the vesicular membrane can change liposomes into transfersomes [Planas et al, 1991] [Cevc et al, 1995] [Paul et al., 1998].

d) Ethosomes

Ethosomes are lipid vesicular carriers embodying ethanol in relatively high concentrations for enhanced skin permeation of drugs [Mbah et al, 2014]. Ethosomes are composed mainly of phospholipids, and have high concentration of ethanol and water for greater drug permeation.

The improved permeation helps to release the entrapped drug particles into deeper skin layers and systemic circulation [Uchechi, 2014]. Structurally, it has a phospholipid bilayer and an aqueous inner core carrying the entrapped active ingredient. They are soft, malleable and nanometers in size [Jain et al, 2007]. The high ethanol content gives it a smaller size when compared to a liposome prepared under the same conditions. The size decreases further as alcohol content increases from 20 to 45 % [Touitou, 2000].

e) Dendrimers

Dendrimers consist of structurally and chemically uniform monodisperse populations that can link to numerous functional groups due to the nature of their branches. The number of branches increases exponentially and branch growth is about 1 nm per generation [Svenson and Tomalia, 2005]. Dendrimers have "dendritic voids" that can mimic the molecular recognition performed by natural proteins [Escobar-Chávez et al, 2012].

f) Nanoparticles

Nanoparticles are composed of polymers, lipids, polysaccharides and proteins [Goswami, 2010] [Rodríguez-Cruz et al, 2009] and can be designed to resist pH, temperature, enzymatic attack, or other problems. They are smaller than 1,000 nm. Substances like drugs, proteins, peptides and DNA can be easily inserted into them. Nanoparticles are of two types: nanospheres and nanocapsules with solid and hollow cores respectively [Huang et al, 2009]. The preparation techniques used depend on their physicochemical properties. Some common techniques are: emulsification-diffusion by solvent displacement, emulsification polymerization, in situ-polymerization, gelation, nanoprecipitation, solvent evaporation/extraction, inverse salting out, dispersion polymerization and others derived from these [Escobar-Chávez et al, 2012].

Table 1: Nanocarriers used for drug delivery [Escobar-Chávez et al., 2012]

NANOCARRIER	SIZE RANGE	PREPARATION METHODS	CHARACTERISTICS
Polymeric nanoparticles	10-1000 nm	• In situ polymerization • Emulsification evaporation • Emulsification diffusion • Emulsification diffusion by solvent displacement.	Solid or hollow particles wich have entraped, binded or encapsulated drugs.
Solid lipid nanoparticles	50-1000 nm	• High-pressure homogenization	Similar to polymeric nanoparticles but made of solid lipids
Inorganic Nanoparticles	<50nm	• Sol-gel technique	Nanometric particles, made up of inorganic compounds such as silica, titania and alumina.
Liposomes	25 nm-100 μm	• Sonication • Extrusion • Mozafari method	Vesicles composed of one or more concentric lipid bilayers, separated by water or aqueous buffer compartments.
Dendrimers	3–10 nm	• Polymerization	Macromolecular high branched structures.
Quantum dots	2-10nm	• Colloidal assembly • Viral assembly • Electrochemical assembly	Made up of organic surfactants, precursors and solvents.
Lipid globules	1-100 nm	• Emulsification espontaneous systems	Multicomponent fluid made of water, a hydrophobic liquid, and one or several surfactants resulting in a stable system
Lipid microcylinders	<1 μm	• Self-emulsification	Self-organizing system in which surfactants crystallize into tightly packed bilayers that spontaneously form cylinders.
Ethosomes	<400 nm	• Cold method • Hot method	Non-invasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation
Aquasomes	60-300 nm	• Self-assembling of hydroxyapatite by coprecipitation method	The particle core is composed of non-crystalline calcium phosphate or ceramic diamond, and it is covered by a polyhydroxyl oligomeric film.
Pharmacosomes	<200 nm	• Hand-shaking method • Ether-injection method	Pure drug vesicles formed by amphiphilic drugs
Colloidosomes	200nm – 1.5 μm	• Self-assembly of colloidal particles at the interface of emulsion droplets	Hollow capsules with elastic shells.
Niosomes	10-1000 nm	• Self-assembly of nonionic Surfactant	Bilayered structures made of non-ionic surfactant vesicles.
Nanoemulsions	20-200nm	• High-pressure homogenization • Microfluidization • Phase Inversion temperature	Submicron emulsions o/w or w/o.

2.7 BIOMACROMOLECULES

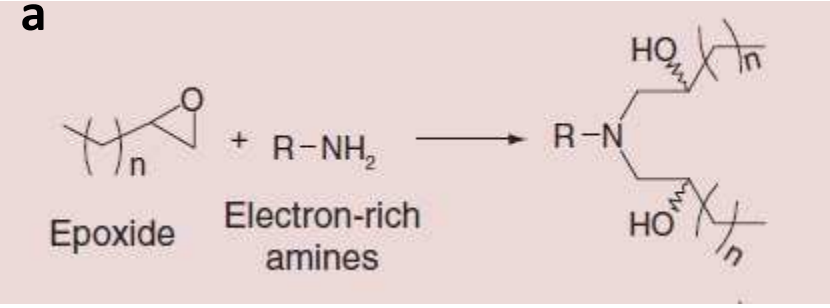
Biomacromolecules refer to large molecules of biological interest with the molecular weights ranging from 10^3 to 10^{12} daltons (Da). Biomacromolecules are considered as single molecules when they are present in a well-defined stoichiometry and when they display little tendency to dissociate spontaneously under physiological conditions. Covalent biomacromolecules are biomacromolecules in which monomer units are linked together by covalent bonds to form giant biomolecules. They include nucleic acids, proteins and polysaccharides [Tsai, 2007].

Polypeptides are linear polymers of α-amino acids, connected by amide bonds (peptide bonds) between the amino group of one monomer unit and the carboxyl group of the following unit, forming a head-to-tail condensation. One end, called the N-terminal (amino-terminal end), has a free α-amino group, whereas the other end, the C-terminal (carboxyl-terminal end), has a free α-carboxyl group. The two types of natural polynucleotides (nucleic acids) are classified according to the sugars they contain. Ribonucleic acid (RNA) contains exclusively β-D-ribose, while the sugar in deoxyribonucleic acid (DNA) is β-2-deoxy-D-ribose. Different nucleic acids can have from around 80 nucleotides (nt), as in transfer RNA (tRNA) to over 10^8 nucleotide-pairs in a single eukaryotic chromosome. The unit size of nucleic acid is the base (for single-stranded species) or base-pair (bp, for double-stranded species) with the unit Kb (thousand base-pairs) and Mb (million base-pairs) [Tsai, 2007].

2.8 LIPID-LIKE NANOPARTICLES (LIPIDOIDS)

Lipid-like nanoparticles are materials or nanoparticles that have the characteristics of a lipid such as the inability to dissolve in water but this lipid-like nanoparticles are amphiphilic, that is they possesses both hydrophilic (water-loving) and lipophilic (fat-loving) properties. These lipid-like

nanoparticles are also referred to as 'lipidoids'. These lipid-like nanoparticles are combinatorially synthesized and a library screening can be used to identify the right particle with the desired properties. The combinatorial method of synthesizing lipidoids is a simple and easy method which involves the reaction of epoxide group with amines. This method eliminates the usage of surfactants, mixed solvents and catalysts, thereby producing only lipidoids. The combinatorial method has significant advantage over the existing techniques for the formulation of nanoparticles. It removes the protection and deprotection, purification or concentration steps [Akinc et al., 2008][Siegwart et al., 2011]. The lipidoids were combinatorially synthesized by mixing 1, 2-epoxyohexadecane and varying amines in a 5 mL Teflon-lined glass screw-top vial at molar ratios (epoxide:amine) of 2.4:1. The reaction took place for two days in the absence of solvent at 80^{o}C. Then it was allowed to cool to room temperature. Flash chromatography on silica gel was used in purifying the reaction mixtures and the characterized using nuclear magnetic resonance spectroscopy (NMR). The library of lipidoids was synthesized through the ring-opening reaction between 1, 2-epoxyhexadecane and amine. The lipidoids are named EC16 followed by the amine number (amine value or amine alkalinity) in the library, where EC16 indicates 1, 2-epoxyhexadecane [Wang et al., 2014]. These lipidoids have excellent properties. They have particle sizes ranging from 50 to 300 nm. The lipid-like nanoparticles are positively charged, cell membranes have large negatively charged domains which will repel the negatively charged nanoparticles.



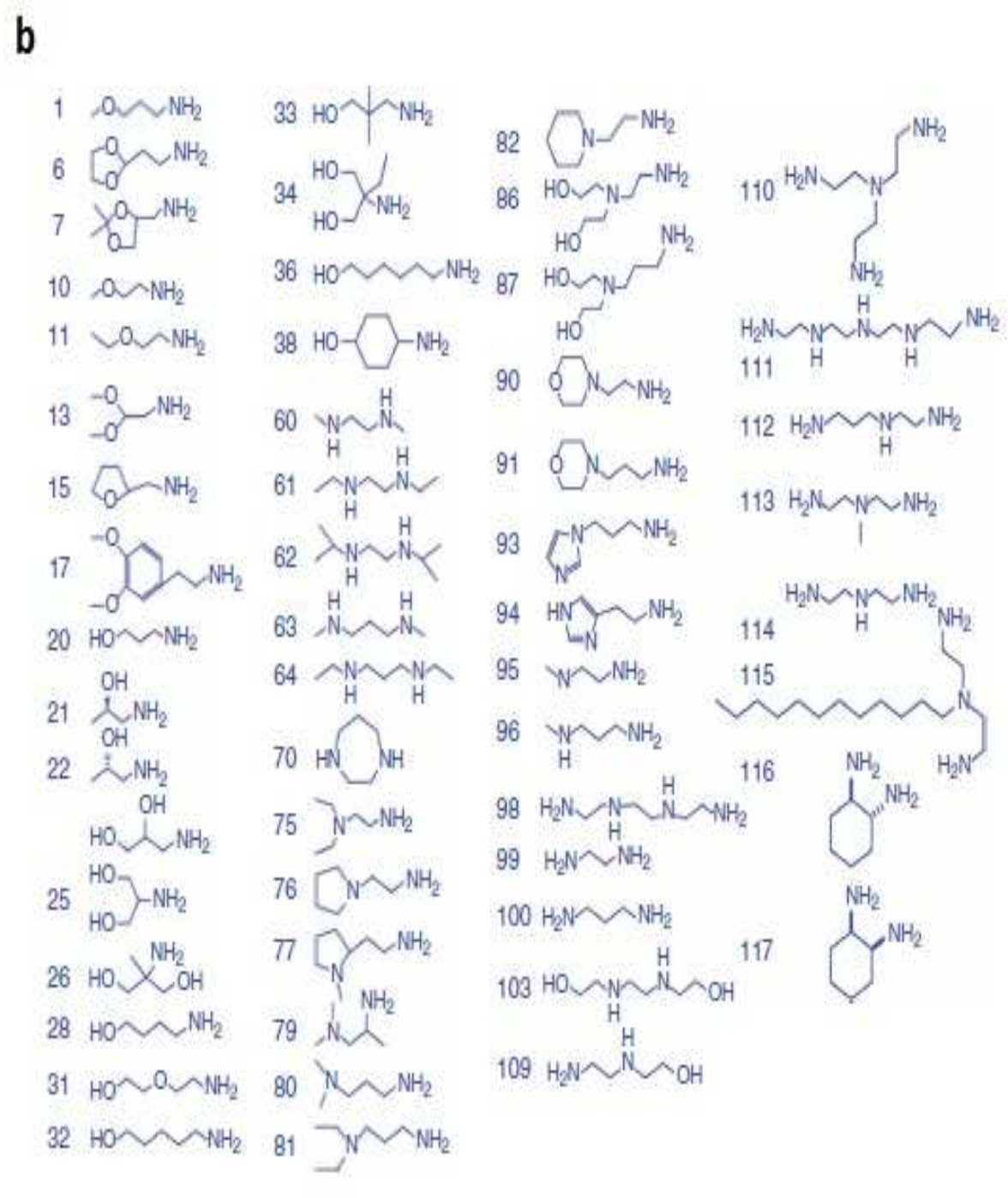


Figure 12: a) Route of synthesis for lipidoids [Altinoglu et al., 2015]. b) The chemical structures of the library of amine used for synthesis of lipidoids [Akinc et al., 2008].

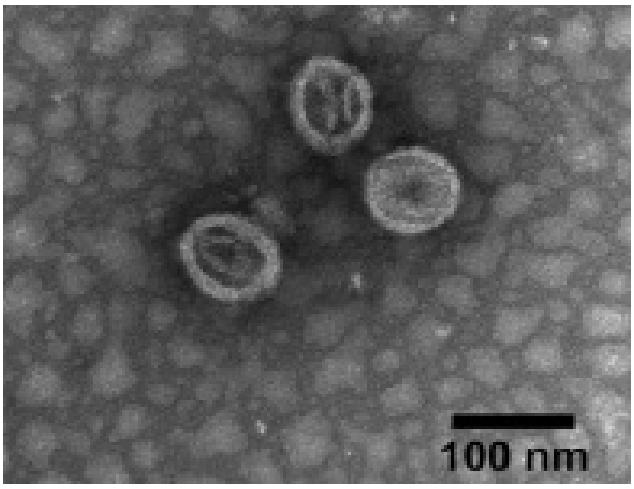


Figure 13: Transmission electron microscopy (TEM) image of lipidoid/protein nanoparticles [Wang et al., 2014].

Synthetic nanoparticles, polymers and inorganic nanoparticles were designed for transdermal delivery but they cannot be used for protein delivery due to complex fabrication procedure and low delivery efficiency [Wang et al., 2014]. To overcome the problem of low delivery efficiency and complicated fabrication procedure, lipid-like nanoparticles were designed. Lipid-like nanoparticles are highly suitable for transdermal delivery of biomacromolecules in many ways. The lipidoids are categorized as vesicular systems (that is have the ability to encapsulate drugs). Cationic nanoparticles have a positive effect on skin permeation thereby promote transdermal permeation. This is because these lipid-like nanoparticles are amphiphilic in nature and their hydrophobic tails can disrupt the hydrophobic skin barrier to enhance the nanoparticle penetration. Also, the charge–charge and hydrophobic interactions between lipidoids and proteins can load proteins into lipidoid nanoparticles and the hydrophobic nature of lipidoid nanoparticles allow easy protein transport through the cell membrane [Wang et al., 2014]. This will help in delivering biomacromolecules to the target organs and can be used for therapy efficiently.

2.9 AIMS OF THE RESEARCH

Biomacromolecules such as nucleic acids and protein have been used for therapeutic purposes but there is the need to deliver these biomacromolecules locally that is exactly to the target organs and cells. Transdermal delivery of these biomacromolecules efficiently will advance the biomacromolecules-based therapeutics. One of the aims of this research is to study lipid-like nanoparticles (lipidoids) for transdermal delivery of biomacromolecules including proteins and nucleic acids. Here, the study will involve penetration and permeation tests to know if the biomacromolecules penetrate through the stratum cornuem, penetration depth in the skin and the amount to biomacromolecules that passes through the skin layers. The other aim of this research is to establish the in vitro model for this study. Here, determining the lipidoid to biomacromolecules weight ratio is essential for high delivery efficiency and the necessary techniques and methods of analyzing the test results. The expected outcome is that the lipidoids will be able to enhance the transdermal delivery of biomacromolecules efficiently.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

Cationic lipid-like nanoparticles (lipidoids), Ec-series (Non-degradable Epoxy base lipidoid) (4 mg/ml): Ec16-63, Ec16-80, Ec16-7, Ec16-10, Ec16-13, Ec16-20, Ec16-22, Ec16-25, Ec16-26, Ec16-31, Ec16-32 (were synthesized by Wang et al.); Cholesterol (10 mg/ml in $CHCl_3$; MW: 386) (Sigma-Aldrich, Sigma, St. Louis, MO, USA); Dioleoylphosphatidylethanolamine, DOPE (5 mg/ml); 1,2 Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene glycol)-2000], DSPE-PEG 2000 (8 mg/ml in distilled water; MW: 2,805.54) (Avanti® Polar Lipids Inc., Alabaste, AL, USA); Green Fluorescent Protein, GFP (18 mg/ml); Fluorescein isothiocyanate Conjugate bovine, FITC-BSA (3 mg/ml) (Sigma-Aldrich, Sigma, St. Louis, MO, USA); Slide-A-Lyzer® 3.5k Dialysis Cassette (Thermo Scientific, Rockford, IL, USA); Phosphate buffer, pH 7.4 (HyClone™ GE Healthcare Life Sciences, Logan, Utah, USA); Chloroform, $CHCl_3$ (MW: 119.38 g/mol) (Sigma-Aldrich, Sigma, St. Louis, MO, USA); Ethanol (90 ml EtOH + 1 ml NaOAc (pH 5.2)); Ultrapure™ Distilled Water, diH_2O (Invitrogen, Life Technologies, Grand Island, NY, USA) were used as received; Nuova II stir plate (Thermolyne, a subsidiary of Sybron); Fluorescence Microscope (Leica Microsystems); Confocal Microscope (Leica Microsystems); Scotch Tough duct tape (3M); 24-well plate (Corning Incorporated); Cryostat, Leica CM 1950 (Leica Microsystems); 11.28mm unjacketed 1.0cm^2 Franz diffusion cell with 3ml receptor volume (PermeGear Riegelsville, PA, USA); 0.25% Trypsin-EDTA (Gibco® Life Technologies); F-4500 Fluorescence Spectrophotometer (Hitachi Ltd, Tokyo,

Japan); Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA); SpectraMax M2 Plate Reader (Molecular Devices, Sunnyvale, CA, USA); Pig skin.

3.2 IN VITRO TESTS FOR DERMAL ABSORPTION

In vitro tests help measure chemical penetration into the skin and the subsequent permeation across the skin by utilizing non-viable skin. Permeation and metabolism on skin can also be simultaneously measured using metabolically active, fresh skin [OECD, 2004]. In vitro tests have several advantages over in vivo tests involving experiments on a whole living organism (animals or humans). These include time and cost savings, better result reproducibility, less variations in restricted parameters, ability to use skin from different species, replicate measurements and study extensively metabolized non-radio-labeled test substances. The following factors have to be considered for in vitro tests for dermal absorption: skin source, viability, receptor fluid composition, preparation and calculation of results [Poet and McDougal, 2002].

3.3 METHODS

3.3.1 Fabrication of Penetration Model

The material (Teflon block) used in building the standard Saarbrücken model (SB-M) for penetration experiment is expensive and difficult to machine. So a similar model was fabricated using cheap and easily available materials. Polycarbonate tube was used in fabricating the setup. Then it was placed on a 24-well plate which served as the base or the receptor chamber and the plate cover was used as the cover for the whole setup (shown in figure 15). As the lipidoid/GFP

nanoparticle (NP) solution was sensitive to light, a tape was used in wrapping the outer part of the fabricated penetration model. This fabricated model has an advantage over the SB-M due to the fact that penetration experiments for many skin samples could be performed at the same or different time periods (time of exposure or incubation time) using this setup.

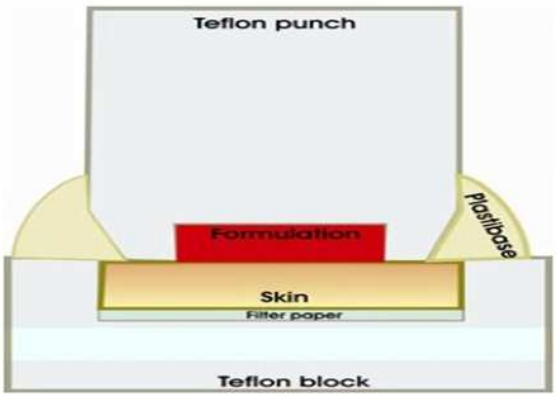
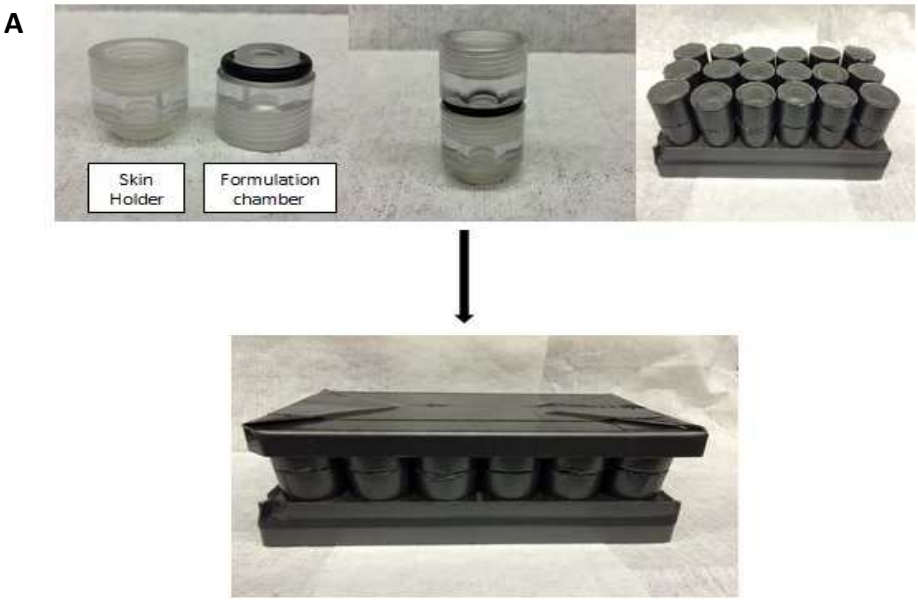


Figure 14: Saarbrücken model (SB-Model) [Contreras, 2007].



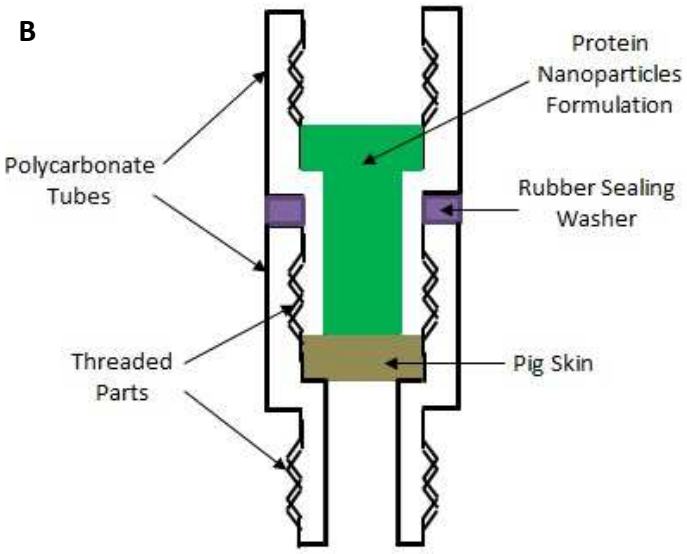


Figure 15: A) Fabricated model (setup) for penetration study. B) Schematic diagram of the setup for penetration experiment using the fabricated model.

3.3.2 Lipidoid/Protein Nanoparticles (NP) Formulation

Nanoparticles (NP) can be made in a multistep process with cholesterol, DOPE and DSPE-PEG. dioleoylphosphatidylethanolamine (DOPE) is a neutral phospholipid for enhancing transfection efficiency [Mochizukia et al., 2013]. Cholesterol is a natural, animal-derived emulsifier for stabilizing lipid dispersion. It forms a monomolecular layer around the emulsion droplet instead of the typical multimolecular layers [UNC Eshelman School of Pharmacy] [Mukherjee et al.]. 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-2000] (DSPE-PEG (2000)) is used for achieving enhanced surface shielding and thus improve pharmacokinetic properties of nanoparticle formulation [Li and Huang, 2009]. Library screening of the lipidoids was used in assessing and selecting the lipidoids that that are suitable for the transdermal delivery of proteins. In 2014, Wang et al. worked on the combinatorially designed lipid-like nanoparticles for intracellular delivery of cytotoxic protein for cancer therapy and

found that the following lipidoids are suitable for protein delivery: Ec16-63, Ec16-80, Ec16-7, Ec16-10, Ec16-13, Ec16-20, Ec16-22, Ec16-25, Ec16-26, Ec16-31, and Ec16-32, that is the reason they are being used for this research. The size of the lipidoids and lipidoid/protein nanoparticles are 129.4 ± 5.4 nm and 131.5 ± 9.7 nm respectively.

Simple mixing of the cationic lipidoid and protein is unstable and leads to aggregating nanoparticles. The cationic lipidoid, cholesterol and DOPE were combined together in a small glass vial, vortexed and left overnight.

Table 2: Materials for the formulation of lipidoid/protein nanoparticles

Substance	**Weight (mg)**	**Volume (µl)**
Cationic Lipidoid	1.6	400
Cholesterol	0.2	20
DOPE	0.1	20
Protein	0.1	5.5
DSPE-PEG	0.2	25
Total	2.2	470.5

A thin film formed at the bottom of the glass overnight. The thin film is rehydrated with 400 µl of ethanol and was allowed to sit for about 2 minutes. The mixture was sonicated vigorously for 5 minutes until the liquid turned cloudy. In another vial, 600 µl of distilled water (diH_2O) was added and then 5.5 µl of GFP (Green Fluorescence Protein) was added to the diH_2O, the mixture was stirred for 4 minutes. The rehydrated thin film was added dropwise to the mixture of diH_2O

and GFP, and the solution became cloudy. This was covered and allowed to sit for 5 minutes. 25 µl of DSPE-PEG was added to the cloudy solution and allowed to sit for 30 minutes to remove the ethanol. Then the solution was dialyzed for 90 minutes.

3.3.3 Dialysis of Lipidoid/Protein Nanoparticles

The Dialysis Cassette is removed from its protective pouch and hydrated before use. This is because the sample volume is small. The cassette is placed in the bench top stand and immersed in a buffer solution for 30 seconds. The cassette is taken out and the excess liquid removed by gently tapping the cassette edge on paper towels. The hypodermic needle's hub is firmly screwed to the Luer-Lok™ fitting of the syringe. The protective sheath is removed from the needle. The syringe is filled with prepared lipidoid/GFP NP by immersing it in the sample and slowly pulling back the syringe piston. The cassette is filled slowly to avoid foaming. This is done by piercing its silicone gasket with the needle through one of the syringe ports at its corner. The cassette is then floated in the dialysis solution of PBS for 90 minutes. After dialysis, the dialyzed sample is drawn through another syringe port at the cassette bottom and collected into a small vial [Bio-Rad AbD Serotec, 2014].

3.3.4 Full-Thickness Skin Preparation

Pig skin was used for the research. The skin was cut into large pieces and a scalpel was used to remove the subcutaneous fatty tissue. The thickness of the fully-thick skin sample was 1756 ± 256 µm. The surface of each specimen was cleaned with water, wrapped in aluminum foil and stored in polyethylene bags at –26°C until used. Previous investigations had shown that a storage time of 6 months did not change the penetration characteristics [Contreras, 2007].

3.3.5 Preparation of Trypsin-Isolated Epidermis Samples

For the purpose of permeation experiments using epidermis samples, the skin pieces were punched out, thawed and cleaned. These were then transferred, dermal side down into filter paper placed in a petri dish which contained 0.25% trypsin solution in PBS buffer and left at room temperature for 12 hours. The procedure was repeated with fresh trypsin solution until the epidermis was isolated. The epidermis was washed three times with PBS buffer and distilled water and then dried at room temperature [Wagner et al, 2001]. The thickness of the epidermis after the procedure was 275 ± 75 µm.

3.3.6 Penetration Study Procedure

For monitoring the penetration of lipidoid/protein nanoparticles (NP), the fabricated setup (figure 15) was used in which the skin itself served as the receptor compartment. Disks of 12 mm diameter were punched out from the frozen skin, thawed and cleaned [Contreras, 2007]. The skin sample (n = 6) was placed into the cavity at the bottom of the polycarbonate tube and the top part of the tube was placed on the bottom part to hold the skin sample in its place and avoid water loss from the skin sample. Infinite dosage which is the application of lipidoid/GFP NP solution onto the skin samples directly without controlling the amount that is being delivered at predetermined time intervals was employed. 300 µl of the solution was filled into the cavity in the top part of the tube. The polycarbonate tube was then placed on the 24-well plate. The setup was covered, placed into a plastic container and transferred into a water bath at (32 ± 1)^{o}C. When the predetermined incubation time was reached, each skin sample was removed from the tube cavity, the lipidoid/GFP NP solution wiped off the skin surface using cotton and then wrapped with aluminum foil [Contreras, 2007].

3.3.7 Permeation Study Procedure

The epidermal and full-thickness skin drug permeation were studied using static Franz diffusion cell (figure 16) technique with a trypsin-isolated epidermis and full-thickness skin samples. The epidermis was positioned between the donor and acceptor compartments. Infinite dosage was employed where 1000 µl of lipidoid/GFP nanoparticles solution was delivered into the donor compartment and a 2 ml of phosphate buffer (pH 7.4) was used as a receptor. The donor compartment was sealed with aluminum foil and the system was maintained at (32 ± 1)°C in a water bath. The acceptor fluid was stirred using magnetic bars at 400 rpm. At predetermined time intervals, samples of 1.0 ml were collected from the acceptor medium and replaced immediately with fresh buffer solution [Contreras, 2007]. Samples were collected until 24 hours and analyzed by BCA assay and Fluorimeter.

Figure 16: Franz diffusion cell.

3.3.8 Cryosectioning

The cryostat was used for the cryosectioning of the frozen samples from the penetration experiment. The samples were sectioned into 50 micron thick slices.

3.3.9 Fluorescence Imaging

To verify the presence and to visualize the distribution of the applied Lipidoid/GFP nanoparticles on the skin, brightfield fluorescence imaging was used.

3.3.10 BCA Assay and Plate Reading

200µL of "A" and 4µL of "B" were mixed together to formulate the working reagent. Using a 96-well plate, 25µL of the sample was pipetted into the microplate well. 200µL of the working reagent was added to each well containing the samples and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37^{o}C for 30 minutes after which it was cooled at room temperature. A plate reader was used in measuring the absorbance which was at 560nm.

3.3.11 Fluorescence Images Analysis

Brightness intensity analysis: Measurement of intensity of the same area in all the samples using ImageJ software [Waseem Raja]. This is shown in figure 17.

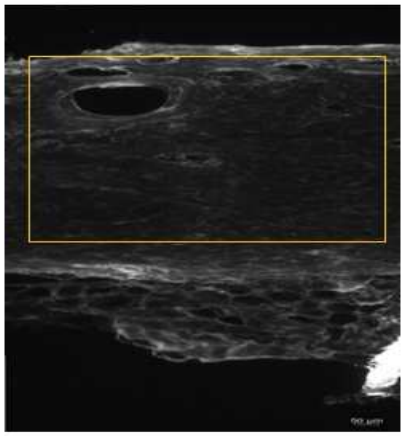


Figure 17: Brightness intensity analysis using ImageJ software.

Penetration depth analysis: Measurement of intensity at each 50µm depth of the same area in all the samples using ImageJ software. This is shown in figure 18. The intensity values gotten from the ImageJ analysis at each depth were normalized with respect to the control values. The normalized intensity values that are more than the normalized control value were considered for the penetration depth analysis. This method does not give the accurate penetration depth but it is used for assuming or predicting the depth that the lipidoid/protein NP has traveled in the skin.

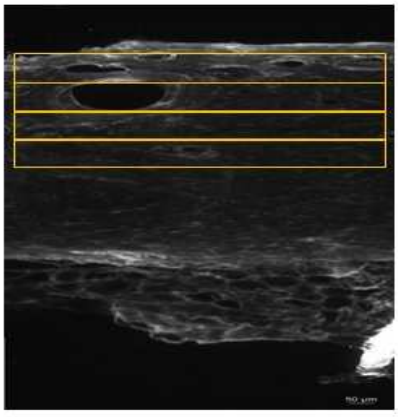


Figure 18: Penetration depth analysis using ImageJ software.

3.3.12 Fluorescence Spectrophotometry

The fluorescence from the receptor solution samples; collected at predetermined time intervals during the permeation experiment; was analyzed using the fluorescence spectrophotometer or fluorimeter for measuring the amount or concentration of the fluorophore (protein) present in the receptor solution.

CHAPTER FOUR

PENETRATION STUDY

4.1 TRANSDERMAL DELIVERY OF PROTEIN

4.1.1 Introduction

In the penetration study of particles through skin (hair follicles was used as the delivery route) done by Walters (ed.) in 2002, efficient penetration occurs when the size of the particles are less than 3 μm. They indicated that smaller the size of the particle, the deeper it travels through the skin. To know how deep protein with a macro size without enhancement will travel through the skin; this penetration experiment was set up. The protein used was Green Fluorescence Protein (GFP). 300 μL of GFP was applied to pig skin with a time of exposure of 6 hours. During histological study of the skin sample, staining was not used because GFP has the ability to fluoresce and can be viewed using fluorescence microscope.

4.1.2 Results

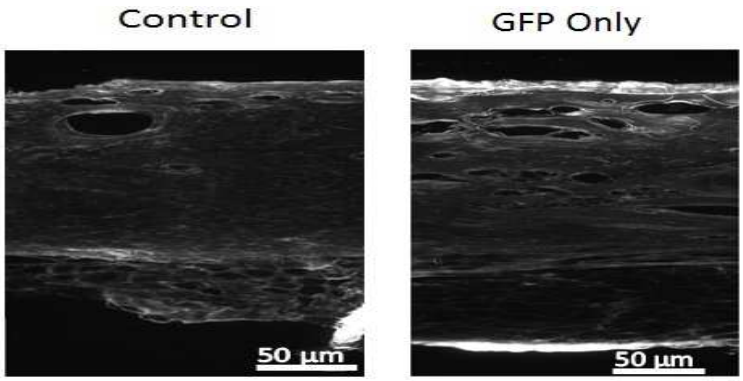


Figure 19: Fluorescence images of the skin samples for both the control (skin sample without any solution or nanoparticles being applied to it) and when GFP was applied to the skin for 6 hr.

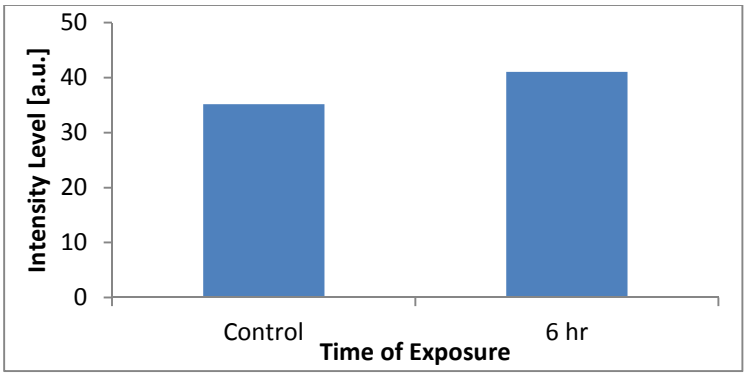


Figure 20: Brightness intensity level of protein (GFP) when applied alone that is without lipidoids onto the skin sample.

4.1.3 Discussion

The microscopic view of the histologic skin specimen after exposure to GFP for 6 hours shows that the brightness of superficial layer of the skin is higher than that of the deep skin layer. The intensity is 41.07 a.u. and the penetration depth is 50 µm.

4.2 STUDYING TRANSDERMAL DELIVERY OF PROTEINS (GFP AND FITC-BSA) USING LIPIDOID (EC16-80)

4.2.1 Introduction

The transdermal protein delivery for 6 hours (described in 4.1) showed that the protein traveled just 50 µm into the skin. The result is not encouraging in the case of target tissue like blood. Many enhancement methods have been used for this such as iontophoresis, ultrasound, microdermal abrasion and chemicals. Here, lipidoids are being studied as enhancers for the delivery of protein through the skin. Ec16-80 was the lipidoid used in the case study. Throughout the course of the research, Ec-series, non-degradable epoxy based lipidoid was the cationic lipid that was used. The proteins used for studying the enhancement of transdermal delivery are green

fluorescence protein (GFP) and FITC- labeled bovine serum albumin (FITC-BSA). The lipidoid was used to encapsulate the two proteins to form two different nanoparticles (NP) which are Ec16-80/GFP and Ec16-80/FITC-BSA NP. These two formed nanoparticles were used for the penetration study. Here, the ratio of weights of the lipidoid to protein is 16:1. This ratio was used because it was the ratio Wang et al has used mostly for encapsulation of particles using lipidoids. 300 µL of Ec16-80/ was applied on the pig skin samples (n = 6) for predetermined time of exposure of 30 minutes, 1 hour, 3 hours, 6 hours, 9 hours and 12 hours.

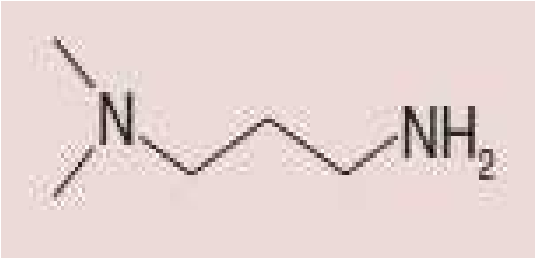


Figure 21: Chemical structure of amine for the synthesis of Ec16-80. The amine number is 80.

4.2.2 Results

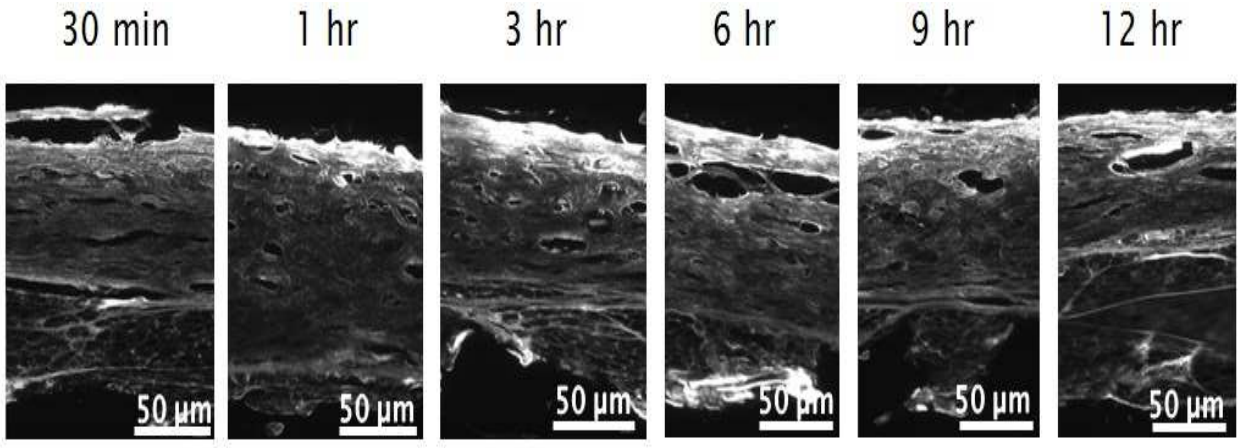


Figure 22: Fluorescence image of the full-thickness skin sample when Ec16-80/GFP NP solution was applied on the skin for predetermined time of exposure.

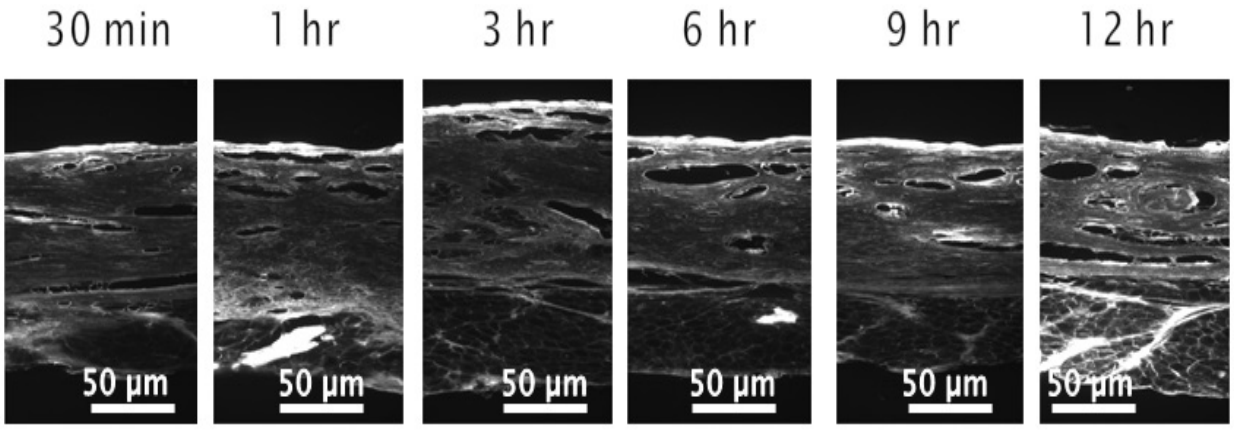


Figure 23: Fluorescence image of the full-thickness skin sample when Ec16-80/FITC-BSA NP solution was applied on the skin for predetermined time of exposure.

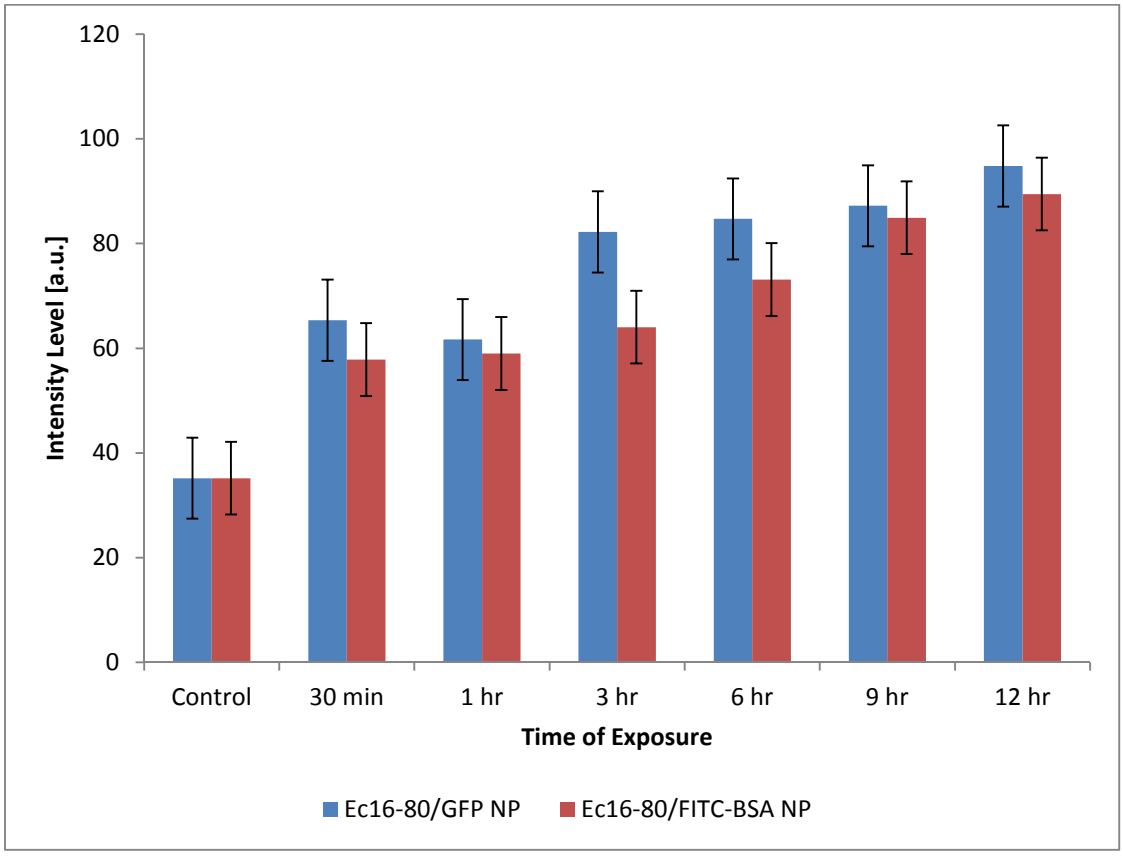


Figure 24: This shows the brightness intensity level of the skin samples both before (Control) and after the Ec16-80/GFP NP and Ec16-80/FITC-BSA NP have been applied to the skin samples at predetermined times of exposure.

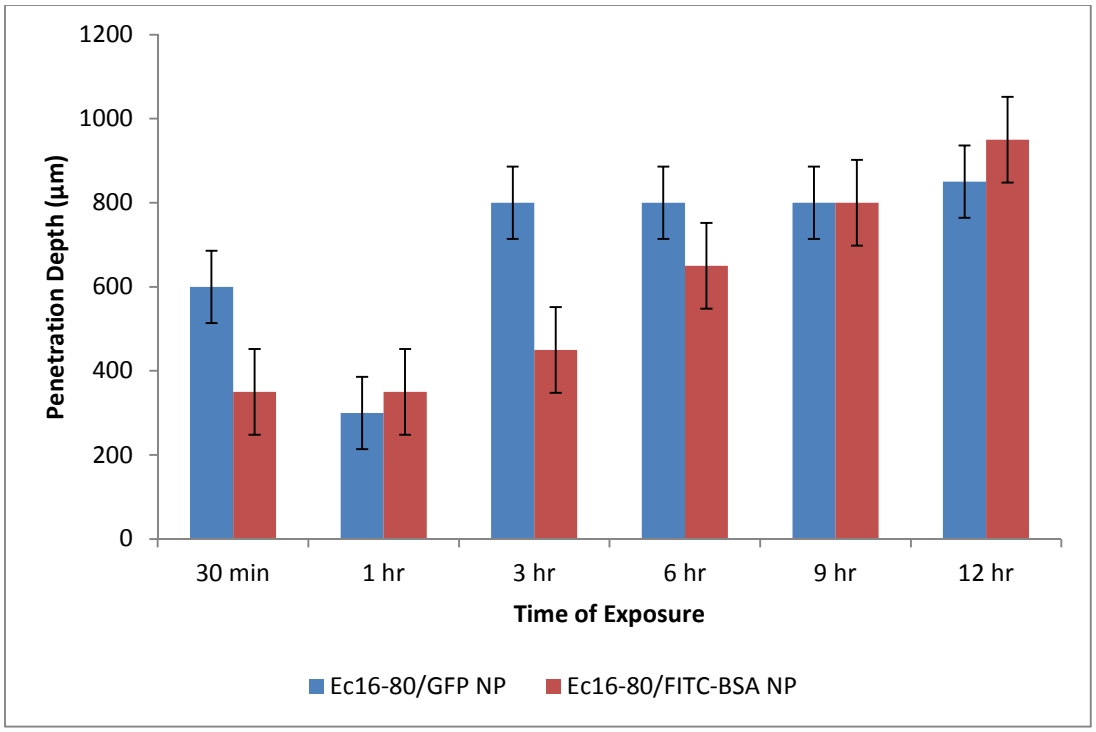


Figure 25: The depth of penetration of the Ec16-80/GFP and Ec16-80/FITC-BSA nanoparticles (NP) in the skin at predetermined time of exposure.

4.2.3 Discussion

The brightness intensity of the histological samples of the skin at predetermined times of exposure increased as the time of exposure increased. The brightness intensity shows the amount of the Ec16-80/GFP NP and Ec16-80/FITC-BSA NP that penetrated through the skin samples. The GFP and FITC-BSA fluorescence increased the brightness intensity of the skin sample when the GFP passed through the skin. The penetration depth of the nanoparticles also increased with the time of exposure. It is expected that the intensity level at 1 hour exposure time should be higher than the intensity level at 30 min exposure time. However it was not so in this case because the skin sample used for the 1 hour exposure time is tougher or harder than the other skin samples used.

Comparing the result from the transdermal delivery of protein (section 4.1) to enhancement of transdermal delivery of GFP using lipidoid(Ec16-80) (section 4.2), the brightness intensity of skin sample at 6 hours exposure time was 41.07 a.u and 84.71 a.u and the penetration depth was 50 µm and 300 µm respectively. This depicted that the amount of protein that passed through the skin sample increased when the lipidoid (Ec16-80) nanoparticles was used as the enhancer. From this penetration experiment, it can be deduced that the lipidoid (Ec16-80) nanoparticles enhanced the transdermal delivery of the proteins. That is, it helped in increasing the quantity of proteins that travel deep into the skin.

4.3 STUDYING TRANSDERMAL DELIVERY OF PROTEINS (GFP AND FITC-BSA) USING LIPIDOID (Ec16-63)

4.3.1 Introduction

Transdermal protein delivery was studied using different lipidoids as enhancers. Initially the lipidoid Ec16-80 was used with the proteins, GFP and FITC-BSA. Here, another lipidoid, Ec16-63 was used with the two proteins, GFP and FITC-BSA in the ratio of weights (Lipidoids:Protein) 16:1. 300 µL of the Ec16-63/protein NP solution was applied to the skin for the penetration experiment for predetermined times of exposure of 30 minutes, 1 hour, 3 hours, 6 hours, 9 hours and 12 hours.

Figure 26: Chemical structure of amine for the synthesis of Ec16-63. The amine number is 63.

4.3.2 Results

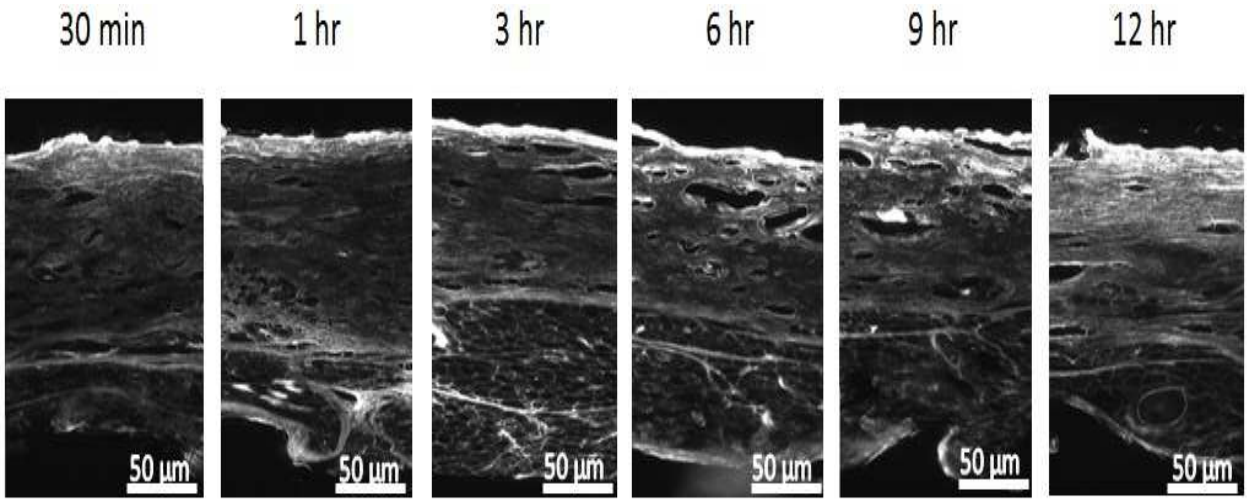


Figure 27: Fluorescence image of the full-thickness skin sample when Ec16-63/GFP NP solution was applied on the skin for predetermined time of exposure and the control.

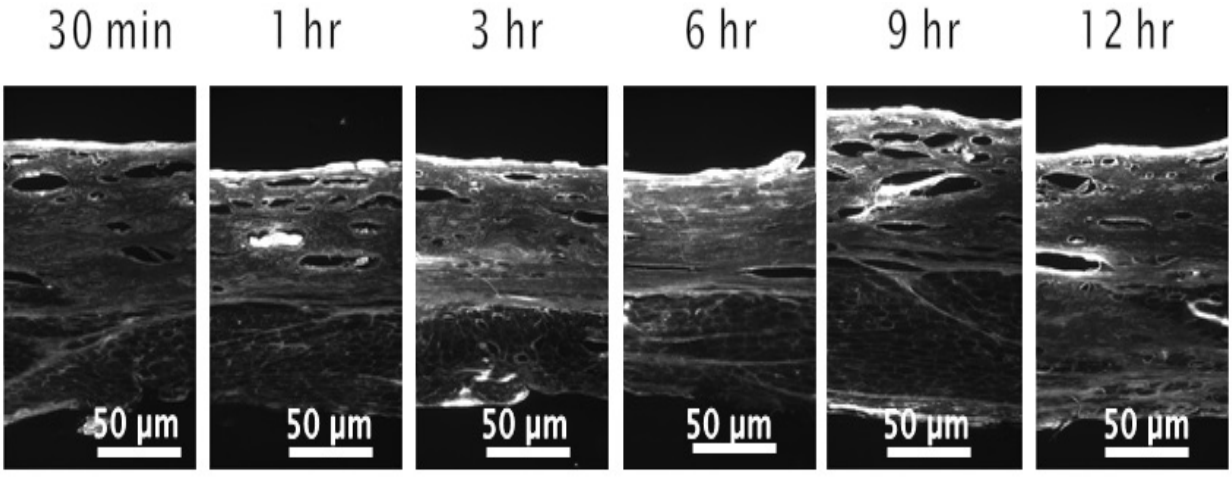


Figure 28: Fluorescence image of the full-thickness skin sample when Ec16-63/GFP NP solution was applied on the skin for predetermined time of exposure and the control.

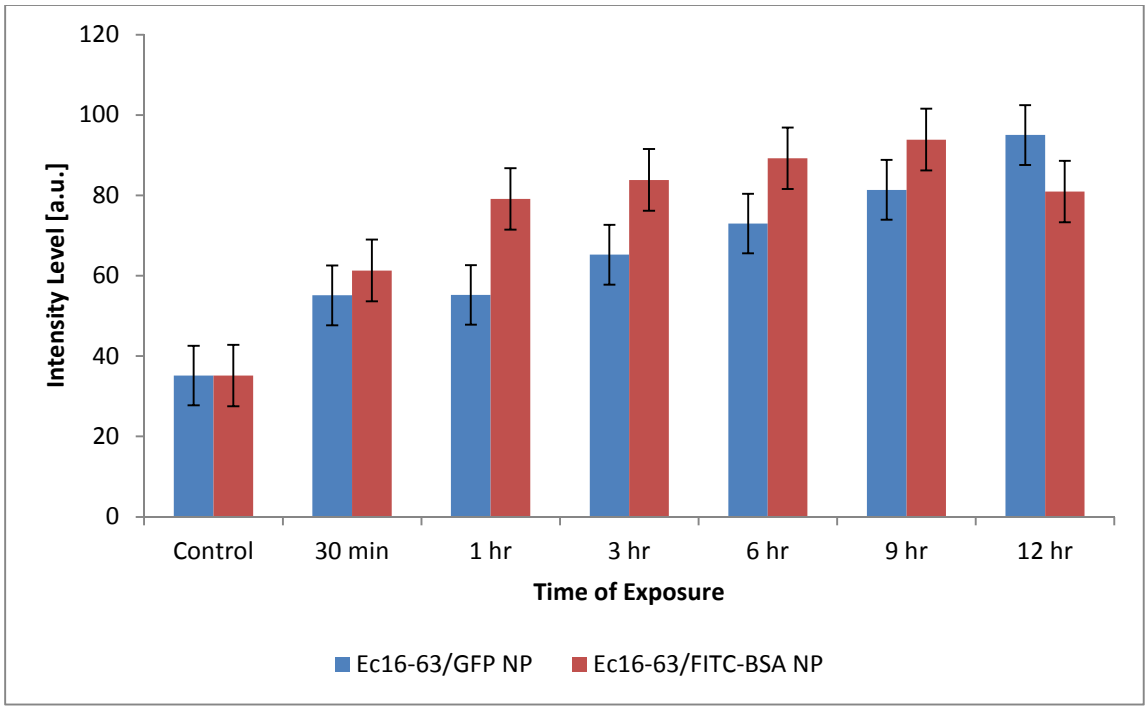


Figure 29: The brightness intensity level of the skin samples both before (control) and after the Ec16-63/GFP and Ec16-63/FITC-BSA NP solutions have been applied to the skin samples at predetermined times.

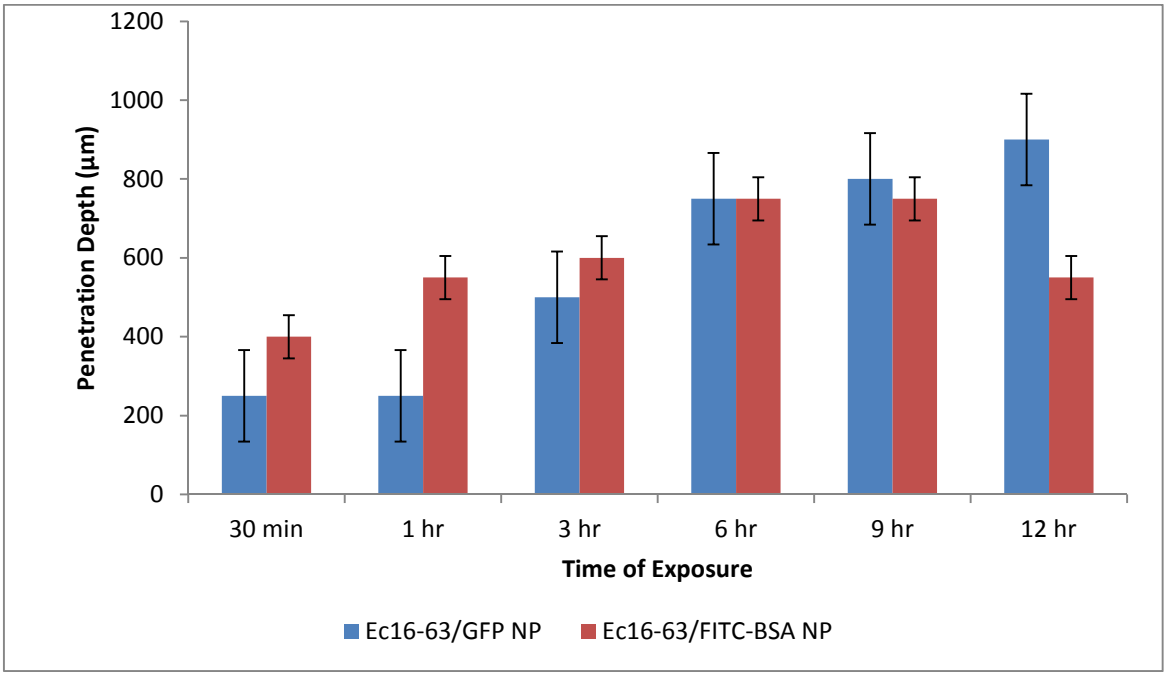


Figure 30: The penetration depth of the Ec16-63/GFP NP and Ec16-63/FITC-BSA in the skin at predetermined time of exposure.

4.3.3 Discussion

Transdermal protein delivery enhancement was studied using Ec16-63 to confirm the results obtained from the use of Ec16-80. The performance of Ec16-63 and Ec16-80 are different may be due to their amine number. The fluorescence from the skin samples showed that the brightness intensity of the skin sample increased as the time of exposure increased. This means that as the time of exposure increased, the amount of protein that travels into the skin, i.e. the penetration depth of protein also increased. This penetration experiment depicted that lipidoid, Ec16-63 can be used as an enhancer for transdermal delivery of protein.

4.4 STUDYING DIFFERENT LIPIDOIDS FOR THE TRANSDERMAL DELIVERY OF PROTEIN (FITC-BSA)

4.4.1 Introduction

Penetration experiment using merely two lipidoids was not enough to prove that lipidoids are good enhancers of transdermal protein delivery. To prove that Ec-series, non-degradable epoxy based lipidoids can be used to enhance transdermal protein delivery; many lipidoids were selected for the penetration experiment. The lipidoids selected were Ec16-7, Ec16-10, Ec16-13, Ec16-20, Ec16-22, Ec16-25, Ec16-26, Ec16-31 and Ec16-32. In this study, FITC-BSA was the protein used instead of GFP. This protein too can fluoresce without staining during histological study. As in the case of the previous penetration experiments, 300 μL of lipidoid/FITC-BSA solution was applied to the skin samples for a period of 6 hours.

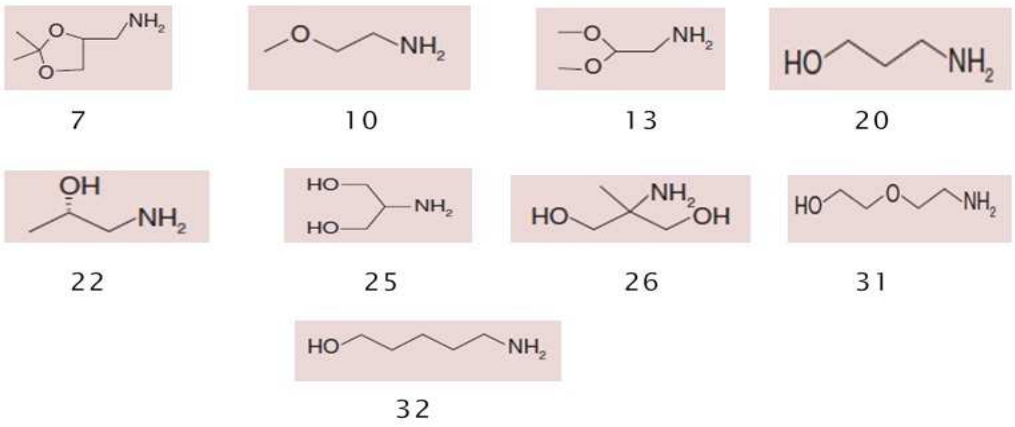


Figure 31: The chemical structures of the different amines used for the lipidoids synthesis [Akinc et al., 2008]. The lipidoids are named Ec16, for 1,2-epoxyhexadecane, followed by the amine number. For example, Ec16-7, Ec16 is 1, 2-epoxyhexadecane while 7 is the amine number.

4.4.2 Results

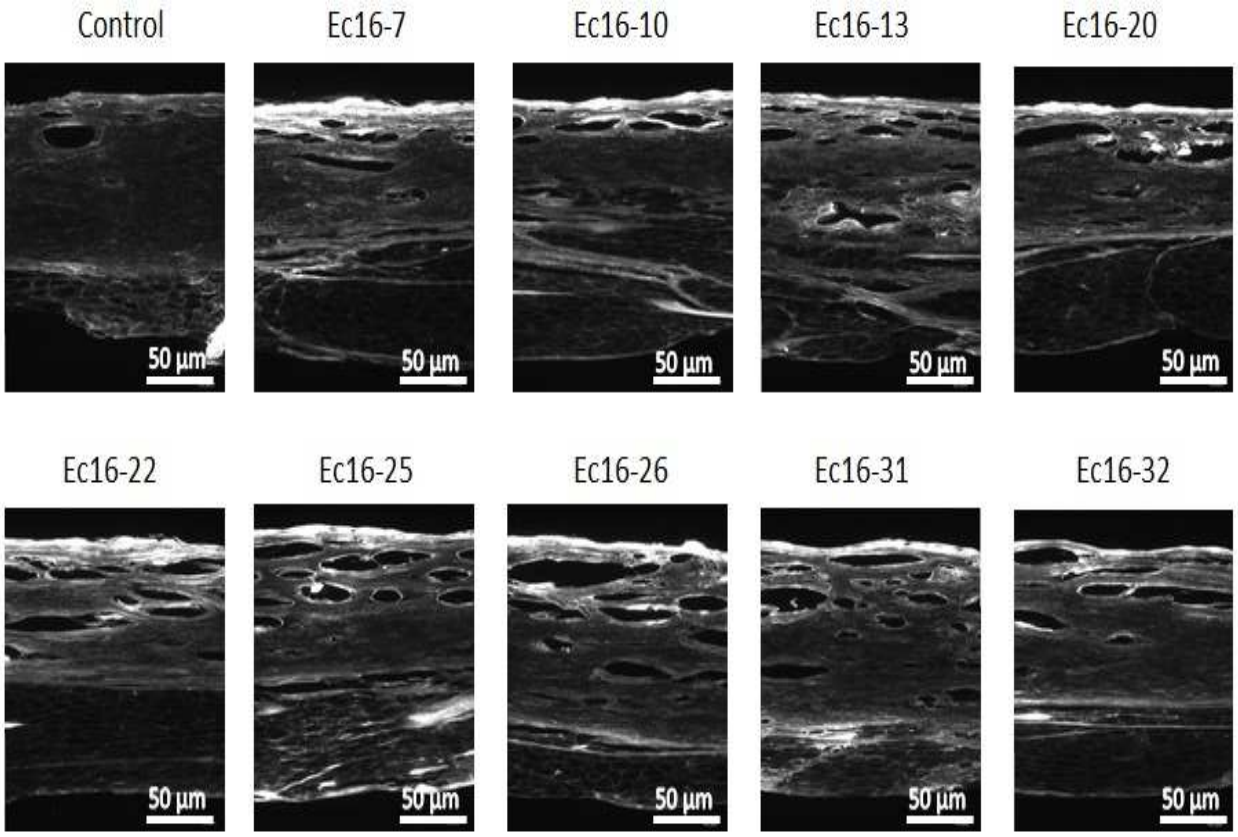


Figure 32: Fluorescence image of the full-thickness skin samples after the application of lipidoid/FITC-BSA NP solution to the skin samples for 6 hr and the control.

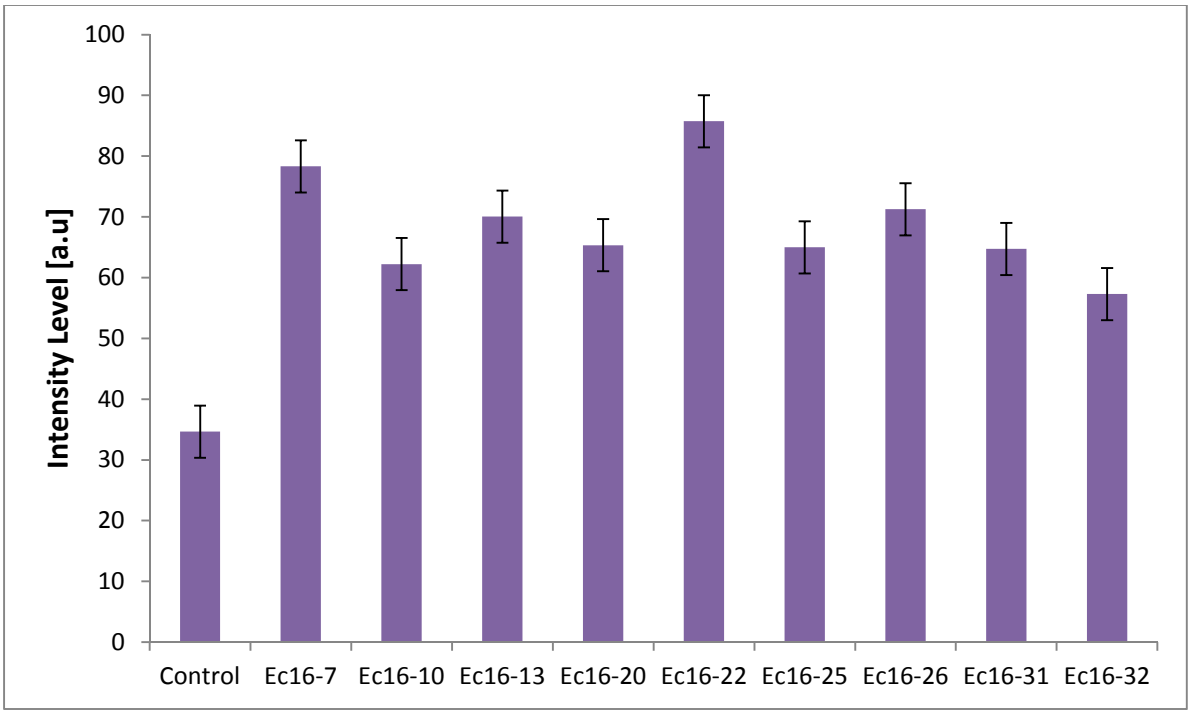


Figure 33: Brightness intensity level of the skin samples of the control and after the lipidoid/FITC-BSA NP solution has been applied to the skin samples for 6 hr.

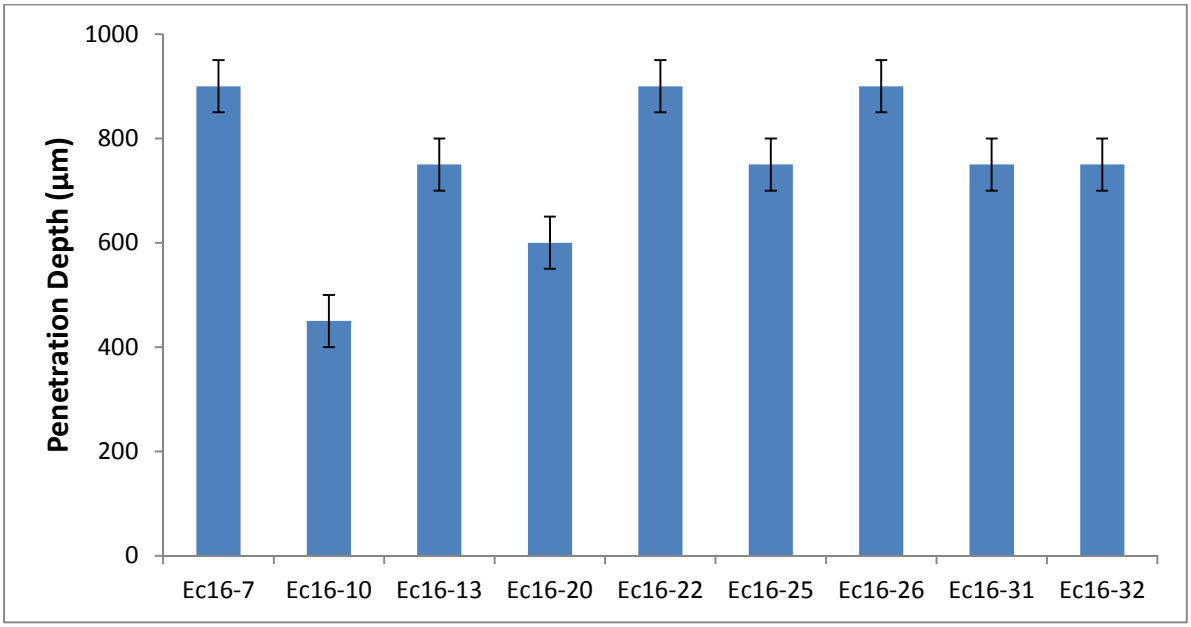


Figure 34: Depth of penetration of the lipidoid/FITC-BSA NP solution into the skin sample after 6 hr of exposure time.

4.4.3 Discussion

The results from this experiment showed that the Ec-series, non-degradable epoxy based lipidoids can be used enhancers for transdermal delivery of protein. The fluorescence from the skin samples showed that the brightness intensity of the skin sample increased as the time of exposure increased. This means that as the time of exposure increased, the penetration depth of protein also increased. All these penetration experiments using different lipidoids showed that lipidoids can be used as enhancers for the transdermal delivery of proteins.

4.5 INVESTIGATING THE EFFECT OF WEIGHT RATIO OF LIPIDOID TO PROTEIN ON TRANSDERMAL DELIVERY

4.5.1 Introduction

For transdermal delivery of proteins using lipidoids, the weight ratio of lipidoids to protein should be such that an optimal enhancement level is achieved. In all the earlier penetration experiments, the weight ratio of lipidoids to the protein was taken as 16:1 because it was the weight ratio that Wang et al. used for some of their work. However, this may not be the optimal weight ratio. So, it was imperative that the optimal weight ratio was investigated and identified. In this experiment, the weight ratios considered were 24:1 and 8:1. The experiment was performed using lipidoids, Ec16-80 and Ec16-63 and the proteins of interest are GFP and FITC-BSA.

4.5.2 Results

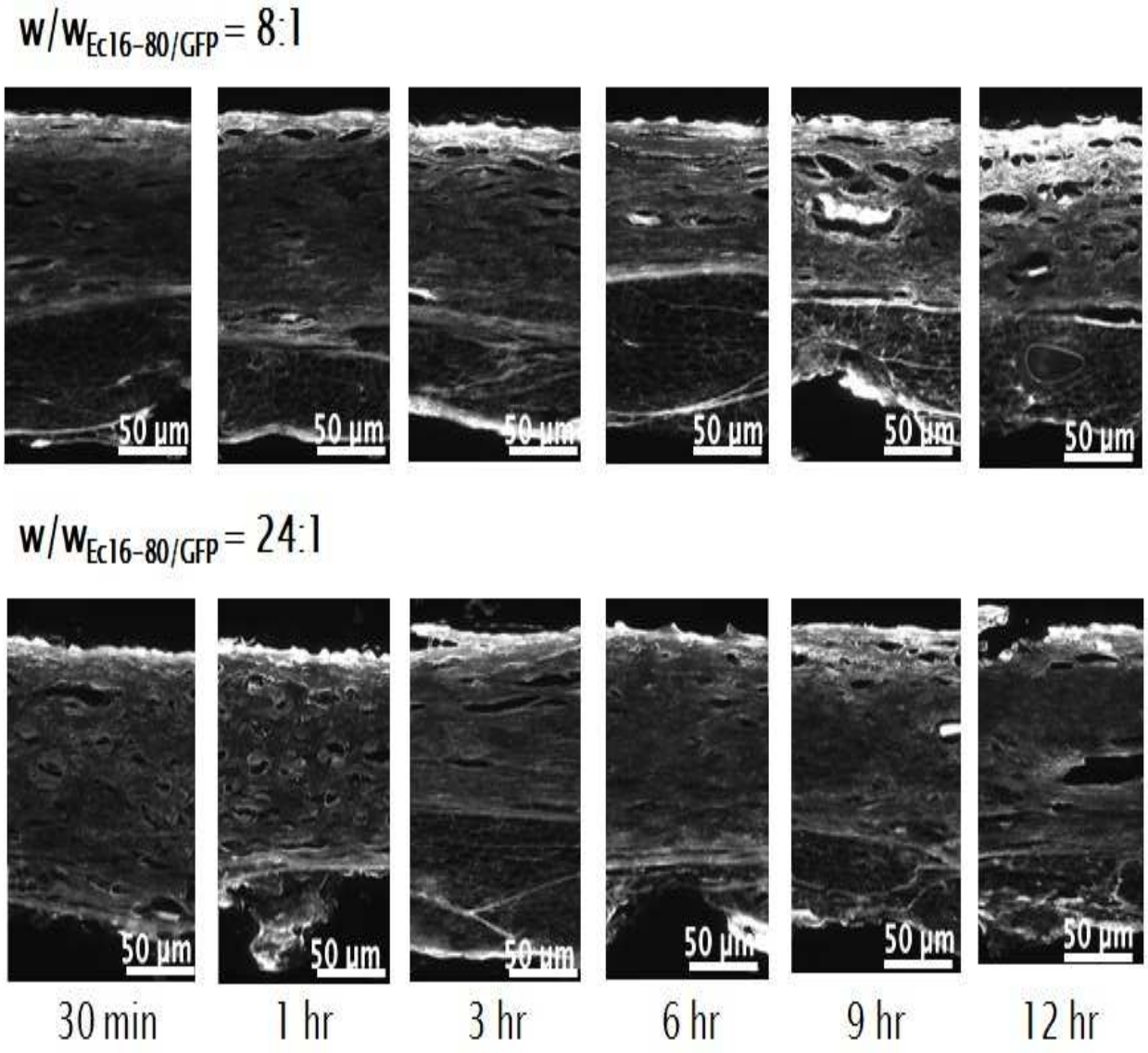


Figure 35: Fluorescence image of the full-thickness skin sample after Ec16-80/GFP NP solution was applied on the skin for predetermined times of exposure and the control. The weight ratio of the Ec16-80 to GFP is 8:1 and 24:1.

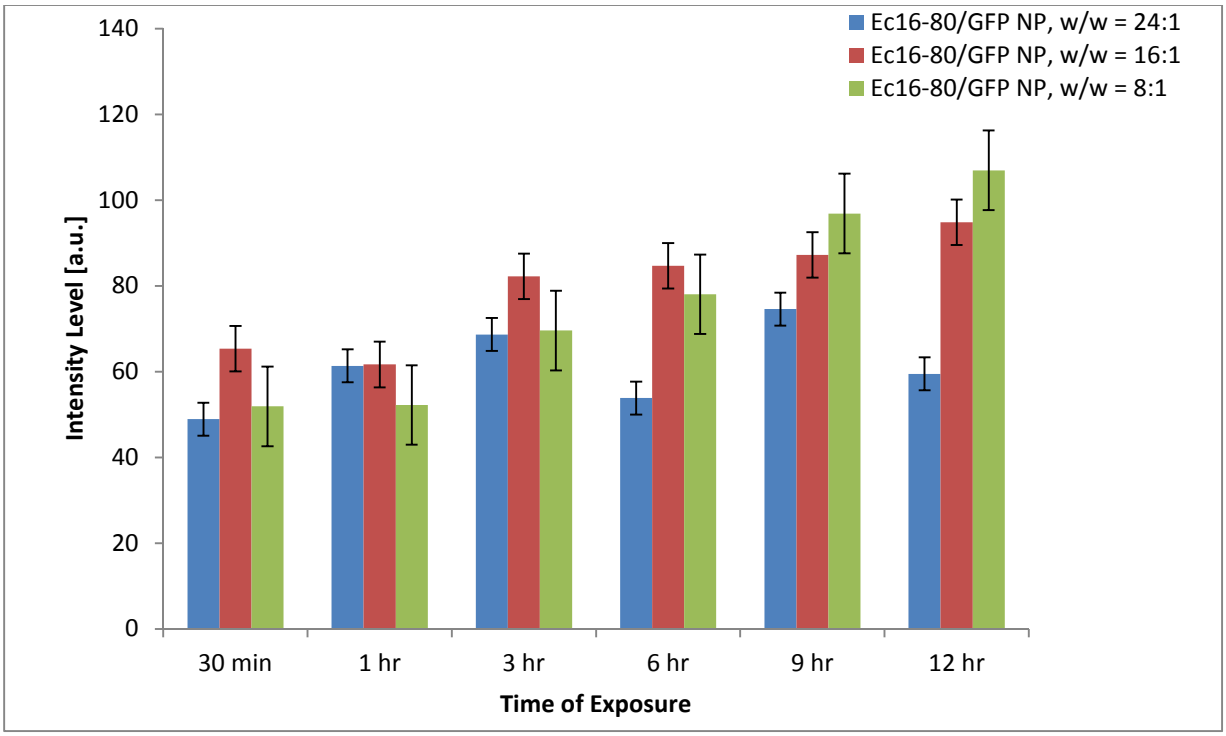


Figure 36: Brightness intensity level of the skin samples when the weight ratio of Ec16-80 to GFP was varied.

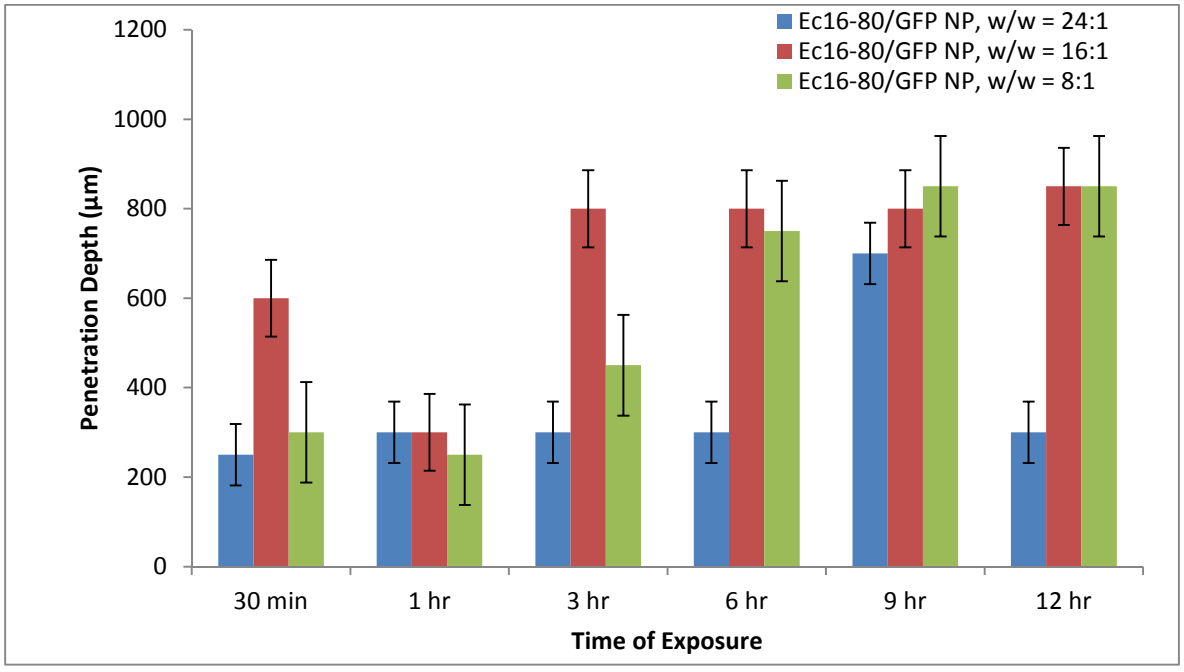


Figure 37: The penetration depth of Ec16-80/GFP nanoparticles in the skin when the weight ratio of Ec16-80 to GFP was varied.

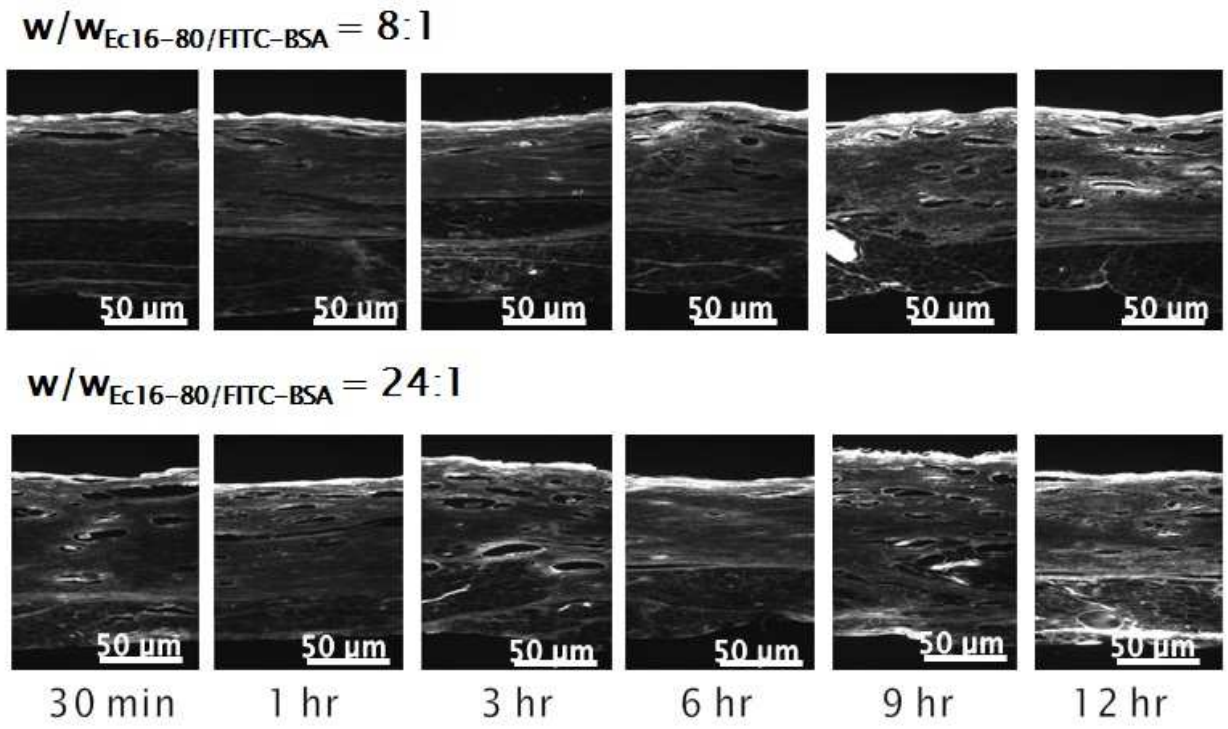


Figure 38: Fluorescence image of the full-thickness skin sample after Ec16-80/GFP NP solution was applied on the skin for predetermined times of exposure and the control. The weight ratio of the Ec16-80 to GFP is 8:1 and 24:1.

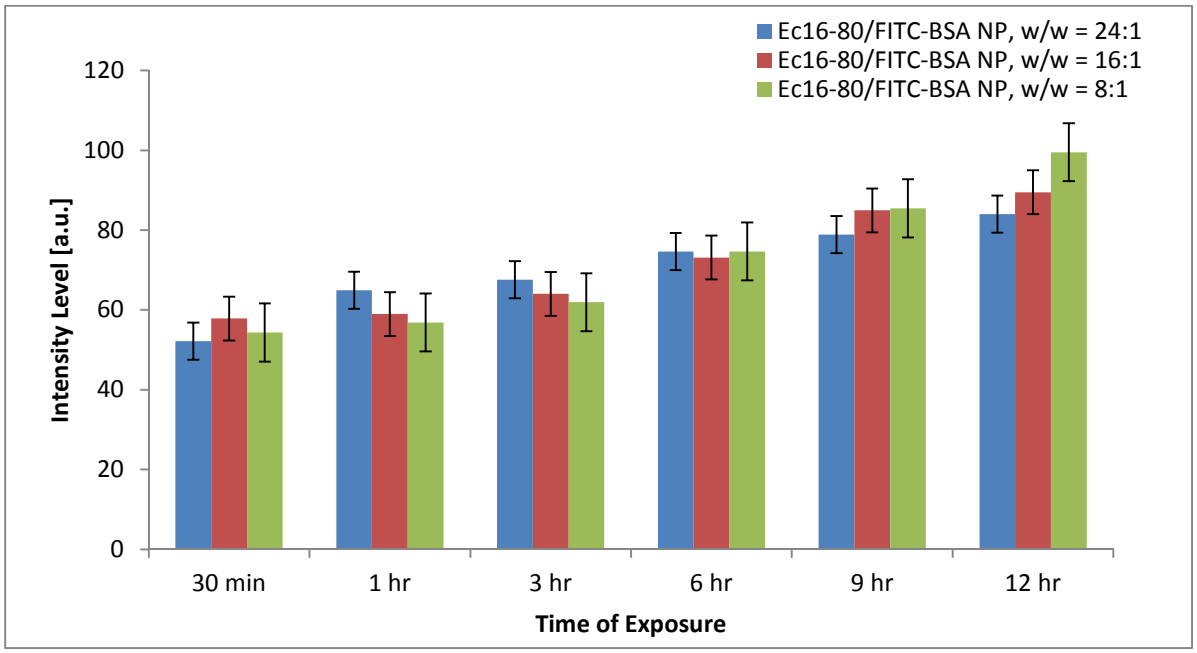


Figure 39: Brightness intensity level of the skin samples when the weight ratio of Ec16-80 to FITC-BSA was varied.

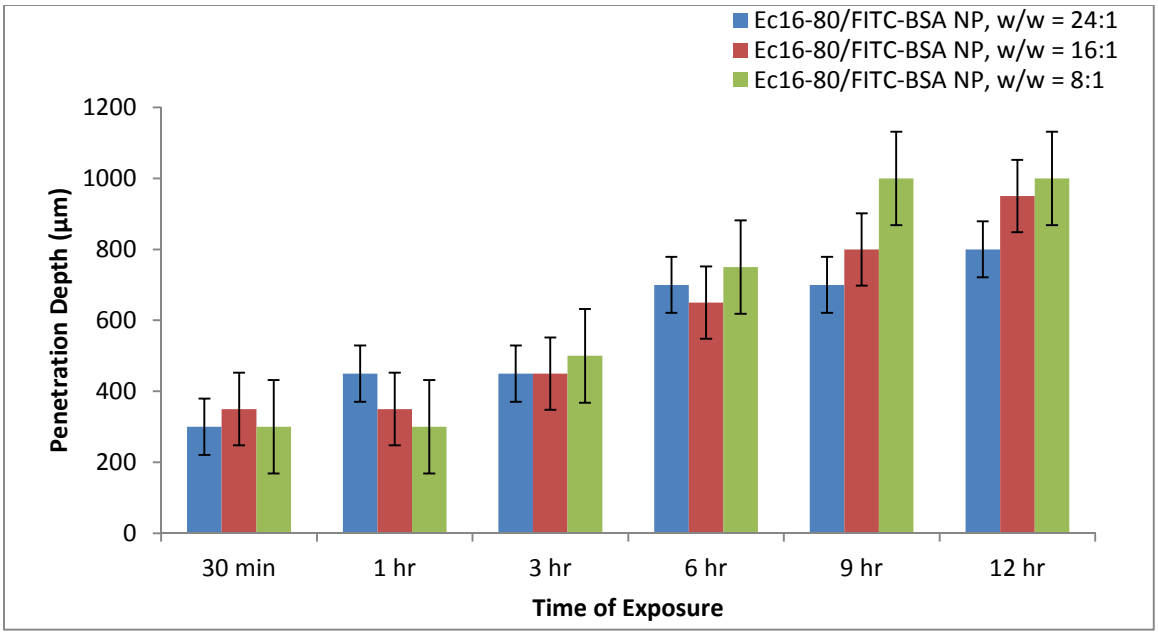


Figure 40: The penetration depth of Ec16-80/FITC-BSA NP in the skin when the weight ratio of Ec16-80 to FITC-BSA was varied.

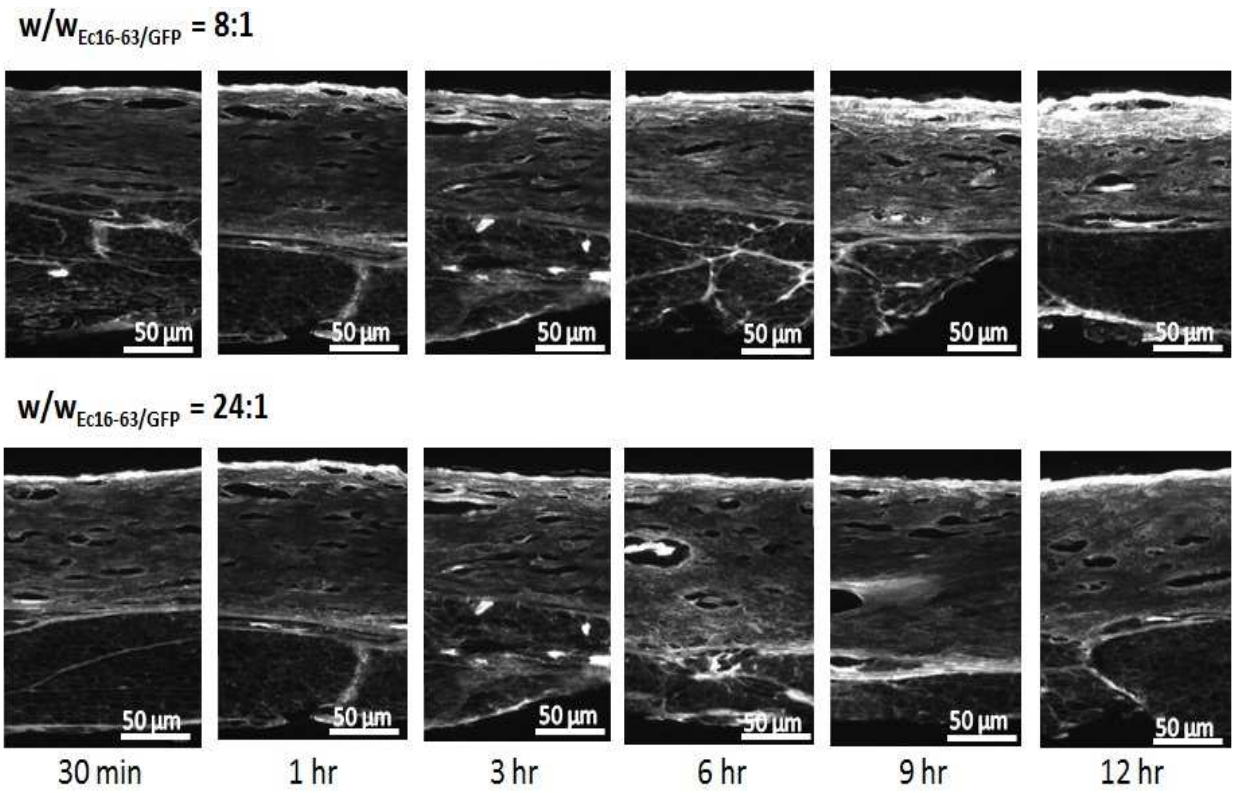


Figure 41: Fluorescence image of the full-thickness skin sample after Ec16-63/GFP NP solution was applied on the skin for predetermined times of exposure and the control. The weight ratio of the Ec16-63 to GFP is 8:1 and 24:1.

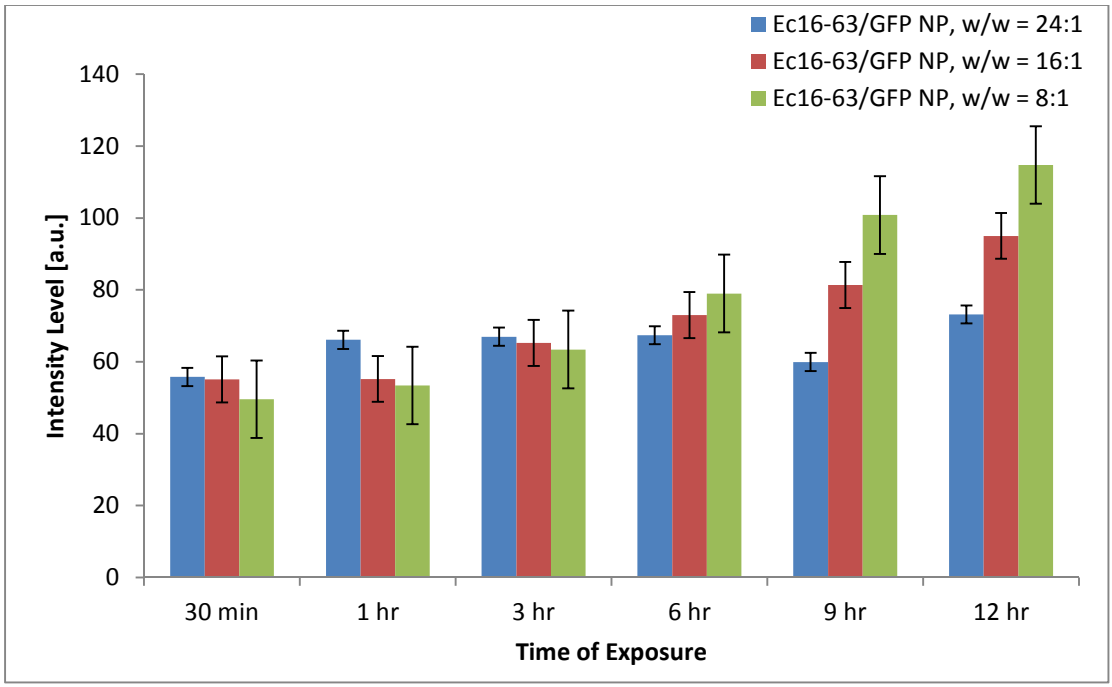


Figure 42: Brightness intensity level of the skin samples when the weight ratio of Ec16-63 to GFP was varied.

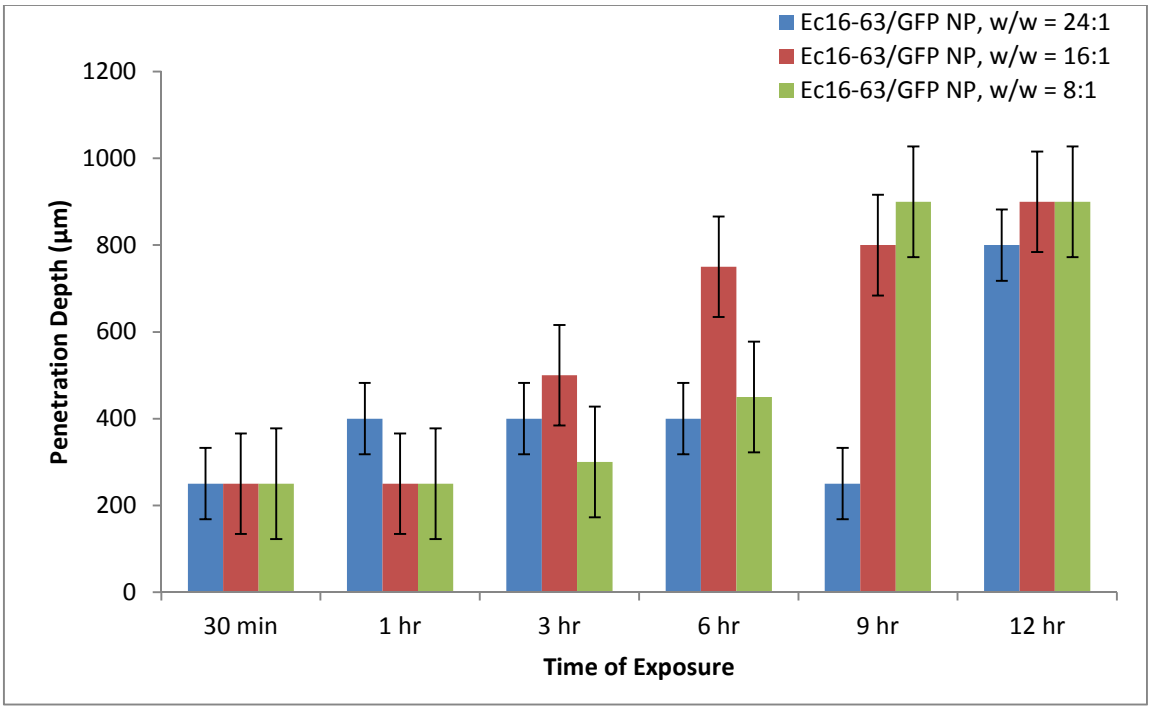


Figure 43: The penetration depth of Ec16-63/GFP NP in the skin when the weight ratio of Ec16-63 to GFP was varied.

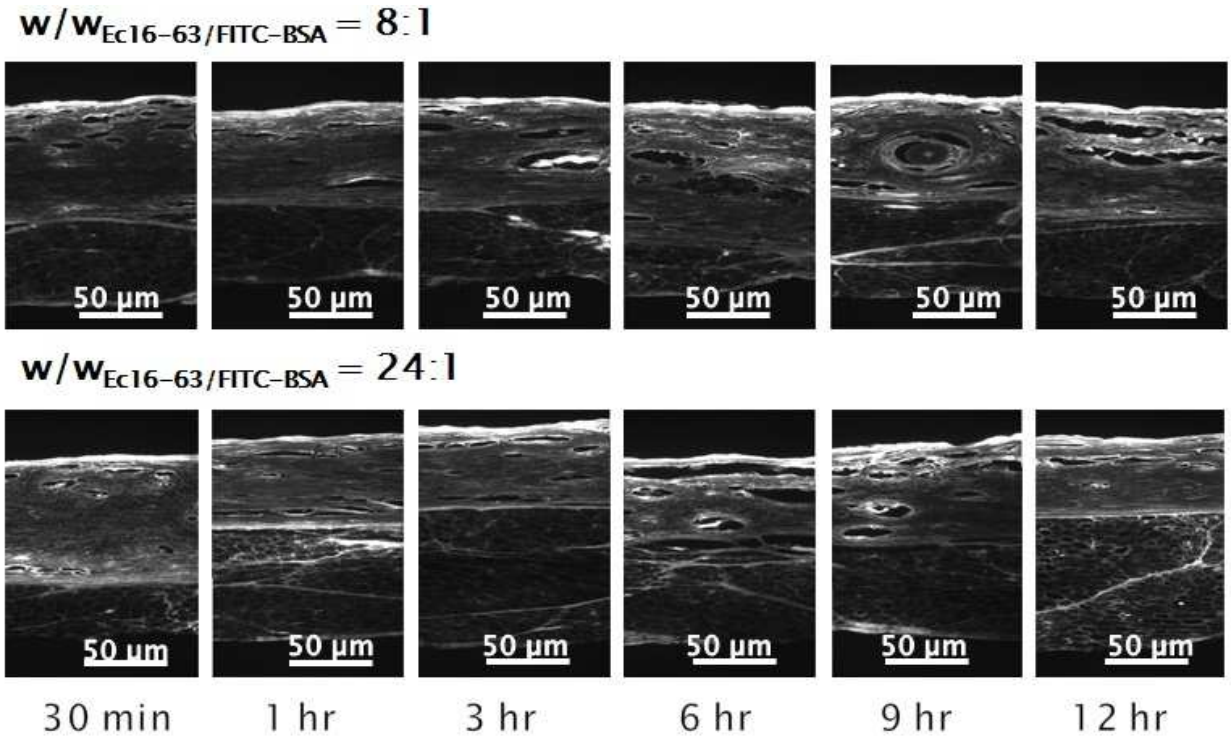


Figure 44: Fluorescence image of the full-thickness skin sample after Ec16-63/FITC-BSA NP solution was applied on the skin for predetermined times of exposure and the control. The weight ratio of the Ec16-63 to FITC-BSA is 24:1 and 8:1.

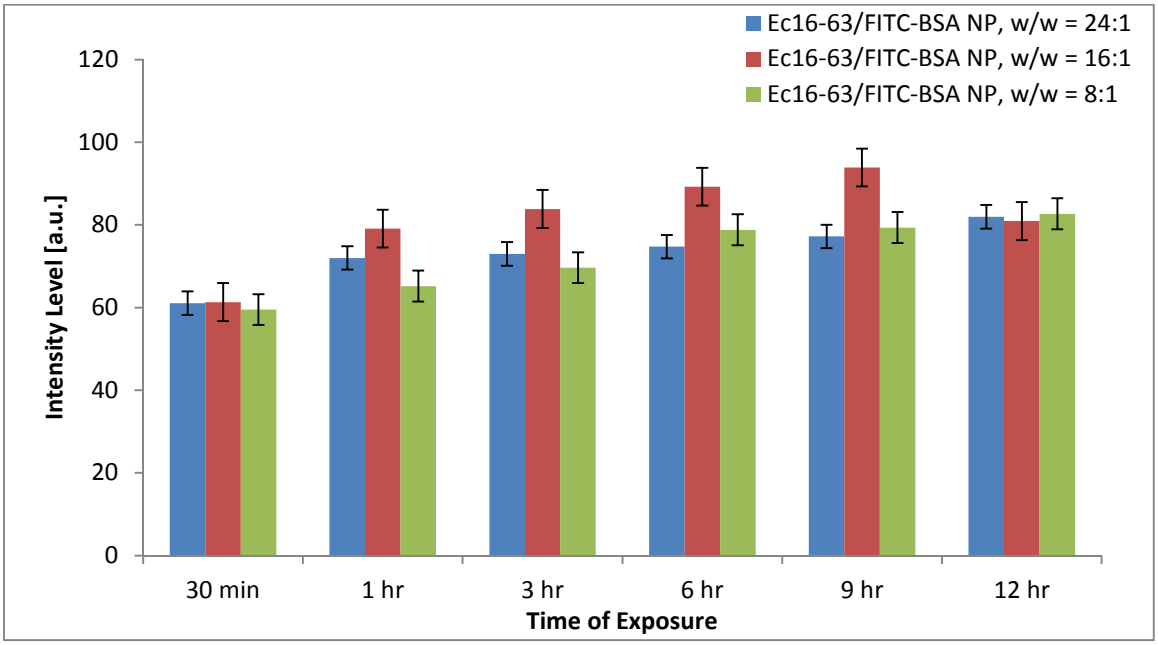


Figure 45: Brightness intensity level of the skin samples when the weight ratio of Ec16-63 to FITC-BSA NP was varied.

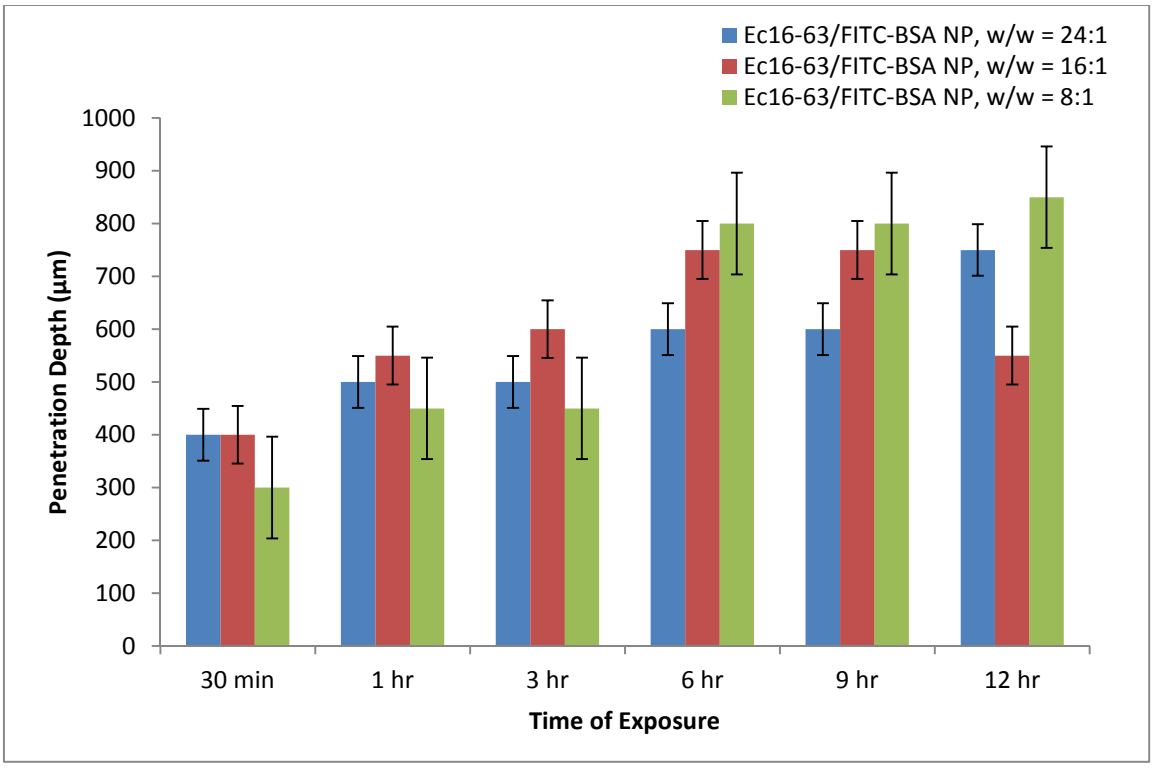


Figure 46: The penetration depth of Ec16-63/FITC-BSA NP in the skin when the weight ratio of Ec16-63 to FITC-BSA was varied.

4.5.3 Discussion

The lipid-protein weight ratio was altered for all the lipidoid/protein NP formulations to identify the optimal weight ratio that gives the best results in enhancing protein delivery through the skin. The fluorescence images of the skin samples at various weight ratios were compared for the lipidoid/protein NP formulations, Ec16-80/GFP NP, Ec16-80/FITC-BSA NP, Ec16-63/GFP NP and Ec16-63/FITC-BSA NP. It was seen that at all the weight ratios investigated, the brightness intensity of the skin samples increased as the time of exposure increased. The few inconsistencies observed were due to the skin structure and thickness. Some of the skin samples were harder than the others.

Comparing the intensity level of the skin samples at the different weight ratios of lipidoid to protein, the brightness intensity of the samples increased as the weight ratio of lipidoid to protein reduced. From the results, the weight ratio 8:1 yielded a consistent increase in the amount of protein as the time of exposure increased. This indicated that transdermal delivery of protein is enhanced better when the weight ratio is 8:1. The lower the weight ratio; the more consistent the increase in the depth of penetration of protein into the skin. From, the weight ratios used for the formulation of the protein nanoparticles, 8:1 is the most efficient of them all.

This research was for studying lipidoids for transdermal delivery of biomacromolecules, including siRNA and proteins and to establish the in vitro model for this study. The penetration experiment was used to prove that lipidoids can be used for the enhancement of the transdermal delivery of protein. So far, the weight ratio considered to be the most efficient for the delivery is 8:1.

CHAPTER FIVE

PERMEATION STUDY

5.1 MEMBRANE DIFFUSION STUDY OF LIPIDOID (Ec16-80)/FITC-BSA NANOPARTICLES THROUGH THE EPIDERMIS OF THE SKIN SAMPLE

5.1.1 Introduction

This experiment is for investigating the amount or the concentration of protein that travels through the epidermis to other layers of the skin. Franz diffusion cell was used for the permeation experiment and the membrane is the epidermis layer of the pig skin. Ec16-80 is used for this study and due to the results from the penetration experiments; the weight ratio of lipidoid to protein 24:1 was eliminated from the list of the weight ratio to be considered for the permeation study. Here, weight ratios 16:1 and 8:1 were considered and FITC-BSA was the protein used.

5.1.2 Results

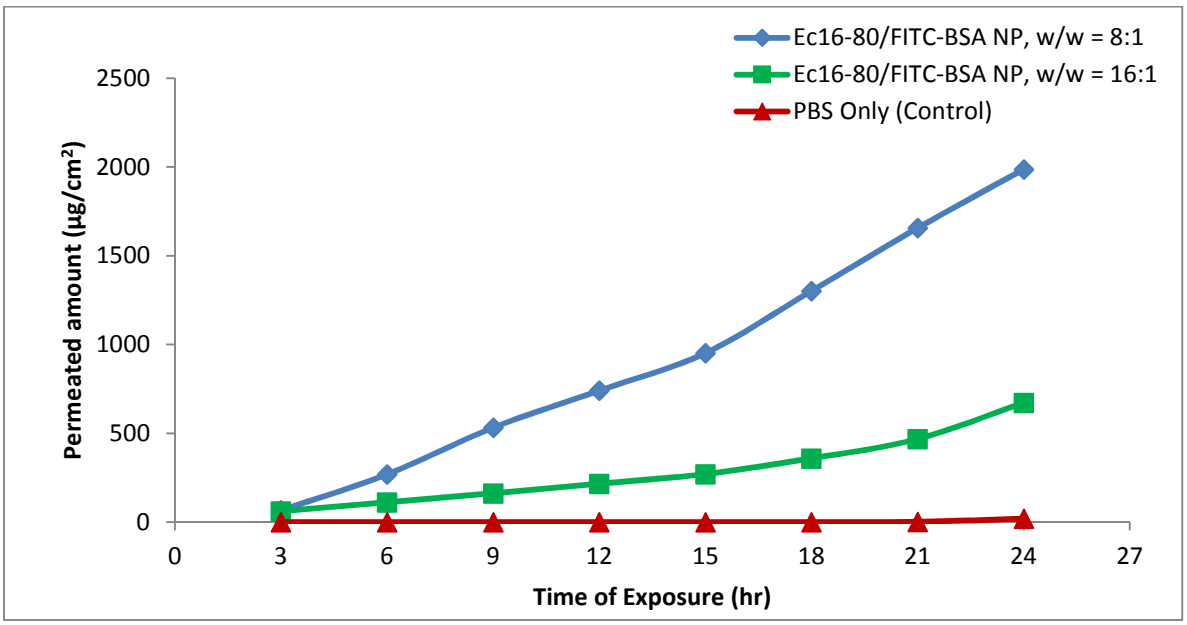


Figure 47: Permeated protein with different weight ratio of Ec16-80/FITC-BSA nanoparticles.

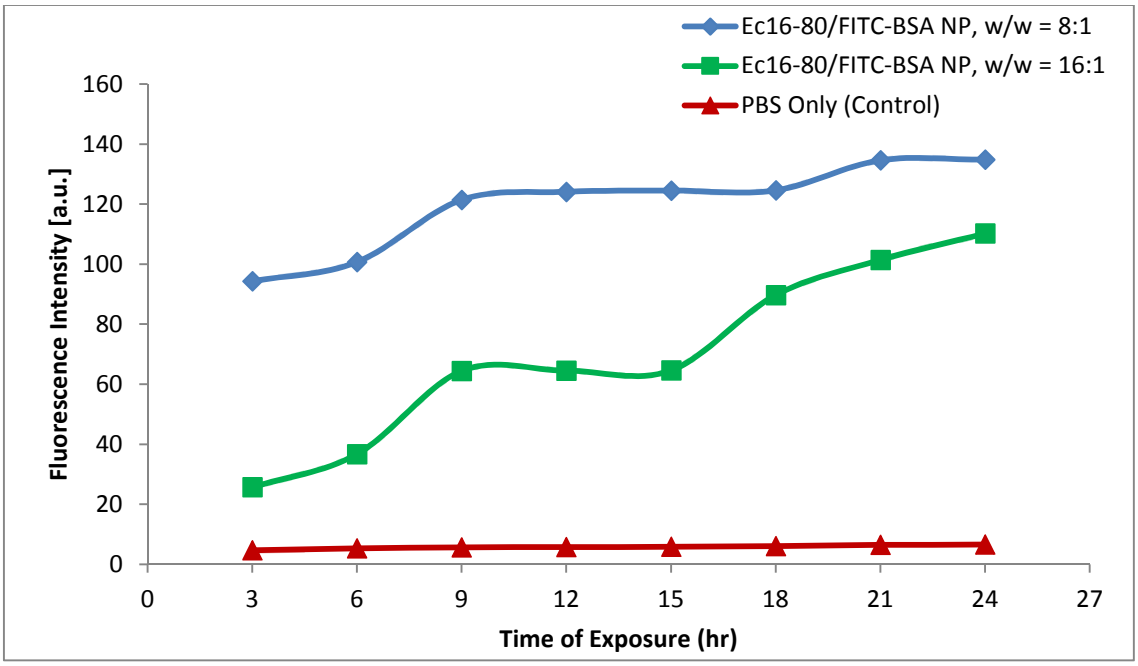


Figure 48: Fluorescence intensity of permeated protein with different weight ratio of Ec16-80/FITC-BSA nanoparticles.

5.1.3 Discussion

The results (figures 47 and 48) from protein permeation through the epidermis showed that a considerable amount of permeation occurred into the deeper layers of the skin. The concentration of the protein in the lipidoid/protein nanoparticles that traveled through the epidermis increased as the time of exposure increased and the peaks of fluorescence intensity spectra of the protein in the lipidoid/protein nanoparticles proved that the intensity of the protein increased as the time of exposure increased When the results from the two (16:1 and 8:1) weight ratios were compared, the weight ratio 8:1 was seen to be ideal because the concentration of the protein in the lipidoid/protein nanoparticles solution that passed through the epidermis was higher for it at the predetermined times of exposure.

When only FITC-BSA was used for the permeation experiment, the receptor solution (PBS) remained the same after 12 hours exposure time.

5.2 MEMBRANE DIFFUSION STUDY OF LIPIDOID (Ec16-63)/FITC-BSA NANOPARTICLES THROUGH THE EPIDERMIS OF THE SKIN SAMPLE

5.2.1 Introduction

The previous experiment has shown that the lipidoid enhanced the permeation of protein through the epidermis using Ec16-80. To test for the permeation using other lipidoids, Ec16-63 was used for this experiment. The weight ratio considered was 16:1.

5.2.2 Results

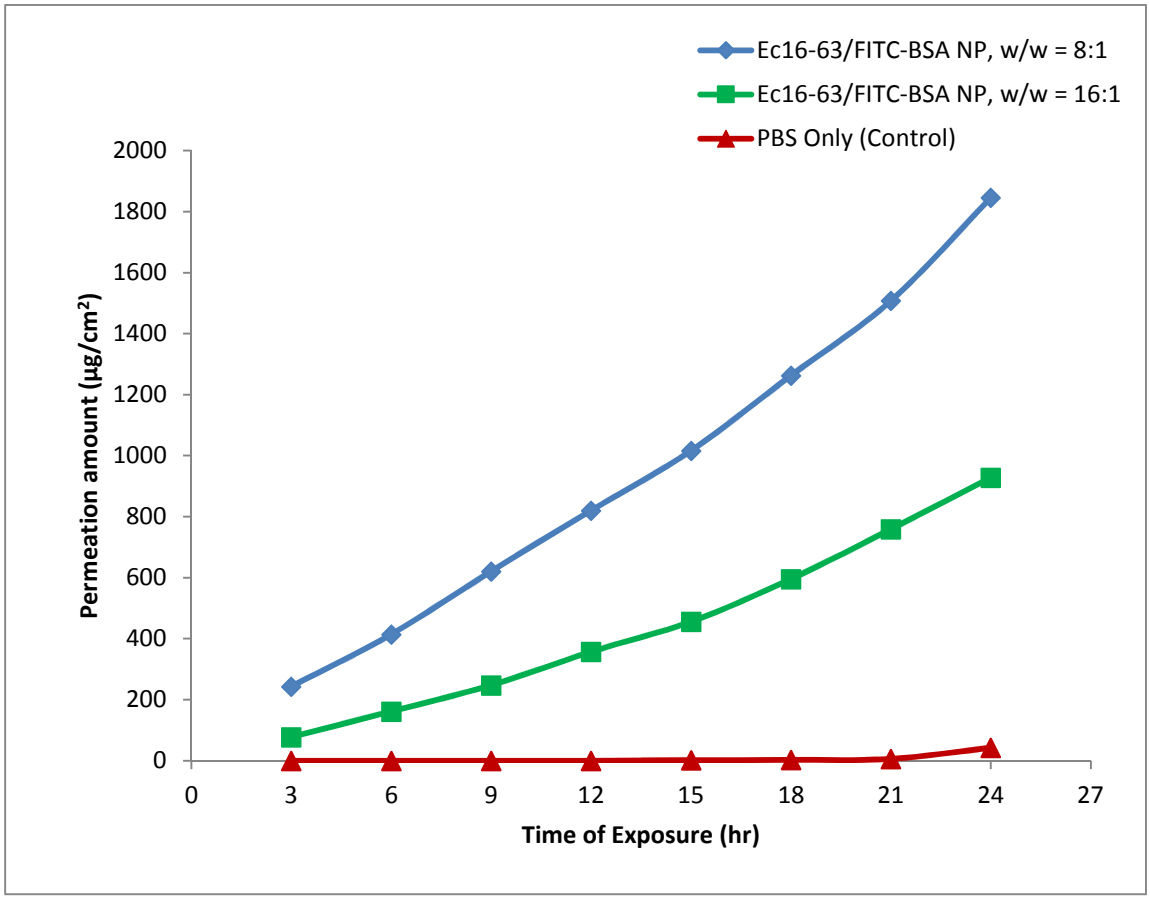


Figure 49: Permeated protein with different weight ratio of Ec16-63/FITC-BSA nanoparticles.

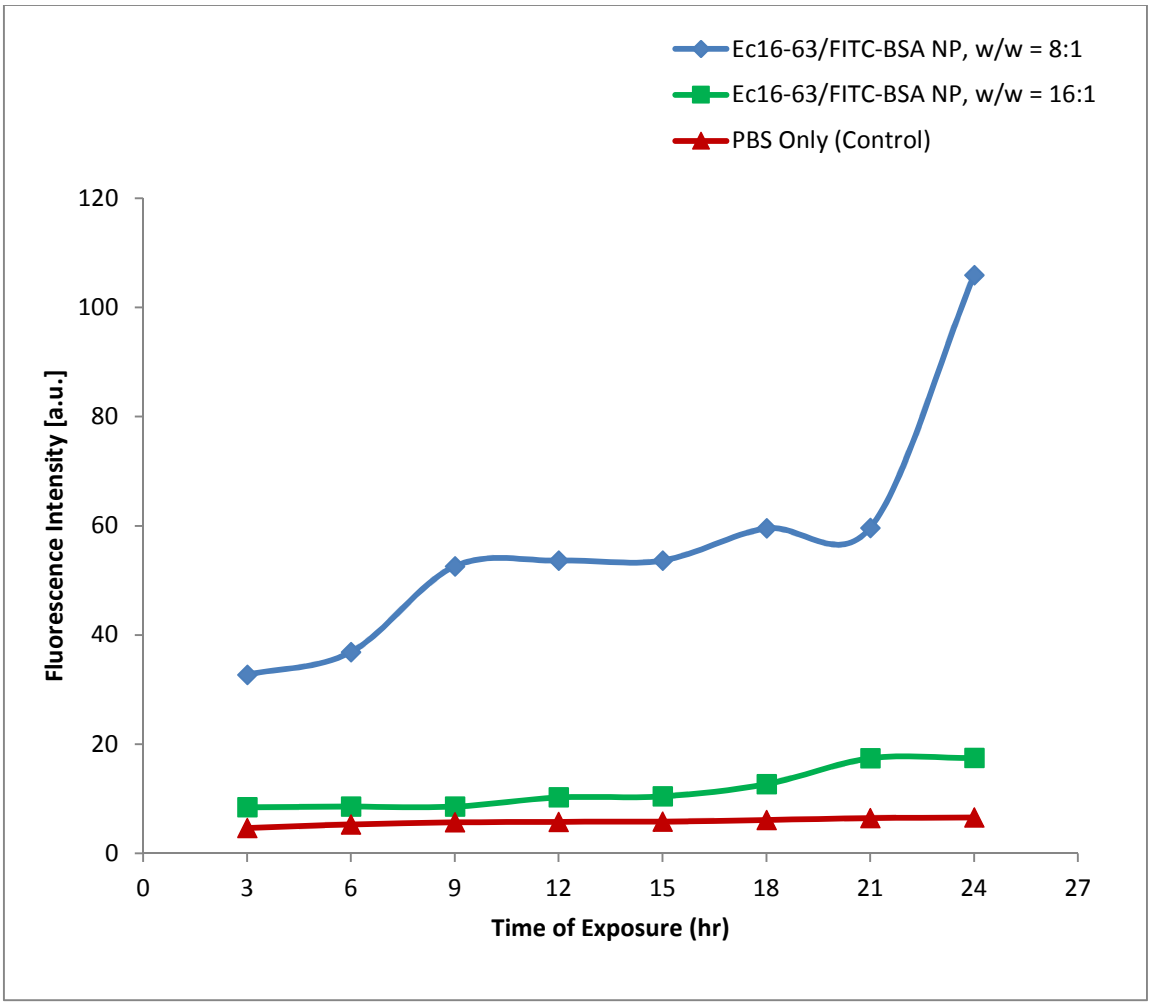


Figure 50: Fluorescence intensity of permeated protein with different weight ratio of Ec16-63/FITC-BSA nanoparticles.

5.2.3 DISCUSSION

The results from this experiment indicated that as the time of exposure of the epidermis to the lipid/protein nanoparticles solution increased, the concentration of the protein that passed through the epidermis also increased because the intensity of the protein also increased.

5.3 MEMBRANE DIFFUSION STUDY OF LIPIDOID (Ec16-80)/FITC-BSA NANOPARTICLES THROUGH THE FULL-THICKNESS SKIN SAMPLE

5.3.1 Introduction

One of the purposes of enhancing transdermal delivery is to be able to get the drugs to the target cell, tissue and organs. This calls for studying the amount or the concentration of protein that travels through the full-thickness skin sample at a predetermined time. Here, Franz diffusion cell and FITC-BSA (protein) were used. The weight ratio considered is 8:1. The aforementioned studies have shown that weight ratio 8:1 is more efficient than other weight ratios (24:1 and 16:1) considered. The experiment was monitored for 24 hr.

5.3.2 Results

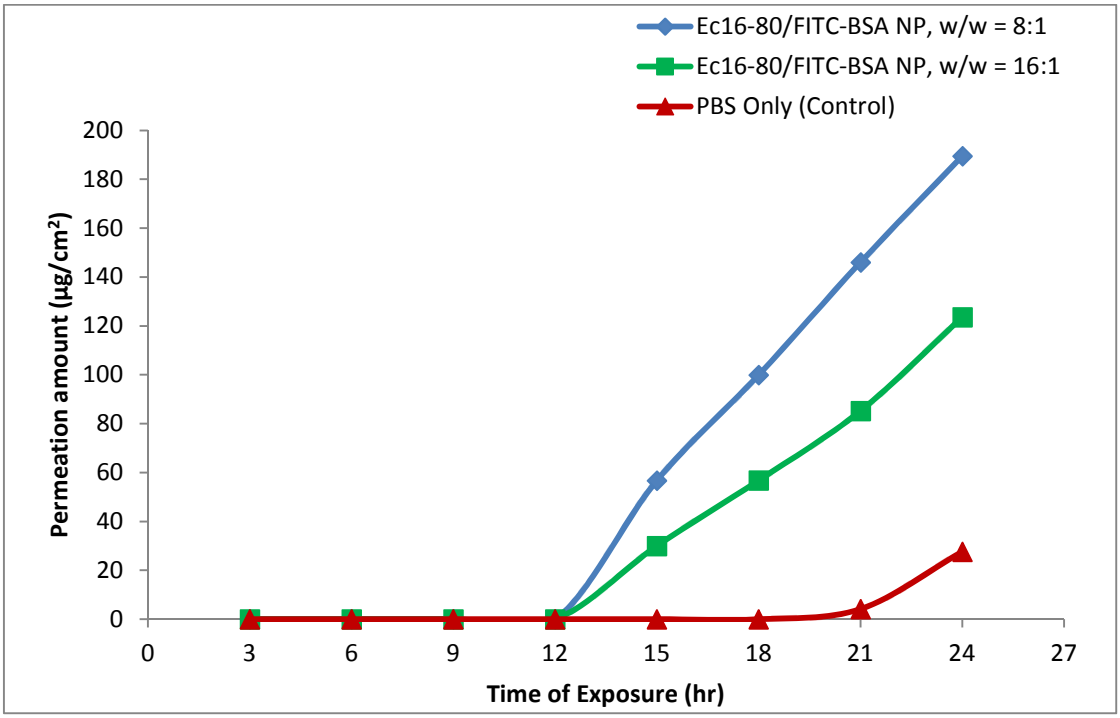


Figure 51: Permeated protein with different weight ratio of Ec16-80/FITC-BSA nanoparticles.

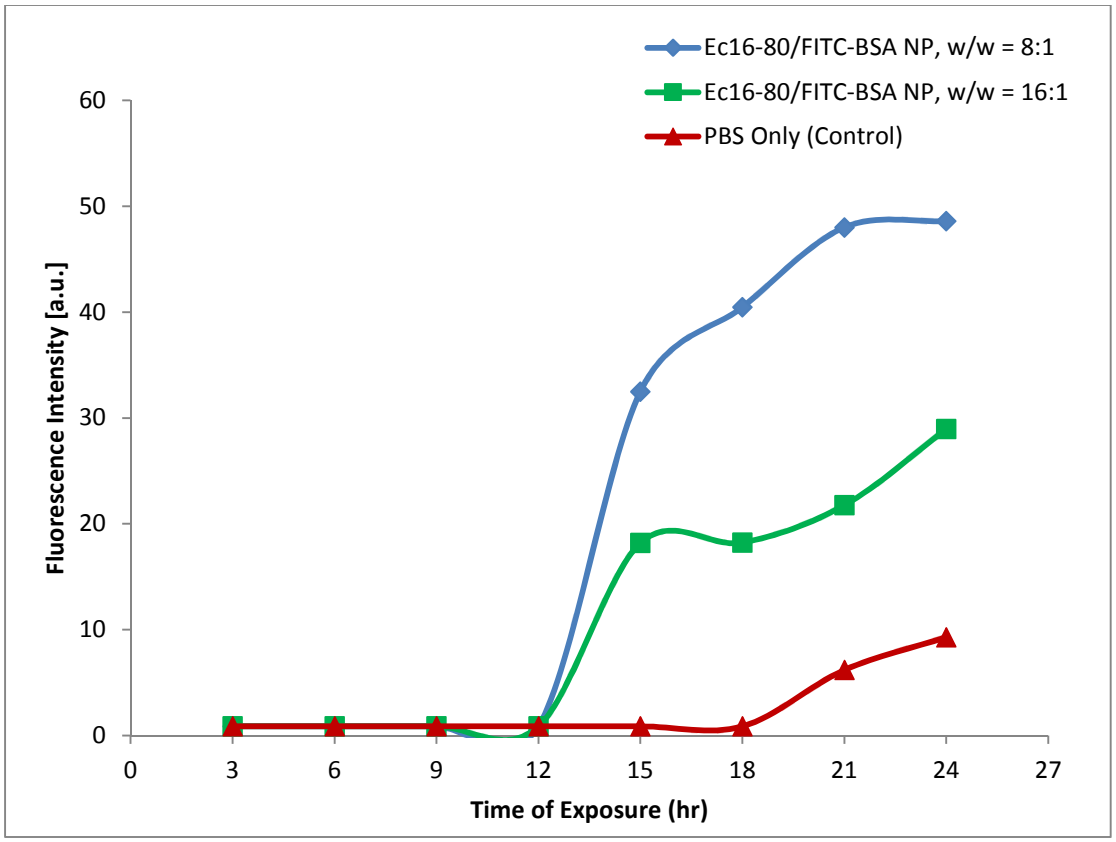


Figure 52: Fluorescence intensity of permeated protein with different weight ratio of Ec16-80/FITC-BSA nanoparticles.

5.3.3 Discussion

The permeation study of the full-thickness skin sample shows that the receptor fluid collected at predetermined time intervals, up to 12 hr did not contain any significant amount of protein. Protein was significantly detected in the receptor fluid collected at 15 hr to 24 hr. This means it took 15 hr for the protein to pass through the full-thickness skin sample. In this study, the weight ratio of 8:1 has a better delivery efficiency compare to weight ratio 16:1. The study has proven that lipidoids can be used in the transdermal delivery of protein and this will enhanced delivery of protein to the target organs and be used for therapy purposes.

CHAPTER SIX

CONCLUSION AND FUTURE DIRECTIONS

Penetration and permeation experiments are the experiments being used for the study of transdermal delivery. This research is for studying the lipid–like nanoparticles (lipidoids) for the transdermal delivery of biomacromolecules including siRNA and protein. The research is also to establish the in vitro model of the study. Protein is the biomacromolecules that is being used as the case study for other biomacromolecules. Both the penetration and permeation experiments being used for this study has shown that the lipidoids enhances the transdermal delivery of protein because it helps in carrying the protein through the stratum corneum (which has being acting as the barrier to the transdermal passage of particles or molecules into the body). Due to the many lipidoids being considered for the penetration experiment, it can be deduced that the Ec-series, non-degradable epoxy lipidoids, can be used for the enhancement of transdermal delivery of protein. The formulation of protein nanoparticles (lipidoid/protein) is another crucial part of this enhancement method to be considered. The weight ratio of the lipid to protein during protein nanoparticles formulation was also study. From the weight ratio considered, the weight ratio 8:1 is best because it yielded the most amount of protein that passes through the stratum corneum and even deeper into the skin. The permeation experiment also proves that protein was able to pass through the epidermis with the aid of the lipidoids and the concentration of the protein passing through the epidermis increases as the time of exposure increases.

Conclusively, with the stratum corneum posing as barrier to the transdermal delivery of protein and the micro size of protein, the lipidoids can be used as an enhancer for the transdermal delivery of protein. The model established in this research is that the weight ratio (lipidoid to

protein) 8:1 can be considered for the formulation of the protein nanoparticles. With protein used as the case study, lipidoids (lipid-like nanoparticles) can be used for the enhancement of the transdermal delivery of biomacromolecules; thereby delivering the biomacromolecules to the targeted cells and tissues. This is desirable for the advancement of therapeutics.

There are still more work to be done to really ascertain the enhancement of transdermal delivery of biomacromolecules using lipidoids. The future work includes studying the delivery using different types of protein like insulin and other biomacromolecules such as nucleic acids, using finite drug dosage, finding out if weight ratio (lipidoid to protein) lower than 8:1 will give a better result and in vivo study may also be considered.

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