In vitro biocompatibility of transferosomes, ethosomes and transethosomes

M Volkwyn

orcid.org/ 0000-0002-3177-5904

Dissertation submitted in fulfilment of the requirements for the degree Master of Science in Pharmaceutics at the North West University

Supervisor: Prof L du Plessis

Co-supervisor: Dr JM Viljoen

Graduation: November 2018

Student number: 23482877

ABSTRACT

Lipid nanocarriers (LN) for transdermal drug delivery, have gained more interest in recent years, due to their rapid penetration into the skin. Liposomes were investigated in the past with the goal of transdermal drug delivery, yet studies confirmed they were not able to achieve transdermal delivery, and should rather be considered for topical delivery. Focus moved to the ultradeformable lipid carriers due to their ability to penetrate the skin barrier without compromising the skin structure. Transferosomes are ultradeformable vesicles (UDV), which consist of a lipid and edge activator, and are the first generation of the elastic LN. Ethosomes are UDVs consisting of a lipid and ethanol, which acts as a membrane modulator, whereas transethosomes consist of a lipid and both an edge activator and ethanol.

LN resemble cell organelles due to their dimensions and content, therefore, a risk of potential cytotoxicity occurs. The first step in determining the biocompatibility of these UDVs was to prepare and optimize LN formulations, including the UDVs (transferosomes, ethosomes and transethosomes) and liposomes as a control. After preparation of these vesicles, each system was characterized utilizing the standardized method of dynamic light scattering (DLS), measuring vesicle diameter, PDI and zeta potential. Quantitative image analysis, utilizing specific shape and size parameters have not been established for LN, due to being mainly used to characterize powder particles in the past. The size and shape parameters of each LN were established by means of image analysis with the Malvern Morphologi G3, including intensity mean, diameter of an equivalent circle (CE diameter), solidity, elongation, convexity, circularity and aspect ratio. The LN were fairly solid, and low levels of elongation were observed, as well as high levels of convexity. The circularity of the LN, however, were varied. It was concluded that elongation, convexity and circularity were parameters that could be utilized for characterization, complementary to DLS. The stability of each system was also observed for 90 days.

The next step in determining the *in vitro* biocompatibility of the UDVs was to observe the effects they had on cells, by means of Thiazolyl blue tetrazolium bromide (MTT) and Trypan Blue dye exclusion assays, utilizing human malignant melanoma cells (A375) and primary epidermal keratinocytes (HaCat). Previous studies have suggested interference of the lipid content in liposomes with absorbance values as determined by the MTT assay, and it was investigated in this study whether this problem also occurred with UDVs.

The effects of vesicle concentration on cell viability was investigated by means of MTT assay. A correlation between lipid content and high absorbance values was observed, therefore, confirming the interference of lipid content of the UDV with accurate cell viability results. The effects of treatment time on cell viability was also investigated, this time utilizing MTT, as well as Trypan Blue dye exclusion assay. No toxicity was observed for the A375 cells, even after the

48 h treatment period, however, cytotoxicity was observed when the HaCat cells were treated for periods longer than 48 h. Both the MTT and Trypan Blue method showed accurate results when determining cell viability, despite having different mechanisms through which they determine viability. The *in vitro* biocompatibility was therefore confirmed for the UDVs, although longer treatment periods may lead to cytotoxicity.

Keywords: Lipid nanocarrier; characterization; Image analysis, Malvern Morphologi G3, Elongation, Convexity, Circularity; Liposomes, Ultradeformable vesicles, Cell viability; MTT; Trypan Blue, Skin toxicity, Biocompatibility

TABLE OF CONTENTS

PREFACE AND DISCLAIMER

This dissertation is presented in article form, therefore, for the purpose of publication, United States English will be used as per the author guidelines of the scientific journals. The articles are longer than the author guidelines prescribed, but will be retained as is for the sake of the dissertation. When the articles will be submitted for publication, they will be condensed to the appropriate length, as the author guidelines prescribed. The reference style is a numbered style, for ease of reading this dissertation.

Author contribution and permission statements

I, Michelle Volkwyn, am the main researcher responsible for the proposal, planning and execution of this study, along with (i) extensive review of the relevant literature, (ii) assessment and optimization of the bulk of the experimental protocol and methods, (iii) collection, analysis, interpretation and presentation of data, (iv) design, planning and writing of research articles, and (v) writing of all sections of this thesis.

Prof. Lissinda H. Du Plessis

Supervisor responsible for guidance, intellectual input and evaluation of research outputs.

Dr. J.M. Viljoen

Co-supervisor responsible for guidance, intellectual input and evaluation of research outputs.

Sonette du Preez

Colleague and co-author responsible for the analysis of samples by the Malvern Morphologi G3, as well as critical input reading.

Statement by co-authors

I hereby confirm that I approve the publication of the aforementioned manuscript(s) and that my role related to the completion of the dissertation, *In vitro* biocompatibility of transferosomes, ethosomes and transethosomes, is representative of my contribution. I give my consent that the M.Sc student Michelle Volkwyn, may include the manuscript(s) as part of her dissertation.

Prof. L.H. du Plessis

(Supervisor)

Dr. J.M. Viljoen

(Co-supervisor)

Me. S. du Preez

(Co-author)

"Data is not information
Information is not knowledge
Knowledge is not wisdom"
~Ian Lowe

ACKNOWLEDGEMENTS

Firstly, I would like to thank the Lord for giving me the opportunity to follow my dreams in pursuing a scientific career, as well as giving me the knowledge and guidance I need in this endeavor, as well as life.

I would like to acknowledge the following people for their guidance, love, understanding and motivation:

My dearest husband, Martin Volkwyn, for every cup of coffee you brought me while I was writing and every single word of support. Thank you for always being interested in my study, even though it isn't even closely related to your field of work. Thank you for always keeping me company when I had late or early laboratory sessions. You are the love of my life and I appreciate everything you do for me. I could not have done this without your help and support. I love you!

My mother, Erika Nortje, you have always supported me in everything I do. You have sacrificed so much for me to achieve what I have achieved thus far. There is nothing I could do to encompass my gratitude. I love you so much Mom!

My siblings, Monique, Marizanne and Eriko, you always keep life interesting. Your support and love is appreciated so much. I love you guys so much!

My father- and mother-in-law, Martin and Lizette Volkwyn, thank you so much for your support over the past two years.

My friends, Arina, Simoné, Michaela and Leroux, thank you for all the times I could unpack my frustrations of failed experiments. Thank you for all the uplifting cups of coffee and words of comfort. I really appreciate all your support.

My supervisor, Prof Lissinda du Plessis, without you this would not even be possible. Thank you for taking in an unsure newly postgraduate student and giving me the chance to learn so much from you. Thank you for guiding me throughout this study, and helping me to learn to think outside the box, as well as how to think scientifically. I appreciate every single hour of time that you have spent on me and with me throughout this journey.

My co-supervisor, Dr. Joe Viljoen, thank you so much for your help throughout this study. Thank you for your uplifting words

To Mrs. Hester de Beer, thank you so much for handling the administration of the Postgraduate students so well. You have made it so much easier to navigate the new and 'scary' world of the postgraduates.

To Dr. Lindi van Zyl, thank you for helping me learn the ropes of the formulation laboratory. You have helped me so much in creating these lipid nanocarriers. Without your know-how I would have still struggled to create fully formed vesicles.

To Dr. Jaco Wentzel, thank you for teaching me how to conduct the assay experiments in my study. I really appreciate your help.

To Dr. Wihan Pfeiffer, thank you for the cell culture training. Without you, I would not have realized that I had a knack for culturing cells.

To Sonette du Preez, thank you for helping me analyzing my vesicles with the Malvern Morphologi G3. I appreciate that you made time for me throughout the last phases of your doctoral dissertation. Thank you so much!

I want to especially thank the **National Research Foundation (NRF)** and the **Centre of Excellence for Pharmaceutical Sciences**, North-West University, Potchefstroom, for the funding and support of this study.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PDI	Polydispersity index
GRAS	Generally regarded as safe
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay / Thiazolyl blue tetrazolium bromide assay
A375 cells	Human malignant melanoma cells
HaCat cells	Human primary epidermal keratinocytes
UDV	Ultradeformable vesicles
GI	Gastro-intestinal
SLN	Solid lipid nanoparticles
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
SUV	Small unilamellar vesicle
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EA	Edge activator
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organization for Economic Co-operation and Development
FDA	US Food and Drug Administration
ECVAM	European Centre for the Validation of Alternative Methods
ROS	Reactive oxygen species
AOPs	Adverse outcome pathways
MIE	Molecular initiation events
LDH	Lactate dehydrogenase
ATP	Adenosine triphosphate
EU	European Union
LN	Lipid nanocarriers
PBS	Phosphate buffered saline
DOF	Depth of field
CE diameter	Diameter of an equivalent circle
TEM	Transmission electron microscopy
DMSO	Dimethylsulphoxide
DMEM	Dulbecco’s Modification of Eagle’s Medium
FBS	Fetal bovine serum
NEAA	Non-essential amino acids
ATCC®	American Type Culture Collection
mV	Milli-Volt
nm	Nanometer

SD	Standard deviation

CHAPTER 1 - INTRODUCTION

1.1. Introduction

The skin as an application route for cosmetics and pharmaceuticals remains at the forefront of research. Transdermal delivery is defined as “the entire process of transport of a substance that is applied to the skin surface,” therefore this process includes absorption through the skin, as well as uptake through microcirculation with the goal of reaching systemic circulation [1, 2]. Topical delivery is defined as “the application of a formulation to the skin, to create a localized effect in the specific area of the skin” [3-6]. Transdermal, as well as topical delivery were developed in ancient times by the Chinese, Egyptians, as well as the Romans. More recently, in the 1970s, large scale development commenced for use of drug-delivery vehicles [1-3]. Both these routes are utilized in cosmetic and pharmaceutical applications [7, 8].

Successful novel formulations and alternatives to simple lotions and creams have been developed, yet there are still some concerns regarding toxicity in cosmetics and pharmaceutics [9, 10]. For a great part in history, human risk assessment has been tested in animal models. This resulted in an enormous amount of animals needed in laboratories. The great numbers of animals, as well as distress caused by the tests caused a great deal of debate on the ethical, scientific and financial feasibility of these testing procedures [11-13]. Another issue concerning animal studies is the fact that there is always a degree of uncertainty when testing on animals, for humans and animals differ vastly in terms of kinetic and dynamics of drugs [14, 15]. Due to this, the EU Cosmetics Regulation (EC 1223/2009) foresaw a ban on the use of animals for testing of cosmetic ingredients in 2013, requiring the use of alternative *in vitro* methods for toxicology analysis [16-18].

Colloidal carriers have proven successful in nanotechnology in recent years. Colloidal carriers are defined as “particulate or vesicular dosage forms that serve as superficial drug reservoirs” [19, 20]. They are divided into micro colloidal carriers and nanoparticles, the nanoparticles are in a nano size range, while the micro colloidal carriers are in the micro size range [1, 21]. Investigations of nanoparticles have been performed on the delivery of drugs to specific sites, focusing on lipid nanocarriers (LN) [22]. These LN include solid lipid nanoparticles (SLN), and polymeric nanoparticles (NP) [22]. Lipid nanocarriers are colloidal systems that could readily deliver drugs in effective concentrations [23-25]. These nanocarriers are lipid droplets filled with an aqueous phase enclosed in a lipid membrane and can be as small as the nano range [23-28]. LN are divided into liposomes, ultra-deformable vesicles (UDV) and SLN. However, when LN is mentioned in this study, there will only be referred to liposomes and UDV.

Initially, development of lipid nanocarriers focused primarily on parenteral and oral use, however, the use of LN in the skin has acquired increased attention in the field of drug-delivery research,

because they have the capacity for controlled release by creating a localized depot in the skin [24, 25]. Several LN have been developed as an alternative to improve skin delivery, for use in pharmaceutics, as well as cosmetics, to ensure controlled and reliable release [21, 29]. These systems include liposomes, transferosomes, ethosomes and niosomes [29].

Bangham *et al.* [30] discovered and investigated liposomes as vesicular LN to improve delivery of therapeutic ingredients at specific sites in 1970; and reports were published more often in the 1980's [1, 29, 31]. Liposomes are self-assembled hollow lipid colloidal particles consisting of a phospholipid bilayer surrounding an aqueous core that encapsulates the active ingredient, on a microscopic level [23, 32-39]. The drug is encapsulated either in the core or in the bilayer depending on the solubility of the drug, as well as the process used to encapsulate it [35, 39]. The drug will be found in the bilayer if it is lipid soluble and in the aqueous core if it is water soluble. Encapsulation of drugs or other active ingredients help to provide a controlled release from the liposomes [37]. They aid localized effects when they are applied to the skin, by depositing the encapsulated active component in the subcutaneous tissue and acting as a drug reservoir [1, 40]. However, liposomes are unstable and it causes increased degradation and aggregation during storage [41]. When degradation of liposomes occurs, oxidation transpires additional to the hydrolysis process of degradation [42]. This indicates that the degradation of liposomes causes it to have redox-active properties. Since this carrier is so unstable, with poor permeability through the skin, it is mostly used for topical drug delivery [40].

Due to above mentioned challenges with liposomes, research has moved towards other types of colloidal carriers, such as transferosomes, ethosomes and transethosomes. In the early 1990s, researchers developed new novel LN, ultra-deformable vesicles (UDV) that demonstrated more deformability than liposomes to enhance their ability to deliver drugs transdermally, as well as easier manufacturing processes to upscale [40, 43-45].

Transferosomes, which were introduced in the 1990s by Cevc and Blume [44], are also known as ultra-deformable liposomes. They are the first generation of elastic LN that were hypothesized to penetrate intact skin spontaneously and can mediate site-specific drug delivery by design [43, 44, 46, 47]. These results could be compared to subcutaneous administration of the same drug [48-51]; fundamentally, it consists of phospholipids and surfactants [40, 52]. A positive feedback mechanism allows transferosomes to attain ultra-high deformability so that the vesicle can change its shape easily and reversibly [47].

There are currently three means by which transferosomes attain drug delivery, i.e., by creating drug depots in the skin, transportation of the drug into the systemic circulation, or by delivering the drug deep beneath the skin [28, 46-48, 53-57]. Various applications in the topical and transdermal field are demonstrated by transferosomes, which are mostly used for transporting

extremely small molecular drugs through the *stratum corneum*, to target specific peripheral tissue [29]. Although transferosomes show promise in the world of transdermal and topical delivery, there are still disadvantages that can create limitations with this carrier, i.e., chemical instability of the carrier can cause it to be prone to oxidative degradation, difficulty loading hydrophilic drugs into the carrier system, costly preparation, and occasional fluctuation in skin permeation, especially when concerning certain hydrophilic drugs, such as 5-fluorouracil [28, 40, 58, 59]. Presently, toxicology studies have shown that transferosomes show a relative lack of toxicity [27, 28].

Ethosomes are also ultra-deformable vesicles that consist of high quantities of ethanol, as well as phospholipids and water [51, 60]. Ethosomes were developed by Touitou *et al.* [60], and are mainly used for transdermal delivery of drugs [29, 40, 51, 61-63]. These vesicles are known to comprise of a very small size relative to liposomes, without taking size reducing steps [51, 62].

The use of ethanol in the preparation of ethosomes confers a negative surface net charge, which is the reason for the decrease in size of the vesicles; for this reason ethanol is able to enhance topical delivery [51, 60, 64-66]. The cell membrane's lipid malleability is influenced by ethanol, causing it to be more fluid, and therefore triggering enhanced permeation [60, 61, 63, 67-69]. However, an increase above 45% in ethanol, may cause the vesicle's membrane to become leaky, which will subsequently lead to lower entrapment efficiency [70, 71]. Drugs incorporated into ethosomes are not limited by their hydrophilicity or lipophilicity, both types can be incorporated into ethosomes [29, 60]. Challenges concerning ethosomes include problems with purity, as well as costly production costs [45, 72]. Extensive toxicological studies have shown that ethosomes are safe to cultured cells [70, 71, 73-75].

Transethosomes, conversely, are lipid vesicles that contain components of both ethosomes and transferosomes which Song *et al.* [76] introduced in 2012. They consist of phospholipids, ethanol, water and surfactants or permeation enhancers [40, 76, 77]. Transethosomes are more deformable than both ethosomes and transferosomes because they contain ethanol, as well as a surfactant [40, 76]. Transethosomes have shown improved skin permeation compared to both ethosomes and transferosomes, due to their irregular spherical shape and high vesicle elasticity, as well as their smaller particle size [40, 76, 77]. They contain components of both ethosomes and transferosomes; therefore challenges affecting transethosomes may be similar to challenges affecting them. Little information, since their discovery in 2012, is available concerning the disadvantages of transethosomes, and there is not much information on their toxicological profile, as toxicology studies are currently being performed [77].

Although LN possess numerous advantages, they are bound to have some disadvantages as well. The most general limitations occur during the design and characterization of LN [19, 78].

The ability to reproduce a specific shape of the carrier has proven to be a limiting factor in the design of these carriers [78, 79]. Other difficulties during design include loss of function, when applied to skin, due to enzymatic function, and changes in isothermal phases causing segregation from a vesicle [19, 78, 80]. These problems are all a consequence of the biological and physical instability of LN, especially liposomes [23], high manufacturing costs and impure phospholipid content may also affect the use of LN [23, 29, 81].

The relationship between the lipid nanocarriers' physiochemical properties and the interaction the carriers have on biological environments, is complex [82]. LNs resemble cell organelles considering their dimensions [82, 83], and mainly consist of lipids and surfactants [82, 83]. Thus, the resemblance in size can cause the carriers to interfere with the cell's vital functions, which in turn can result in potential cytotoxicity [82, 83]. Additionally, LN are formulated using excipients that are generally recognized as safe (GRAS), however the effects of nanotoxicity have not yet been researched satisfactory and nanotoxicity is not yet predictable when utilizing current toxicological methods. This area of research is nonetheless a rapidly developing and emerging subject in the nanomedicine research field [82]. Different parameters of LN may affect their toxicity, such as their shape, size, surface area and ability to create reactive oxygen species (ROS), therefore toxicity studies, such as oxidative stress, should be performed to establish whether these parameters are causing toxicity [82, 84]. Using novel pharmaceutical nanotechnology, these grey areas can be investigated and the desired outcomes can be achieved, because highly adaptable complex LN requires impartiality during testing and application design [19, 23].

According to Kohane and Langer [85] biocompatibility is defined as "an expression of the benignity of the relation between a substance and its biological environment." A substance can be inappropriate, even if it has a mostly benign reaction with biological tissue. The cell's ability to endure in the presence of substances can roughly be determined by *in vitro* studies [85]. The evaluation of safety and biocompatibility is a prerequisite when new substances or carriers are introduced onto the market [13]. Toxic effects are usually related to the concentration of drug *in vitro*, in this case the concentration of the colloidal carrier [14]. There are three ways by which the free concentration of colloidal carrier can affect the cell in a toxicological manner, namely: a concentration dependent impairment of mitochondrial function; cell membrane integrity is affected by LN, especially liposomes, which induce the release of lactate dehydrogenase (LDH); by affecting oxidative stress markers in cells [36]. Therefore, toxicological risk assessment or biocompatibility, has become a key aspect during development of novel treatments.

In skin toxicity assays, the measurement of necrosis is classified as *in vitro* skin corrosion potential [86]. *In vitro* models with specific endpoints, based on clinical or histopathological endpoints, can determine general mechanisms of toxicity from drug delivery systems [14, 36, 87, 88].

Mitochondrial activity is one of these endpoints, because nanoparticles have a pronounced influence on it [36, 89, 90].

There are different guidelines published to aid in biocompatibility testing. These include the International Standard – Biological evaluation of medical devices [91], OECD guidelines for the testing of chemicals [92], FDA Clinical Trials Guidance Documents and ECVAM Guidelines (European Union Reference Laboratory for alternatives to animal testing). The first step in toxicological risk assessment is the selection of the cellular system. The cellular system should be chosen according to what needs to be evaluated. In the case of mitochondrial damage evaluation, cell types that naturally endowed a high number of mitochondria should be selected. Secondly, the types of cytotoxic assays should be selected. The viability assay most commonly used is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that measures mitochondrial activity. The MTT is taken up by live cells and converted by the mitochondrial dehydrogenase enzymes to the product, formazan that can be quantified spectrophotometrically [13, 93].

The mitochondria play an important role in apoptosis and necrosis of cells, and interference into their function can lead to impaired cellular energy as well as lipid metabolism that can lead to release of cell death mediators [94, 95]. The role of the mitochondria in cell death is depicted in Fig. 1.1. One of the main key events in necrosis or apoptosis of cells is the mitochondrial membrane permeability, which triggers most forms of cell death [95, 96]. Low mitochondrial membrane potential can signal mitochondrial dysfunction, which can ultimately lead to necrosis or apoptosis [36].

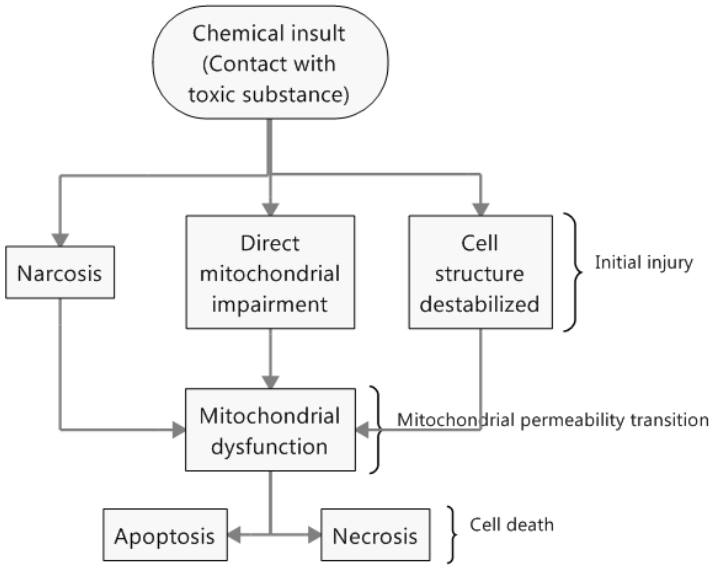


***Figure 1.1**: Outcome pathway from chemical insult to cell death. Initial injury because of contact with potential toxic substances can cause narcosis (damage to the cell's plasma membrane induced by chemicals), direct inhibition of the mitochondria or destabilization of the*

cell's structure. This causes a mitochondrial permeability transition, which will finally lead to apoptosis or, in the case of this study, necrosis

(Figure adapted from Vinken and Blaauboer [13])

A study by Angius and Floris [97] indicated that liposomes interfere with the MTT assay. They attributed this interference to the high affinity of lipid droplets to the MTT reagent, averting the MTT forming formazan intracellularly. The MTT-formazan continues to aggregate within the cells due to its highly lipophilic nature [97]. It was also suggested that the redox processes that are induced by liposomal formulations could affect this assay [97]. The most commonly utilized vital dye exclusion method is the Trypan Blue dye exclusion assay [98, 99]. Trypan blue is a dye that would not be taken up by viable cells, therefore, these would not be stained. Non-viable cells, on the other hand, would be stained due to their membranes that are not in tact [98]. The authors concluded that the use of vital dye exclusion as an additional method to the MTT is highly recommended. No studies up to date have investigated whether UDV, which also contain lipids also interferes with the MTT assay.

1.1.1. Research problem

Lipid nanocarriers (LN) other than liposomes are becoming more popular and effective for topical and transdermal delivery, but it is necessary to determine the biocompatibility, as well as the characterization of these systems before they can be used clinically. These LN have been characterized in the past using dynamic light scattering (DLS) characterization, including the vesicle diameter, polydispersity index (PDI) and zeta potential. However, novel means of characterization, such as the use of image analysis with the Malvern Morphologi G3 (Malvern Instruments Ltd, Malvern, Worcestershire, UK), have not yet been investigated.

Image analysis makes use of static images to quantify size and shape parameters in addition to particle morphology [100]. This study characterized liposomes, transferosomes, ethosomes and transethosomes using standardized characterization methods, utilizing DLS. In addition, the LN were characterized using the Malvern Morphologi G3 system (Malvern Instruments Ltd., Malvern, Worcestershire, UK), and determined whether this method could be utilized as a supplementary method for characterization. Characterization of the vesicles is of particular importance in biocompatibility studies, as certain parameters including size and shape of LN have been found to have effects on cytotoxicity [101-103].

These carriers are formulated with excipients generally regarded as safe (GRAS) [104, 105], however, they contain high amounts of surfactants and ethanol [106]. The skin's biocompatibility of these systems is poorly characterized. The first step in biocompatibility testing is determining the cytotoxicity of these carriers. The golden standard for testing cytotoxicity is the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Due to its lipophilic nature, the MTT-formazan accumulates in the lipid droplets of the carrier systems, as well as the plasma membranes, and this could lead to unreliable results [97, 107]. The MTT-formazan is also influenced by other nanoparticles, pyruvate analogues, polyphenols, acidic pH and processes such as reduction [108].

Due to liposomes' instability, cytotoxic effects can often be exerted by the liposomes themselves, and should therefore be tested by the MTT assay. The MTT assays are, however, often affected by the reactive oxygen species released by degenerating liposomes which may lead to unreliable results. Interactions from liposomes in the MTT assays have been scrutinized, because cell viability experiments are normally comparing liposomes containing active ingredients and "empty" liposomes, not to cell viability in untreated cells [97].

There is uncertainty whether transferosomes, ethosomes and transethosomes will also cause this problem with the MTT assay due to the presence of the ethanol and surfactants in the lipid-based formulations, and this must therefore be established. If the same problem occurs, alternative assays must be investigated. These assays will focus on *in vitro* skin toxicity parameters, where necrosis is linked to skin corrosion.

1.2. Aim and objectives of study

1.2.1. Aims of this study

The aim of this study was to determine the *in vitro* biocompatibility of transferosomes, ethosomes and transethosomes. Particular emphasis was placed on the possible interference the LN might have with the standardized MTT assay.

In addition, the secondary aim of this study was to investigate whether quantitative image analysis could be used complementary to standardized DLS characterization of LN.

1.2.2. Objectives of this study

The specific objectives of this study were to:

- Formulate and optimize LN, including liposomes as a control, and the UDVs: transferosomes, ethosomes and transethosomes.
- Characterize the LN systems with DLS using the parameters: particle size, PDI and zeta-potential.
- Characterize the LN systems with image analysis using the parameters: CE diameter, intensity mean, solidity, elongation, convexity, circularity and aspect ratio.

- Cultivate human malignant melanoma cells (A375) and primary epidermal keratinocytes cells (HaCat) as the *in vitro* model for the skin biocompatibility evaluation.
- Investigate the effect of different concentrations of the UDVs on cell viability with the MTT assay, to establish half maximal inhibition concentrations (IC_{50}) as a measure of biocompatibility.
- Investigate the possible interference of the UDVs with the MTT assay by comparing it to a vital dye exclusion assay (Trypan Blue)

A literature study is provided in Chapter 2. Chapter 3 contains the article on the characterization study of the LN by means of DLS and image analysis. Chapter 4 contains the article on the study of the biocompatibility of the LN, as well as the possible interference of these LN on the accuracy of the MTT assay in comparison with the Trypan Blue dye exclusion assay, and finally, Chapter 5 provides an overreaching discussion and conclusion of the study. A discussion on the limitations of the study and recommendations for future studies are made.

1.3. References

1. Bhushan, B., Dermal and transdermal delivery, in *Encyclopedia of nanotechnology*, B. Bhushan, Editor. 2012, Springer Science & Business. p. 517-525.
2. El-Kattan, A., C.S. Asbill, and S. Haidar, Transdermal testing: Practical aspects and methods. *Pharmaceutical science & technology today*, 2000. **3**(12): p. 426-430.
3. Aulton, M.E. and K.M.G. Taylor, Aulton's pharmaceutics: The design and manufacture of medicines. 4th ed. 2013: Elsevier Ltd. 894.
4. Chen, H. and J. Fang, Therapeutic patents for topical and transdermal drug delivery systems. *Expert opinion on therapeutic patents*, 2005. **10**(7): p. 1035-1043.
5. Osborne, D.W. and A.H. Amann, Topical drug delivery formulations. *Drug and the pharmaceutical sciences: A series of textbooks and monographs*, ed. J. Swarbrick. Vol. 42. 1990, New York: Marcel Dekker Inc. 420.
6. Singh, D., N. Mital, and G. Kaur, Topical drug delivery systems: A patent review. *Expert opinion on therapeutic patents*, 2016. **26**(2): p. 213-228.
7. Nounou, M.*, et al.*, Liposomal formulation for dermal and transdermal drug delivery: Past, present and future. *Recent patents on drug delivery & formulation*, 2008. **2**(1): p. 9-18.
8. Toll, R.*, et al.*, Penetration profile of microspheres in follicular targeting of terminal hair follicles. *Journal of investigative dermatology*, 2004. **123**(1): p. 168-176.
9. Pimentel, F.*, et al.*, Macroalgae-derived ingredients for cosmetic industry —an update. *Cosmetics*, 2017. **5**(1).
10. Chang, R.K.*, et al.*, Generic development of topical dermatologic products: Formulation development, process development, and testing of topical dermatologic products. *AAPS Journal*, 2013. **15**(1): p. 41-52.
11. Blaauboer, B.J., Biokinetic modeling and in vitro-in vivo extrapolations. *Journal of toxicology and environmental health, Part B, Critical Reviews*, 2010. **13**(2-4): p. 242-252.
12. Kramer, N.I.*, et al.*, Biokinetics in repeated-dosing in vitro drug toxicity studies. *Toxicology in vitro*, 2015. **30**(1 Part A): p. 217-224.
13. Vinken, M. and B.J. Blaauboer, In vitro testing of basal cytotoxicity: Establishment of an adverse outcome pathway from chemical insult to cell death. *Toxicology in vitro*, 2017(39): p. 104-110.
14. Blaauboer, B.J.*, et al.*, The use of biomarkers of toxicity for integrating in vitro hazard estimates into risk assessment for humans. *ALTEX*, 2012. **29**(4): p. 411-425.
15. Renwick, A.G. and N.R. Lazarus, Human variability and noncancer risk assessment — an analysis of the default uncertainty factor. *Regulatory toxicology and pharmacology*, 1998. **27**: p. 3-20.
16. Coecke, S.*, et al.*, Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicology in vitro*, 2013. **27**(5): p. 1570-1577.

17. Punt, A.*, et al.*, Evaluation of research activities and research needs to increase the impact and applicability of alternative testing strategies in risk assessment practice*. Regulatory toxicology and pharmacology*, 2011. **61**: p. 105–114.
18. Spielmann, H.*, et al.*, Successful validation of in vitro methods in toxicology by Zebet, the National Centre for Alternatives in Germany at the BFR (Federal Institute for Risk assessment)*. Experimental and toxicologic pathology*, 2008. **60**(2-3): p. 225-233.
19. Cevc, G., Lipid vesicles and other colloids as drug carriers on the skin*. Advanced drug delivery reviews*, 2004. **56**(5): p. 675-711.
20. Sharma, J.*, et al.*, Colloidal drug carriers*. The internet journal of family practice*, 2009. **9**(2).
21. Cevc, G. and U. Vierl, Nanotechnology and the transdermal route: A state of the art review and critical appraisal*. Journal of controlled release*, 2010. **141**(3): p. 277-299.
22. Alvarez-Roman, R.*, et al.*, Skin penetration and distribution of polymeric nanoparticles*. Journal of controlled release*, 2004. **99**(1): p. 53-62.
23. Alexander, A.*, et al.*, Recent expansion of pharmaceutical nanotechnologies and targeting strategies in the field of phytopharmaceuticals for the delivery of herbal extracts and bioactives. *Journal of controlled release*, 2016. **241**: p. 110-124.
24. Kumar, L.*, et al.*, Fatty acid vesicles acting as expanding horizon for transdermal delivery*. Artificial cells, nanomedicine, and biotechnology*, 2017. **45**(2): p. 251-260.
25. Neubert, R.H., Potentials of new nanocarriers for dermal and transdermal drug delivery*. European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
26. Honeywell-Nguyen, P.L. and J.A. Bouwstra, Vesicles as a tool for transdermal and dermal delivery*. Drug discovery today: Technologies*, 2005. **2**(1): p. 67-74.
27. Priyanka, R. and D. Shipra, Transferosomes - a novel carrier for transdermal drug delivery system*. International journal of pharmacy & technology*, 2012. **4**(1): p. 1854-1865.
28. Vinod, K.R.*, et al.*, Critical issues related to transferosomes – novel vesicular stystem*. Acta scientiarum polonorum - Technologia alimentaria*, 2012. **11**(1): p. 67-82.
29. Akhtar, N., Vesicles: A recently developed novel carrier for enhanced topical drug delivery*. Current drug delivery*, 2014. **11**(1): p. 87-97.
30. Bangham, A.D., M.M. Standish, and J.C. Watkins, Diffusion of univalent ions accross the lamellae of swollen phospholipids*. Journal of molecular biology*, 1965. **13**: p. 238-252.
31. Goyal, P.*, et al.*, Liposomal drug delivery systems – clinical applications*. Acta Pharmaceutica*, 2005. **55**: p. 1–25.
32. Barry, B.W., Novel mechanisms and devices to enable successful transdermal drug delivery*. European journal of pharmaceutical science*, 2001. **14**: p. 101-114.
33. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Can drug-bearing liposomes penetrate intact skin? *Journal of pharmacy and pharmacology*, 2006. **58**(4): p. 415-429.

34. Hillaireau, H. and P. Couvreur, Nanocarriers' entry into the cell: Relevance to drug delivery. *Cellular and molecular life sciences*, 2009. **66**(17): p. 2873-2896.
35. Lasic, D.D., *et al.*, Gelation of liposome interior: A novel method for drug encapsulation. *Federation of European Bioclaemical Societies Letters*, 1992. **312**(2, 3): p. 255-258.
36. Roursgaard, M., *et al.*, In vitro toxicity of cationic micelles and liposomes in cultured human hepatocyte (HepG2) and lung epithelial (A549) cell lines. *Toxicology in vitro*, 2016. **36**: p. 164-171.
37. Saraf, S., *et al.*, Advancements and avenues in nanophytomedicines for better pharmacological responses. *Journal of nanoscience and nanotechnology*, 2015. **15**(6): p. 4070-4079.
38. Torchilin, V.P., Structure and design of polymeric surfactant-based drug delivery systems. *Journal of controlled release*, 2001. **73**: p. 137-172.
39. Zamboni, W.C., Liposomal, nanoparticle, and conjugated formulations of anticancer agents. *Clinical cancer research*, 2005. **11**(23): p. 8230-8234.
40. Ascenso, A., *et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
41. Simoes, M.C., J.J. Sousa, and A.A. Pais, Skin cancer and new treatment perspectives: A review. *Cancer letters*, 2015. **357**(1): p. 8-42.
42. Fordada, M., *et al.*, Chemical degradation of liposomes by serum components detected by NMR. *Chemistry and physics of lipids*, 2000. **104**: p. 133-148.
43. Ascenso, A., *et al.*, In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. *European journal of pharmaceutics and biopharmaceutics*, 2014. **88**(1): p. 48-55.
44. Cevc, G. and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochimica et Biophysica Acta*, 1992. **1104**: p. 226-232.
45. Manosroi, A., *et al.*, Novel elastic nanovesicles for cosmeceutical and pharmaceutical applications. *Chiang Mai journal of science*, 2009. **36**(2): p. 168-178.
46. Cevc, G., A. Schäitzlein, and G. Blume, Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. *Journal of controlled release*, 1995. **36**: p. 3-16.
47. Cevc, G., Transdermal drug delivery of insulin with ultradeformable carriers. *Clinical pharmacokinetics*, 2003. **41**(5): p. 461-474.
48. Cevc, G. and G. Blume, New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, transfersomes. *Biochimica et Biophysica Acta*, 2001. **1514**: p. 191-205.

49. Cevc, G. and G. Blume, Biological activity and characteristics of triamcinolone-acetonide formulated with the self-regulating drug carriers, transfersomes®. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2003. **1614**(2): p. 156-164.
50. Cevc, G. and G. Blume, Hydrocortisone and dexamethasone in very deformable drug carriers have increased biological potency, prolonged effect, and reduced therapeutic dosage*. Biochimica et Biophysica Acta*, 2004. **1663**(1-2): p. 61-73.
51. Elsayed, M.M*., et al.*, Lipid vesicles for skin delivery of drugs: Reviewing three decades of research*. International journal of pharmaceutics*, 2007. **332**(1-2): p. 1-16.
52. Abdellatif, A.A. and H.M. Tawfeek, Transfersomal nanoparticles for enhanced transdermal delivery of clindamycin*. AAPS PharmSciTech,* 2016. **17**(5): p. 1067-1074.
53. Cevc, G*., et al.*, The skin: A pathway for systemic treatment with patches and lipid-based agent carriers. *Advanced drug delivery reviews*, 1996. **18**: p. 346-378.
54. Cevc, G., Drug delivery across the skin*. Expert opinion on investigating drugs*, 1997. **6**(12): p. 1887-937.
55. Cevc, G., G. Blume, and A. Schäitzlein, Transfersomes-mediated transepidermal delivery improves the regio-specificity and biological activity of corticosteroids in vivo*. Journal of controlled release*, 1997. **45**: p. 211–226.
56. Simoes, S.I*., et al.*, Developments in the rat adjuvant arthritis model and its use in therapeutic evaluation of novel non-invasive treatment by SOD in transfersomes*. Journal of controlled release*, 2005. **103**(2): p. 419-434.
57. Zheng, W.S*., et al.*, Preparation and quality assessment of itraconazole transfersomes*. International journal of pharmaceutics*, 2012. **436**(1-2): p. 291-298.
58. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Skin delivery of oestradiol from deformable and traditional liposomes: Mechanistic studies*. Journal of pharmacy and pharmacology*, 1999. **51**: p. 1123-1134.
59. Ita, K.B*., et al.*, Dermal delivery of selected hydrophilic drugs from elastic liposomes: Effect of phospholipid formulation and surfactants*. Journal of pharmacy and pharmacology*, 2007. **59**(9): p. 1215-1222.
60. Touitou, E*., et al.*, Ethosomes — novel vesicular carriers for enhanced delivery characterization and skin penetration properties*. Journal of controlled release*, 2000. **65**: p. 403-418.
61. Thadanki, M. and A.K. Babu, Review on ethosomes: A novel approach of liposomes*. International journal of pharmacy & life sciences*, 2015. **6**(1): p. 4171-4176.
62. Dayan, N. and E. Touitou, Carriers for skin delivery of trihexyphenidyl HCl: Ethosomes vs. Liposomes*. Biomaterials*, 2000. **21**: p. 1879-1885.
63. Verma, P. and K. Pathak, Therapeutic and cosmeceutical potential of ethosomes: An overview. *Journal of advanced pharmaceutical technology & research*, 2010. **1**(3): p. 274-282.

64. Bouwstra, J.A. and P.L. Honeywell-Nguyen, Skin structure and mode of action of vesicles. *Advanced drug delivery reviews*, 2002. **54**(1): p. S41–S55.
65. Lopez-Pinto, J.M., M.L. Gonzalez-Rodriguez, and A.M. Rabasco, Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *International journal of pharmaceutics*, 2005. **298**(1): p. 1-12.
66. Marto, J.*, et al.*, Ethosomes for enhanced skin delivery of griseofulvin. *Colloids and Surfaces B: Biointerfaces*, 2016. **146**: p. 616-23.
67. Abdulbaqi, I.M.*, et al.*, Ethosomal nanocarriers: The impact of constituents and formulation techniques on ethosomal properties, in vivo studies, and clinical trials. *International journal of nanomedicine*, 2016. **11**: p. 2279-2304.
68. Ainbinder, D. and E. Touitou, Testosterone ethosomes for enhanced transdermal delivery. *Drug delivery*, 2005. **12**(5): p. 297-303.
69. Limsuwan, T. and T. Amnuaikit, Development of ethosomes containing mycophenolic acid. *Procedia chemistry*, 2012. **4**: p. 328-335.
70. Jain, S.*, et al.*, Formulation and evaluation of ethosomes for transdermal delivery of lamivudine. *AAPS PharmSciTech*, 2007. **8**(4): p. E1-E9.
71. Rakesh, R. and K.R. Anoop, Ethosomes for transdermal and topical drug delivery. *International journal of pharmacy & pharmaceutical sciences*, 2012. **4**(3): p. 17-24.
72. Manosroi, A., P. Jantrawut, and J. Manosroi, Anti-inflammatory activity of gel containing novel elastic niosomes entrapped with diclofenac diethylammonium. *International journal of pharmaceutics*, 2008. **360**(1-2): p. 156-163.
73. Nandure, H.P.*, et al.*, Ethosome: A novel drug carrier. *International journal of pharmaceutical research & allied sciences*, 2013. **2**(3): p. 18-30.
74. Paolino, D.*, et al.*, Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers. *Journal of controlled release*, 2005. **106**(1-2): p. 99-110.
75. Touitou, E.*, et al.*, Intracellular delivery mediated by an ethosomal carrier. *Biomaterials*, 2001. **22**: p. 3053-3059.
76. Song, C.K.*, et al.*, A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation. *Colloids and Surfaces B: Biointerfaces*, 2012. **92**: p. 299-304.
77. Chen, Z.X.*, et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers. *European journal of pharmaceutical science*s, 2017. **99**: p. 240-245.
78. Prow, T.W.*, et al.*, Nanoparticles and microparticles for skin drug delivery. *Advanced drug delivery reviews*, 2011. **63**(6): p. 470-491.
79. Caldorera-Moore, M.*, et al.*, Designer nanoparticles: Incorporating size, shape and triggered release into nanoscale drug carriers. *Expert opinion on drug delivery*, 2010. **7**(4): p. 479-495.

80. Bower, C.K.*, et al.*, Activity losses among T4 lysozyme charge variants after adsorption to colloidal silica*. Biotechnology and bioengineering*, 1999. **64**(3): p. 373-376.
81. Wang, X.*, et al.*, Advances of cancer therapy by nanotechnology*. Cancer research and treatment*, 2009. **41**(1): p. 1-11.
82. Zhang, X.Q.*, et al.*, Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine*. Advanced drug delivery reviews*, 2012. **64**(13): p. 1363-1384.
83. Shvedova, A.A., V.E. Kagan, and B. Fadeel, Close encounters of the small kind: Adverse effects of man-made materials interfacing with the nano-cosmos of biological systems*. Annual review of pharmacology and toxicology*, 2010. **50**: p. 63-88.
84. Xia, T., N. Li, and A.E. Nel, Potential health impact of nanoparticles*. Annual review of public health*, 2009. **30**: p. 137-150.
85. Kohane, D.S. and R. Langer, Biocompatibility and drug delivery systems*. The royal society of chemistry*, 2010. **1**: p. 441–446.
86. Hulzebos, E.*, et al.*, Use of structural alerts to develop rules for identifying chemical substances with skin irritation or skin corrosion potential*. QSAR & combinatorial science*, 2005. **24**(3): p. 332-342.
87. Nel, A.*, et al.*, A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment*. Perspectives - American chemical society*, 2013. **7**(8): p. 6422–6433.
88. Nel, A.*, et al.*, Nanomaterial toxicity testing in the 21st century: Use of a predictive toxicological approach and high-throughput screening*. Accounts of chemical research*, 2013. **46**(3): p. 607-621.
89. Hall, A.*, et al.*, Polyethylenimine architecture-dependent metabolic imprints and perturbation of cellular redox homeostasis*. Biochimica et Biophysica Acta*, 2015. **1847**(3): p. 328-342.
90. Jantzen, K.*, et al.*, Oxidative damage to DNA by diesel exhaust particle exposure in co-cultures of human lung epithelial cells and macrophages*. Mutagenesis*, 2012. **27**(6): p. 693-701.
91. ISO10993, Biological evaluation of medical devices - part 5: Tests for in vitro cytotoxicity. ISO 10993, 2009. **5**.
92. OECD, OECD guidelines for the testing of chemicals. 2001, Organisation for Economic Co-operation and Development (OECD).
93. Fotakis, G. and J.A. Timbrell, In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride*. Toxicology letters*, 2006. **160**(2): p. 171-177.
94. Jones, D.P.*, et al.*, Mechanisms of pathogenesis in drug hepatotoxicity: Putting the stress on mitochondria. *Molecular interventions*, 2010. **10**(2): p. 98-111.

95. Tsujimoto, Y. and S. Shimizu, Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis*, 2007. **12**(5): p. 835-840.
96. Pessayre, D., *et al.*, Central role of mitochondria in drug-induced liver injury. *Drug metabolism reviews*, 2012. **44**(1): p. 34-87.
97. Angius, F. and A. Floris, Liposomes and MTT cell viability assay: An incompatible affair. *Toxicology in vitro*, 2015. **29**(2): p. 314-319.
98. Louis, K.S. and A.C. Siegel, Cell viability analysis using trypan blue: Manual and automated methods. *Methods in molecular biology*, 2011. **740**: p. 7-12.
99. Jones, K.H. and J.A. Senft, An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *Journal of histochemistry and cytochemistry*, 1985. **33**(1): p. 77-79.
100. Malvern Instruments Limited, Morphologi G3 user manual, M.I. Limited, Editor. 2015, Malvern Instruments Limited: Malvern, Worcestershire, UK.
101. Krupa, A., *et al.*, Preparation of solid self-emulsifying drug delivery systems using magnesium aluminometasilicates and fluid-bed coating process. *Powder technology*, 2014. **266**: p. 329-339.
102. Peltonen, L., Practical guidelines for the characterization and quality control of pure drug nanoparticles and nano-cocrystals in the pharmaceutical industry. *Advanced drug delivery reviews*, 2018. **131**: p. 101-115.
103. Jindal, A.B., The effect of particle shape on cellular interaction and drug delivery applications of micro- and nanoparticles. *International journal of pharmaceutics*, 2017. **532**(1): p. 450-465.
104. Pardeike, J., A. Hommoss, and R.H. Muller, Lipid nanoparticles (sln, nlc) in cosmetic and pharmaceutical dermal products. *International journal of pharmaceutics*, 2009. **366**(1-2): p. 170-184.
105. Chen, M.L., Lipid excipients and delivery systems for pharmaceutical development: A regulatory perspective. *Advanced drug delivery reviews*, 2008. **60**(6): p. 768-777.
106. Lachenmeier, D.W., Safety evaluation of topical applications of ethanol on the skin and inside the oral cavity. *Journal of occupational medicine and toxicology*, 2008. **3**: p. 26.
107. Stockert, J.C., *et al.*, MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica*, 2012. **114**(8): p. 785-796.
108. Wang, S., H. Yu, and J.K. Wickliffe, Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale tio2. *Toxicology in vitro*, 2011. **25**(8): p. 2147-2151.

CHAPTER 2 – LITERATURE REVIEW

2.1. Lipid nanocarriers promising novel formulations for transdermal and topical route

Transdermal delivery is defined as “the entire process of transport of a substance that is applied to the skin surface.” Absorption through every layer of the skin, as well as the uptake through microcirculation and distribution through the systemic circulation are all part of the transdermal delivery process [1, 2]. Topical delivery is defined as “the application of a formulation to the skin, to create a localized effect in a specific area of the skin” [3-6]. Transdermal, as well as topical delivery were first modelled by the ancient Chinese, Egyptians and Romans [1-3]. Another report on how the transdermal or topical route of delivery were used, was the use of a mercury ointment to treat syphilis from the 1910’s to the 1950’s, where after development of transdermal and topical delivery systems commenced actively in the mid-1970’s when many advances were made concerning transdermal delivery vehicles [1-3, 7-9]. The topical and transdermal routes are both used in cosmetic and pharmaceutical applications [10, 11].

The skin is an exceptional site for delivery of drugs, because it is easily accessible, along with avoiding degradation of molecules through the gastrointestinal tract (GI) [12]. Traditional formulations used for transdermal delivery, include ointments, gels, creams and medical plasters that may contain natural compounds, as they are considered reasonably safe [13, 14]. These traditional formulations, however, did not effectively overcome the skin barrier to ensure systemic effects [13]. In 1981, Azla developed the first transdermal patch, *Transderm-Scop*, which is still on the market [2, 15].

The transdermal route became more relevant during the early 1980’s because it displayed a wide range of advantages [2]. Since then, many other advantages have been established, including: avoidance of the GI tract; averting hepatic first-pass effects. improvement of patient compliance by lowering the dosing schedule, provision of a steady plasma profile by providing sustained delivery of drugs and therefore side effects as well as toxicity will be minimized, rapid termination of drug effects, extension of the half-lives of drugs that have short half-lives, and many more [2, 12, 14, 16-21]. Due to the aforementioned advantages, the administration of peptides and proteins plays a significant role in the relevance of the transdermal route, for it is easily degraded inside the GI tract, and low uptake into the systemic circulation from the GI tract [22].

During the early 1920’s Rein hypothesized that the layer of cells, that connect the *stratum corneum* to the epidermis, were the principle resistance that faced transdermal transport [23, 24], however through Scheuplein’s research, it was determined that the *stratum corneum* itself limits transdermal transport [23, 25, 26]. Other challenges regarding the transdermal route are interpatient variation in penetration, effectiveness of the skin’s barrier system, hydrolysis of drugs,

formulation issues and stability challenges [14, 17, 19, 27]. Only a small number of drug molecules have been successfully delivered transdermally because of the limitations of transport through the *stratum corneum* [21, 23]. The topical route's challenges are similar to those of the transdermal route, especially when the structure of the skin is involved, where the major challenge is permeability to the targeted sites on the skin [6].

Successful novel formulations and alternatives to simple lotions and creams have been developed, yet there are still some concerns regarding toxicity in cosmetics and pharmaceutics. Throughout history, human risk assessment has been tested in animal models, which resulted in an enormous amount of animals needed in laboratories. This high numbers of animals, as well as the distress caused by these experiments, initiated a great deal of debate on the ethical, scientific and financial feasibility of these testing procedures [28-30]. Another issue concerning animal studies is the fact there is always a degree of uncertainty when testing, for humans and animals differ vastly in terms of kinetic and dynamics of drugs [31, 32]. Due to these reasons, the European Cosmetics Regulation (EC 1223 / 2009) foresaw a ban on the use of animals for testing of cosmetic ingredients in 2013, and requires the use of alternative methods for toxicology analysis [33, 35, 36].

2.2. Lipid colloidal carrier systems

Novel formulations that have proven successful over the past ten years in nanotechnology, are colloidal carriers. Colloidal carriers are defined as "particulate or vesicular dosage forms that serve as superficial drug reservoirs" [37, 38]. These carrier systems can be divided into micro colloidal carriers and nanoparticles. The size range of nanoparticles is smaller than 1 µm, whereas micro colloidal carriers are larger than 1 µm [1, 39]. Nanoparticles have been investigated for delivery of drugs to specific sites, especially lipid nanocarriers, such as solid lipid nanoparticles (SLN), and polymeric nanoparticles [40]. Lipid nanocarriers are colloidal systems that have a particle or droplet size as small as 500 nm, with the ability to deliver drugs in effective concentrations [41-43]. They are water filled vesicles with a lipid membrane [44-46], and are divided into liposomes, ultra-deformable vesicles (UDV) and SLN. However, when lipid nanocarriers are mentioned in this chapter, they will only be referred to as liposomes and UDVs.

Initially, development of lipid nanocarriers focused primarily on parenteral and oral use, however, the use of thereof in the skin acquired increased attention in the field of drug-delivery research, because they have the capacity for controlled release by creating a localized depot in the skin [42, 43]. Several lipid nanocarriers have been developed as an alternative to improve skin delivery; for use in pharmaceutics, as well as cosmetics, to ensure controlled and reliable release [39, 47]. These systems include liposomes, transferosomes, ethosomes and niosomes [47].

2.2.1. Lipid nanocarrier advantages

Reports have shown that lipid nanocarriers have a promising future in the topical and transdermal delivery of various drugs; they will deliver drugs more effectively and *in vivo* drug release will be more easily attained [47, 48]. The use of liposome-based products for clinical application was promoted by the encouraging results obtained during research on experimental animals [49]. Econazole was the first drug that used colloidal carriers on the skin, and was introduced commercially shortly before 1990, but several patents of lipid nanocarriers were submitted, one of which included a liposomal lidocaine product [37].

Most lipid nanocarriers can penetrate rapidly into the skin, especially through pilosebaceous units and wounds [37, 50-54]. Bilayer vesicles are highly adaptable because they achieve bilayer elasticity, causing them to be more deformable [37]. Ultradeformable lipid nanocarriers can achieve penetration without compromising the skin barrier due to their extreme versatility [37, 55-60]. Other advantages for lipid nanocarriers are the transport of drugs of various molecular size and weight, as well as structure and polarity, across the skin; the controlled release of drug from the carrier through the skin, albeit a localized depot in the skin can also be achieved; targeted delivery through the skin; non-toxicity of these carriers; relative thermodynamic stability and incorporation of both hydrophilic and hydrophobic drugs into these carriers [37, 61-63].

2.2.2. Liposomes

Overcoming the skin barrier, as well as obtaining controlled release using liposomes provoked great interest in the scientific community for a few decades after their discovery [64]. Liposomes were first identified by Alec Bangham, in 1964, who described them as closed vesicles, consisting of phospholipids, formed in an excess of a watery phase [65]. This description was improved later on, defining liposomes as colloidal particles capable of encapsulating drugs, which are formed as concentric bio-molecular layers [56, 63, 66-72]. Only during the 1980's did Mezei and Gulasekharam [72] report the potential of skin delivery through the use of liposomes [66, 67, 72-76]. At first, conventional liposomes were investigated because of their drug permeation enhancing properties across the skin, as well as drug retention capability within the skin [74, 77, 78].

2.2.2.1. Structure of liposomes

Liposomes consist of one or more concentric amphiphilic lipid bilayers that enclose an aqueous compartment within, without surface tension [10, 39, 47, 49, 53, 55, 79-85]. These bilayers resemble biological membranes [71]. Liposomes form spontaneously when a lipid is brought into contact with an aqueous phase and becomes hydrated [47, 62, 71, 86]. With conventional liposomes, these bilayers are usually stiff, preventing undesirable leakage of drugs from the

vesicles [39]. These artificial vesicles are microscopic and usually nearly spherical [10, 39, 41, 47, 84, 87, 88].

Liposomes are typically prepared from phospholipids, which are natural components of the cell membrane [37, 70, 74, 82, 83, 87, 89-92]. The use of other amphiphilic components is also possible [82, 91]. Electrolytes may also be added, and will enhance lipid bilayer formation, as well as provide isotonicity [86]. The most common phospholipid used in formulating liposomes is phosphatidylcholine (PC), obtained from egg yolk or soya bean, although phospholipids such as phosphatidylethanolamines (PE) and phosphatidylserines (PS) can also be used [66, 83]. Phospholipids are employed because of their ability to form bilayers, which is due to their amphiphilic nature [10, 71, 83, 86]. Therefore, the vesicles show lipophilic behavior within the bilayer, whereas a hydrophilic environment is maintained within the vesicles, as well as the suspension [83, 86, 93]. The use of cholesterol in addition to PC is for its stabilizing effects on the structure of the vesicles, thus for its improvement of bilayer characteristics [67, 92, 94]. The materials used in forming liposomes are relatively biocompatible, biodegradable and non-toxic [49, 95].

The size of liposomes may vary notably depending on the ingredients and methods employed during formulation, with a relatively large average size of 75 nm in diameter, but can reach sizes as small as 20 nm and as large as a few micrometers in diameter [37, 39, 41, 47, 67, 85-87, 95]. The types of lipids used during formulation, along with the preparation method, may also influence other characteristics of liposomes, such as liposome charge, membrane fluidity, particle size distribution, charge surface hydration, and clearance of the drug-carrier system [86, 94]. Liposomes are considered a favorable carrier system for hydrophilic, as well as lipophilic drugs, due to their size and amphiphilic character [87].

Liposomes can be categorized, on the basis of lamellae, size, as well as composition [70, 83, 95]. When a liposome consists of a single bilayer surrounding the aqueous compartment, it is referred to as a unilamellar lipid vesicle, where after it is further classified according to its size, i.e. small unilamellar vesicles (SUV), or large unilamellar vesicles (LUV) [86]. SUV have an average size of ≤0.1 µm, whereas the LUV have a size of >0.1 µm [93, 95]. Liposomes consisting of multiple bilayers enclosing the aqueous compartments, are referred to as a multilamellar vesicles (MLV) [71, 86, 95], which have an average size of >0.1 µm [95]. A summary of the characteristics of SUVs, LUVs and MLVs are listed in Table 2.1.

***Table 2.1**: Characteristics of liposomes classified by size and number of lamellae*

Type	***Characteristics***
SUV (Small unilamellar vesicles)	• Single bilayer • Thermodynamically unstable, therefore, less suitable for drug delivery • Susceptible to aggregation or fusion • Long circulation half-life • Low aqueous volume to lipid ratio (0.2:1.5 mole lipid) [83, 93, 95]
LUV (Large unilamellar vesicles)	• Single bilayer • Useful for hydrophilic drugs • High aqueous volume to lipid ratio (7:1 mole lipid) [83, 95]
MLV (Multilamellar vesicles)	• Multiple bilayers • Improved encapsulation of lipophilic drugs • Moderate aqueous volume to lipid ratio (4:1 mole lipid) • Mechanically stable during long-term storage [83, 95]

Liposomes may also be classified based on their composition and method of drug delivery as: conventional liposomes, pH-sensitive liposomes, cationic liposomes, long circulating liposomes, immuno-liposomes, heat sensitive liposomes and magnetic liposomes [83, 95]. The characteristics of these liposomes are listed in Table 2.2.

***Table 2.2**: Characteristics of liposomes classified based on composition and method of drug delivery*

Type	*Characteristics*
Conventional liposomes	• Composed of neutral or negatively charged phospholipids and cholesterol • Short circulation half-life • Dose dependent pharmacokinetics [83, 95]
pH-sensitive liposomes	• Composed of phospholipid such as phosphatidylethanolamine (PE) • Subject to coated pit endocytosis at low pH • Suitable for intracellular delivery of macromolecules and weak bases • Pharmacokinetics are similar to those of conventional liposomes [83, 95]
Cationic liposomes	• Composed of cationic lipids • May fuse with cells or endosome membranes • Suitable for negatively charged macromolecules, such as DNA and RNA • Mainly used for local administration • Structurally unstable • Toxic at high doses [83, 95]
Long circulating liposomes	• Also known as stealth liposomes • Composed of neutral high transition temperature lipids and cholesterol • Long circulation half-life • Dose independent pharmacokinetics • Surface have hydrophilic coating [83, 95]
Immuno-liposomes	• Conventional or long circulating liposomes with attached antibody or recognition sequence • Subject to receptor mediated endocytosis • Cell-specific binding [83, 95]
Heat sensitive liposomes	• Composed of dipalmitoyl phosphatidylcholine • Maximum release will be obtained at 41°C, which is the phase transition temperature of the lipids [83]
Magnetic liposomes	• Composed of phosphatidylcholine, cholesterol and a small amount of colloidal particles of magnetic iron oxide [83]

Liposomes can encapsulate hydrophilic compounds within their aqueous core, or intercalate lipophilic drugs within the lipid bilayer [49, 68, 86, 93, 95-97]. They are unique in their ability to accommodate a wide variety of drugs, with different physiochemical properties [79].

2.2.2.2. Efficacy of liposomes

As stated previously, liposomes have the potential to be drug carriers for a variety of drugs, encapsulating small molecular weight drugs, as well as diagnostic agents and therapeutic proteins, with seemingly superior delivery in certain fields [41, 71, 93]. Liposomal systems have shown pronounced systemic efficacy; they have the ability to enhance capillary permeability of hydrophilic drugs, therefore, localizing these drugs to their different target areas [94, 98].

It has been claimed that liposomes have the ability to enhance delivery of encapsulated drugs into the skin, suggesting they would be useful as carriers for both dermal and transdermal drug delivery [97, 99, 100]. Reportedly, they improve drug deposition into the epidermis, when compared to a saturated aqueous control [93]. There have been mixed reports regarding the efficacy of transdermal delivery of liposomes, causing a controversy on liposomes acting as dermal and transdermal drug carriers [101].

Mezei and Gulasekharam [72] reported that liposomes could be effectively used for transdermal delivery of triamcinolone acetonide [76, 102]. There are reports that liposomes could deliver drugs transdermally through the hair follicles of the skin, causing the drugs to move deeper into the skin layers [86, 103, 104]. Additional reports suggest that transdermal delivery could have been reached, not only by hair follicles, but also by sebaceous glands [86].

Contradictory to Mezei's reports, there have been studies that show once the liposomes are applied to the skin, they will remain confined within the upper layers of the skin, and act as a drug reservoir, suggesting they are only suitable for topical delivery [63, 67, 87, 105-109]. Although liposomes may enhance skin deposition, minimal penetration to deeper tissues occurs [67, 68, 92, 108, 109]. A possible reason for this is due to the large size of liposomes; they will not fit within the intercellular lipid domains of the *stratum corneum*, and will therefore, not deliver the drug within the deeper skin layers [62, 67, 92, 108]. Therefore, liposomes have little value as transdermal carriers, but have great potential as localizers of topically applied drugs, thereby improving the therapeutic index, and minimizing toxicity [10, 67, 87, 97, 101, 110].

2.2.2.2.1. Mechanism through which liposomes are transported into the skin

Bangham *et al.* [85] investigated and discovered liposomes as vesicular lipid nanocarriers to improve delivery of therapeutic ingredients at specific sites; and reports were published in the 1980's as liposomes gained more popularity [1, 47, 49]. The drug is either encapsulated in the core or in the bilayer depending on the solubility of the drug, as well as the process used to encapsulate it [96, 111]; or it will be found in the bilayer if it is lipid soluble and in the aqueous core if it is water soluble. Encapsulation of drugs or other active ingredients support the provision of a controlled release from the liposomes [112]. They aid localized effects when they are applied

to the skin, by depositing the encapsulated active component in the subcutaneous tissue and acting as a drug reservoir [1, 63].

An advantage of delivery through or into the skin, is that the onset of toxic effects are much slower than that of other delivery methods [113]. A possible site of toxicity in the skin as suggested by Park *et al.* [114], is the keratinocytes within the epidermis, as they are sensitive to nanoparticles (as shown in Fig. 1.1). Other forms of toxicity are skin corrosion and skin irritation, which occurs in the *stratum corneum*, the epidermis or within the dermis and is usually caused by most inorganic acids and bases, as well as strong organic bases and acids [115]. After topical application, liposomes can have diverse mechanisms in terms of drug delivery [62, 116]. They may enhance deposition of drug within the skin, without causing systemic absorption [116]. They can also cause targeted delivery to skin appendages [116]. Although there has been much speculation, there are a few potential mechanisms identified by which liposome deposition within the skin is reached (as depicted in *Figure 2.1*) [82, 92, 93, 104, 117, 118]:

- a free drug process
- permeation enhancement due to interaction with skin lipids
- penetration of the *stratum corneum* by intact liposomes, via pilosebaceous units and by fusing to the skin, releasing the drug into the skin

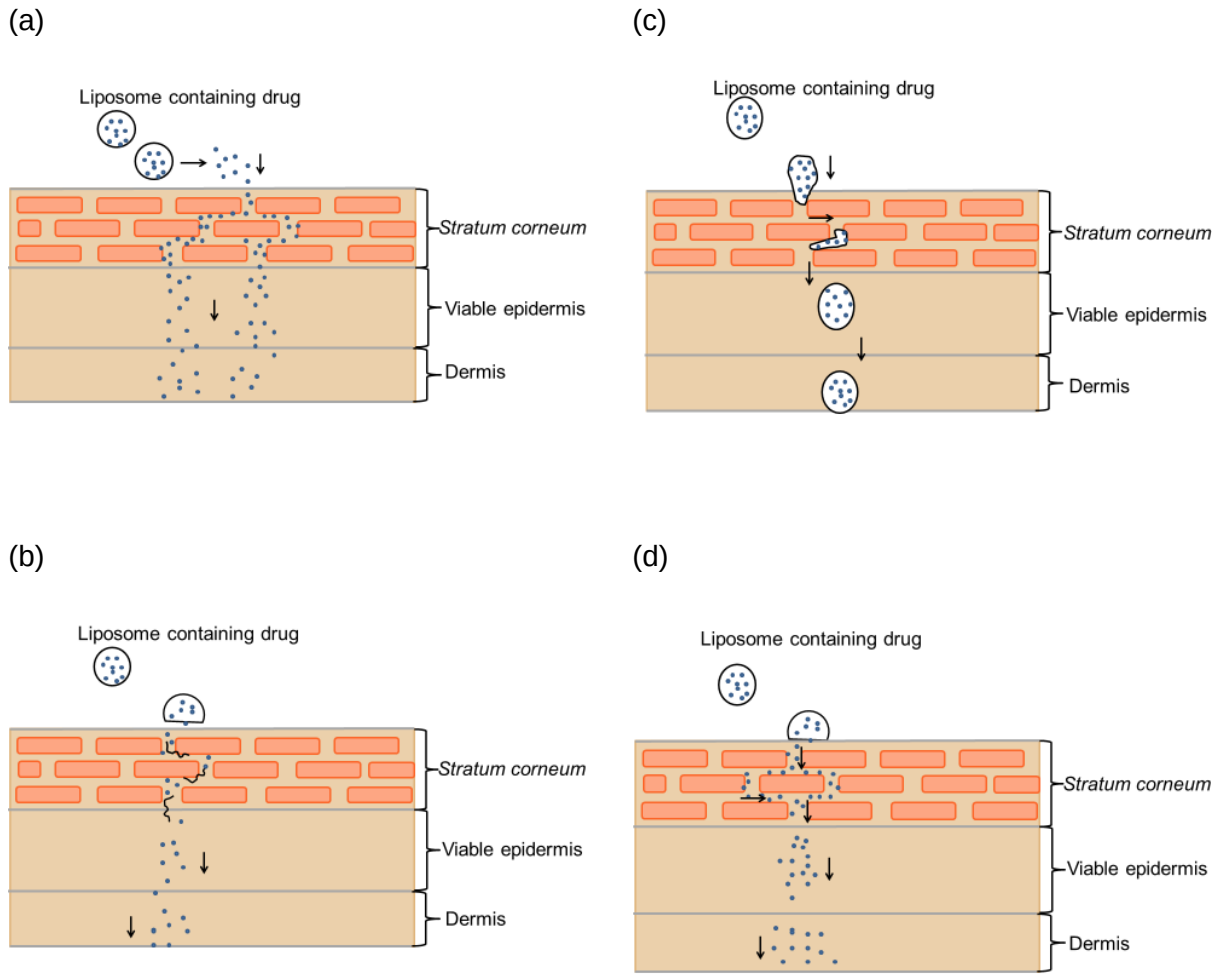


***Figure 2.1**: The mechanisms through which liposomes penetrate into the skin. (a)* A free drug delivery process, (b) Permeation *enhancement due to interaction with skin lipids, (c) Penetration of stratum corneum by intact liposomes, (d) Fusion of liposomes to the skin, causing release of drug into the skin. The possible areas of toxicity within the skin are shown (★)*

Figure adapted from El Maghraby *et al.* [62], [116].

2.2.2.2.1.1. A free drug process

With the free drug process mechanism, liposomes adhere onto the skin, after which the vesicles rupture and the drug is released from the vesicles, permeating through the skin independently from the vesicles, as depicted in *Fig. 2.1 (a)* [10, 62, 82, 119]. The free drug penetrates within the skin via the transcellular or intercellular pathway [10]. Liposomes that deliver drugs via this mechanism can be considered only as carriers that control drug release [62].

2.2.2.2.1.2. Permeation enhancement due to interaction with skin lipids

Enhancement of drug permeation can be caused by the release of lipids from the vesicles, which in turn have an interaction with skin lipids [10, 82, 120]. This interaction may possibly cause

alteration of the skin's barrier function, by inducing structural rearrangement, through loosening, of the top layer of skin, i.e. the *stratum corneum*, because of the lipids' similarity to the lipids of the skin [93, 101, 120, 121]. This mechanism needs the liposomes to fuse with the skin surface, for the lipids to be released into the skin, mix with the *stratum corneum* lipid matrix and enhance drug partitioning into the skin [47]. The effects of skin lipid interaction were first observed in 1987, where it was reported that lecithin lowered the permeability barrier of the skin, causing enhanced transdermal delivery [116, 122]. This mechanism is also known as the molecular mixing mechanism and is illustrated in *Fig. 2.1 (b)* [104].

2.2.2.2.1.3. Penetration of the *stratum corneum* by intact liposomes

Small unilamellar liposomes may penetrate the *stratum corneum* into the intercorneocyte lipid rich regions, leading them to act as microreservoirs for controlled localization and release when they start to degrade within the skin, as depicted by *Fig. 2.1 (c)* [10, 72, 76, 116]. According to De Leeuw *et al.* [93], liposomes can be 'sucked' into the epidermis, through a trans-epidermal osmotic gradient accompanied by hydration force. Optimal conditions can only be obtained by administering the liposomes under occlusion, such as dry skin surface [93, 118, 123], however, this mechanism was disproved by Du Plessis *et al.* [104] for larger liposomes.

2.2.2.2.1.4. Fusion of liposomes to the skin, causing release of drug into the skin

Liposomes are able to fuse to the skin surface, forming an occlusive film and leading to increased skin hydration, in addition to De Leeuw's [93] mechanism [92, 124]. The liposomes "lose" their bilayer membrane by fusing to the skin during this penetration process, before the drug enclosed within is released into the skin, as shown in Fig. 2.1 (d) [47, 121]. The lipid film formed on the skin when the liposomes fuse, lyophilizes the skin surface [125]. Therefore, different sites of possible toxicity exists, but issues with liposome biocompatibility mostly occurs in the upper layer in the *stratum corneum*.

2.2.2.3. Clinical applications of liposomes

Liposomes are used in a variety of applications in the pharmaceutical, as well as cosmetic fields, because of their flexibility and in the case of the pharmaceutical field, for their clinical efficacy [68, 89, 126]. In the cosmetic arena, liposomes are mostly utilized due to their moisturizing and restorative characteristics [86, 127].

The first liposomal formulation marketed was Pevaryl Lipogel, by the Swiss company Cilag A.G. in 1988, and contained 1% econazole [86, 89, 128]. Liposomes have similarly been used in vaccine delivery, gene therapeutics and oral drug delivery [68, 98]. In the treatment of cancer, liposomes have been used intravenously to carry cytotoxic drugs, with the reduction of toxic effects in mind, as well as increased stability of the drug [65, 86, 129].

Although liposomes do not deliver drugs transdermally, they have been widely employed for drug delivery into the skin [130]. They have additionally been used as a topical carrier for lignocaine as an anesthetic, as well as corticosteroids as treatment for atopic eczema, with restricted systemic absorption of these drugs [37, 89, 130-133]. In the treatment of acne, liposomes have been employed to deliver tretinoin [89]. They are also used in the dermal delivery of heparin and diclofenac [91]. Other examples of drugs delivered topically by liposomes include progesterone, doxorubicin and amphotericin B [72, 86, 95].

2.2.2.4. Advantages of liposomes

Liposomes have a wide range of advantages that add to the rationale to develop this drug carrier system even further. They act as drug containing reservoirs, protecting the drug, causing improved stability and applying controlled release, as well as targeted delivery [47, 69, 70, 83, 86, 134-136]. Encapsulation of certain drugs within liposomes, assist in overcoming difficulties linked to these drugs, such as toxicity, solubility, bioavailability, as well as immunogenicity [86, 137]. Side-effects related to toxicity are limited, since a lower concentration of drug is needed when a local effect is required [47, 83, 86, 93, 116, 138-140]. Due to their amphiphilic nature, liposomes can encapsulate both hydrophilic and lipophilic drugs, rendering it possible to deliver a larger variety of drugs [47, 83, 84, 86, 93, 97, 136]. The pharmacokinetics and pharmacodynamics of drugs are furthermore altered when encapsulated in liposomes [83, 135, 141]. These vesicles have shown enhanced entrapment ability, biocompatibility and biodegradability with a non-toxic nature [47, 49, 83, 84, 86, 93].

2.2.2.5. Challenges concerning liposomes

Apart from the above mentioned advantages, liposomes also have limitations. Formulation of liposomes are costly and the purity of ingredients are variable, therefore, drug leakage from the vesicles can sometimes occur [10, 47, 69, 70, 83]. Liposomes are unstable, causing increased degradation, aggregation, sedimentation and crystallization during storage [37, 70, 142, 143]. When degradation of liposomes occurs, oxidation follows in addition to the hydrolysis process of degradation [47, 70, 83, 144-146]. This indicates that the degradation of liposomes causes it to have redox-active properties. Liposomes are thermodynamically unstable [71] and due to this reason, along with poor permeability through the skin, it is mostly used for topical drug delivery [47, 63, 69, 147, 148]. Other motives behind using liposomes mainly as transdermal and topical delivery vehicles, is the conflicting results that have been attained concerning their efficacy to deliver drugs systemically [101, 149]. Skin deposition of liposomes are significantly affected by lipid concentration, technique of preparation and thermodynamic state of the bilayers and are usually confined to the surface layers of the *stratum corneum*, there they dehydrate and fuse with skin lipids, forming a depot [22, 56, 62, 69, 82, 101, 123, 149, 150].

Although mostly used topically, it has been observed that liposomes have increased skin penetration when penetration enhancers are added to the formula [151]. They have likewise been shown to be very valuable in skin delivery, mostly because lipophilic compounds have been shown to possess enhanced skin deposition characteristics in the skin's layers [152].

2.2.3. Ultradeformable vesicles

Due to the above mentioned challenges with liposomes, research has moved towards other types of colloidal carriers, such as transferosomes, ethosomes and transethosomes. In the early 1990's researchers developed new novel lipid nanocarriers, ultra-deformable vesicles (UDV), which demonstrated more deformability than liposomes to enhance their ability to deliver drugs transdermally, as well as easier manufacturing processes to upscale [63, 69, 123, 153].

2.2.4. Transferosomes

Transferosomes, which were introduced in 1992 by Cevc and Blume [123], are also known as ultra-deformable liposomes. They are trademarked by IDEA AG in Munich, Germany, and are most commonly used in drug delivery technology as the first generation of elastic lipid nanocarriers [46, 63, 69, 154, 155]. The name "transferosome" is derived from the Latin word "transferre," which translates to "to carry across", and the Greek word "soma," which translates to "body," providing it the name of "carrying body" [46]. Transferosomes are artificial phospholipid-based elastic nanovesicles that also contain an edge activator such as sodium cholate [46, 63, 65, 69, 155, 156].

2.2.4.1. Structure of transferosomes

Transferosomes, especially the first generation, fundamentally consist of phospholipids, such as phosphatidylcholine (PC), and surfactants, that act as edge activators (EA) [46, 63, 69, 87]. The phospholipids form a bilayer and the EA provides high deformability, to assist the vesicle in changing its shape easily and reversibly [13, 65, 101, 148, 154, 157, 158]. This is attained through a positive feedback mechanism and the elasticity is increased due to redistribution of the lipid bilayer [13, 66, 90, 101].

Second generation transferosomes, introduced in 1998, consist of a basic bilayer builder, such as PC, and no less than two polar lipophilic substances, for example non-ionic surfactants or surfactant mimicking drugs [65, 158-160].

Edge activators are often surfactants consisting of a single chain, that destabilises the lipid bilayers in transferosomes vesicles, hence the extreme deformability of these vesicles, and improved delivery of encapsulated drugs through the skin barrier are easily accomplished [56,

158, 161]. A high deformability allows the vesicles to squeeze through channels and pores, smaller than a tenth of the transferosome's size, in the *stratum corneum [66, 155, 162].*

The malleability of transferosomes causes it to cross the skin barrier, and requires minimum energy to do so [63, 65, 81]. Therefore, transferosomes resemble liposomes in morphology but not in function [163].

2.2.4.2. Efficacy of transferosomes

The efficacy of transferosomes can be attributed to its high vesicle deformability, which results in less energy needed to deform, causing it to react in a self-adapting manner to mechanical stress from its surroundings [46, 58, 164]. They are the first generation of deformable liposomes reported to penetrate intact skin, and hypothesized to cross the skin barrier spontaneously; they can mediate site-specific drug delivery by design [10, 13, 22, 46, 65, 69, 90, 101, 123, 153, 155, 165, 166]. These results are comparable to subcutaneous administration of the same drug, therefore, it is well established that transferosomes are more effectively transported through the skin than conventional rigid liposomes [60, 87, 101, 148, 158, 167-171].

2.2.4.2.1. Mechanism through which transferosomes are transported across the skin

There are multiple mechanisms proposed through which transferosomes attain drug delivery across the skin, depending on their composition [63, 172]. This permeation may be as result of a synergic mechanism between the transferosome's carrier capabilities and its permeation enhancement ability [63].

First, transferosomes may enhance penetration by entering the *stratum corneum*, after which they modify the intercellular lipid lamellae in the skin cells, and therefore, facilitate free drug penetration across the *stratum corneum* [64, 166, 172, 173]. They undertake stress-dependent modifications in their own composition to minimize the resistance of the *stratum corneum [166].* An example of this mechanism is the enhanced deposition of 5-fluorouracil as investigated by El Maghraby *et al.* [64].

Secondly, transferosomes may act as the drug carriers themselves, entering the *stratum corneum* while transporting the drugs inside [172]. Transferosomes may squeeze through channels between the intracellular sealing lipids of the *stratum corneum* that are one-tenth the diameter of the vesicle's size [13, 46, 63, 164, 174]. The means by which the transferosomes achieve this is the drive caused by an osmotic transdermal gradient, but this gradient can only be reached by applying the transferosomes under non-occlusive conditions [13, 63, 65, 101, 174-176]. Occlusion eliminates the hydration gradient and would therefore result in lower amount of the drugs being delivered through the skin [67, 123, 158, 177]. In the delivery of transferosomes, the osmotic gradient can be attained by the evaporation of excess water from the transferosome

formulation, causing the vesicles to “search” for more hydrated spaces, in this case in the *stratum corneum* [60, 63, 123, 162]. These transferosomes cross into the skin in a non-diffusive way, suggesting that the concentration of the drug in the vesicles, as well as the vesicles themselves, will not affect the transport across the skin [56, 63, 65, 167].

Thirdly, it has been hypothesized that transferosomes create a drug depot within the skin [13, 57, 58]. The small drug molecules have a habit of dissociating from the carrier and moving into the blood stream by means of diffusion, while the larger drug molecules will be dispersed within the skin tissue by interstitial flow [13].

Several studies have been performed confirming that transferosomes improve the *in* vitro delivery of several drugs into the skin [64, 173, 176, 178, 179]. The efficacy of this drug carrier opened new possibilities in research to target drugs into peripheral tissues [37, 57, 64].

2.2.4.3. Clinical applications of transferosomes

Various applications in the topical and transdermal field are demonstrated by transferosomes, because of their superiority, concerning drug permeation, to the conventional gel- and liquid- state vesicles, as well as conventional liposomes [63, 180]. It was demonstrated by Elsayed *et al.* [176] that cumulative drug permeation and deposition into the skin, showed pronounced improvement in comparison to a drug formulated in an aqueous solution. Transferosomes may act as a carrier for both high, as well as low molecular weight drug molecules, but they are mostly used for transporting extremely small molecular drugs through the *stratum corneum* to target specific peripheral tissue and in some cases, the systemic circulation [46, 47, 89]. Examples of drugs, that utilize transferosomes as carriers are analgesics, anesthetics, corticosteroids [58, 164, 168], sex hormones such as oestradiol [178], anticancer drugs [163, 181], insulin [13, 22, 182] and other proteins [55, 162] [22, 46, 89, 123, 155].

Transfersomal formulations containing corticosteroid can be applied in lower concentrations, delivering the same effect as conventional topical corticosteroids [167]. They also deliver the corticosteroids to the target site, a deep layer of the skin, providing sustained release [123, 164, 183].

Development of transferosomes is under way for the delivery of tamoxifen as an anticancer drug, as a means to limit the side-effects to a minimum by delivering the drug through the skin, and not through the oral route [164].

Transferosomes can furthermore be used in the development of transcutaneous immunization, to provide a novel needle-free method for immunization and vaccines [69, 164, 176, 184]. They provide a means of spontaneous transport for topically administered antigen solutions and carrier-associated antibodies across the intact skin, with targeted delivery to the lymphatic system [162].

Studies have reported the use of transferosomes for delivery of interleukin-2 and interferon-α, as well as Cyclosporine-A [162, 164, 185].

Clinical studies have furthermore confirmed the success of transfersomal formulations containing non-steroidal anti-inflammatory drugs (NSAIDs) [37, 60, 186]. These drugs are delivered deeper into the skin compared to conventional gel formulations, and the concentration of drug within the skin is at least ten times higher than that of the gel formulation [164].

Proteins can be difficult to deliver into the human body, mostly because of degradation, as well as problematic uptake in the GI tract [164, 182]. Studies have shown that certain proteins, for example insulin, can be delivered within the skin by transferosomes [164, 182]. Insulin deliveries with transferosomes as carriers have already been successfully verified under human clinical studies [37, 57].

The abovementioned examples are but a few of the applications for which transferosomes are used. Therefore, transferosomes offer an opportunity for the transdermal delivery of a wide range of drugs, notwithstanding the size of these drug molecules [22, 164].

2.2.4.4. Advantages of transferosomes

Transferosomes are suitable for easy skin penetration, along the hydration gradient due to their ultra-elasticity [46, 55, 123, 164, 177, 187]. This minimizes the risk of vesicle rupture, therefore not compromising the structure of the skin, resulting in being safe for use on the skin [13, 37, 46, 156]. Transferosomes promote drug retention within the layers of the skin by protecting the drug molecules against clearance to dermal blood vessels [46, 63, 164, 188]. Their ability to create a slow or sustained release is due to the transferosomes acting as a depot in the skin [46, 164]. Reports show that transferosomes' delivery and bioavailability are similar to that of a subcutaneous injection [13, 189-191].

Transferosomes have a high drug entrapment efficiency of drugs, especially with lipophilic drugs [46, 164]. They can accommodate both hydrophilic and lipophilic compounds due to their composition, which consists of hydrophobic, as well as hydrophilic components [164]. An assortment of drugs can be incorporated into transferosomes, independent of molecule size, molecular weight or polarity [13]. They have low toxicity due to their biocompatibility and biodegradability, because they consist of natural lipids [46, 164].

The production of transferosomes is easy to execute on larger scale, because it is a simple process carried out with pharmaceutically acceptable ingredients [37, 46, 164]. Presently, toxicology studies have shown that transferosomes show a relative lack of toxicity [45, 46].

2.2.4.5. Challenges concerning transferosomes

Although transferosomes show such promise in the world of transdermal and topical delivery, there are still disadvantages that can create limitations with this carrier. Difficulty ensues after loading of these vesicles with hydrophobic drugs, because their deformability and elastic properties can be compromised [63]. When formulating transferosomes, the purity of the natural phospholipids used is a continuous problem [46]. These vesicles must be formulated according to the needs and characteristics of each individual drug; therefore, formulation is a costly process [13, 46, 47, 174].

Oxidative degradation of the lipids in the vesicles can furthermore cause transferosomes to be chemically unstable [46, 47]. During application, occasional fluctuation in skin permeation may transpire, especially when concerning certain hydrophilic drugs, such as 5-fluorouracil [192, 193].

2.2.5. Ethosomes

Ethosomes are elastic lipid vesicles first introduced by Touitou *et al.* [194], and tailored especially for transdermal delivery of drugs, mostly because they facilitate non-invasive delivery of drugs deep within the skin layers [47, 63, 101, 195-201]. Development of ethosomes for transdermal delivery was largely focused on improving the transdermal transport of drugs with different physiochemical makeup [199, 202-204]. These vesicles are known to be soft and malleable, as well as comprising a very small size relative to liposomes, without taking size reducing steps [89, 101, 205, 206].

2.2.5.1. Structure of ethosomes

Ethosomes are also ultra-deformable vesicles that consist of high quantities of ethanol (20%-45%), well-known for serving as a penetration enhancer, as well as phospholipids (2-5%) and water [63, 69, 101, 194, 196, 206, 207]. The phospholipids used can range from phosphatidylcholine (PC), phosphatidylserine (PE), phosphatidylethanolamine and phosphatic acid [39, 63, 200, 206]. Ethosomes may be unilamellar or multilamellar through the core [198, 208-210].

Ethosomes differ from liposomes, because of their ethanol content, along with a notably smaller vesicle size, ranging from as small as 150 nm to a few microns in diameter, without size-reducing methods [63, 70, 101, 143, 194, 198, 200]. They possess a higher entrapment efficiency and stability when compared to liposomes [194, 198]. The smaller size of ethosomes, as well as their flexibility, can be attributed to the ethanol in their composition, causing an improved fluidity of the cell membrane lipids [68, 77, 86, 211]. It has been reported that the ethanol concentration is inversely proportional to vesicle size within the concentration range of 20 to 45% [70, 101, 194, 210]. However, an increase above 45% in ethanol concentration, may cause the vesicle's

membrane to become leaky, which will subsequently lead to lower entrapment efficiency [106, 198].

There are numerous mechanisms through which ethanol may enhance delivery of ethosomes. First, ethanol may interact with the polar head groups of the lipid molecules in the *stratum corneum*. This may influence the melting point of these lipids, fluidizing them, and reducing the density of lipids in the cell membrane [63, 69, 101, 157, 194, 196, 198, 206, 211, 212]. This mechanism is commonly known as the 'pull effect' of ethanol [213-215].

Secondly, ethanol confers a negative surface net charge, causing the reduced size of ethosomes [3, 101, 151, 194, 201, 210, 213, 216]. This negative charge is proportional to the ethanol concentration [90, 194, 205]. The negative net charge also causes the vesicle system to have an increased degree of stability against agglomeration [198, 210, 213].

Lastly, an increase of thermodynamic activity is caused by the evaporation of ethanol [213-215]. This is commonly known as the 'push effect' of ethanol [213-215]. Therefore, ethanol provides ethosomes with the needed characteristics to penetrate the skin more easily, reaching the deeper layers underneath the *stratum corneum* [101, 196, 216].

2.2.5.2. Efficacy of ethosomes

As stated, ethosomes deliver substances much deeper into the skin and in higher quantities, making it much more efficient than conventional liposomes [69, 137, 194, 196, 206]. Unlike the conventional liposomes, ethosomes retain a higher transdermal flux, therefore, they can be used more widely [106, 137, 194, 197, 200, 211]. Several studies reported that these vesicles improved skin delivery of several drugs *in vivo* and *in vitro*, such as minoxidil and testosterone, whether these drugs are hydrophilic or lipophilic of nature [86, 92, 101, 137, 157, 176, 194, 199, 204, 205, 216-219]. Ethosomes correspondingly have the ability to deliver drugs under occlusive and non-occlusive conditions, unlike transferosomes that can only obtain delivery under non-occlusive conditions [101, 199, 204, 205, 210, 216, 217, 220].

2.2.5.2.1. Mechanism through which ethosomes are transported across the skin

The mechanisms, by which ethosomes obtain penetration, as well as permeation, are not yet fully understood. As mentioned above, ethanol's synergistic relationship with the phospholipids in the vesicles, as well as the skin, plays a significant role in the penetration of these vesicles through the skin [63, 106, 196, 221]. This is also known as the interdigitating effect of ethanol on the lipid bilayer of the skin, as well as the vesicles [101, 148, 198]. Due to this effect, ethosomes tend to penetrate much deeper into the skin, before the drugs contained in these vesicles, are released [211].

Owing to ethosomes' ability to carry such a diverse group of substances, with different physicochemical characteristics, as well as lipophilicities, a wide range of applications have been developed in various fields, such as biotechnology, pharmaceutics, veterinarian and the cosmetic field [198, 206].

2.2.5.3. Clinical applications of ethosomes

Ethosomal systems are mainly developed for acquiring topical or transdermal delivery [211, 221]. In 2000 commercialization of the ethosome technology commenced and this field is still rapidly evolving [196]. Several *in vivo* and *in vitro* trials have been conducted through which promising results were reported for a large group of drugs, i.e. acyclovir, bacitracin, testosterone, insulin, indinavir, naloxone, methotrexate, minoxidil, erythromycin, ammonium glycyrrhizinate, azelaic acid, antioxidants, trihexyphenidyl hydrochloride and buspirone hydrochloride [67, 196-199, 204, 210, 222-225]. These drugs have shown enhanced flux into the skin compared to conventional liposomes [198, 199, 204, 222-224]. An improved skin deposition profile was moreover observed for some of these drugs [198, 204, 210, 222]. Other clinical applications for ethosomes include transcutaneous immunization against hepatitis and anti-cellulite therapy [196, 198, 206, 226].

2.2.5.4. Advantages of ethosomes

Ethosomes have several advantages. Drugs incorporated into this type of lipid nanocarrier are not limited by their hydrophilicity or lipophilicity; both types can be incorporated into ethosomes [47, 194]. Using ethosomes render delivery of larger molecules, such as proteins possible [196, 197, 206]. Extensive toxicological studies have shown that ethosomes are safe to cultured cells, because the raw materials used to formulate are non-toxic and deemed safe for pharmaceutical use [70, 106, 137, 196, 198, 199, 206]. The manufacturing process of ethosomes is relatively simple, and does not require complicated machinery [101, 196-198, 206, 227].

Enhanced dermal, as well as transdermal delivery are observed when ethosomes are used as carriers [184, 194, 196-198, 206, 227]. Ethosomal systems are usually administered in a semisolid form, such as a cream or gel, causing it to have a relatively high patient compliance, mostly because it is a non-invasive, simple way to deliver drugs through the skin [196-198, 202, 206, 227].

2.2.5.5. Challenges concerning ethosomes

Albeit ethosomes have so many advantages, challenges concerning this delivery system are also bound to occur. The largest concern of ethosomes is the varying purity of the phospholipids that are used during formulation of these vesicles, leading to high costs of high quality phospholipids [69, 134, 198, 228]. According to Romero and Morilla [65] ethosomes are not apropos to entrap hydrophilic solutes [75]. They are also sensitive to light, causing them to undergo oxidative

degradation, therefore, antioxidants such as α-tocopherol are added to reduce oxidation [198, 229].

2.2.6. Transethosomes

Unlike the previously discussed UDV, transethosomes were only introduced more recently, in 2012, by Song *et al.* [148], in attempt to combine the advantageous characteristics of transferosomes and ethosomes [202]. Transethosomes are UDV, comprising of both the components of transferosomes and ethosomes [63, 211, 230, 231]. Transethosomes are more deformable than both ethosomes and transferosomes because they contain ethanol, as well as a surfactant [63, 148].

2.2.6.1. Structure of transethosomes

Transethosomes, as mentioned previously, are comprised of the components of transferosomes and ethosomes, therefore containing a phospholipid such as phosphatidylcholine, an edge activator such as Span 80 ® or a permeation enhancer such as oleic acid and ethanol [63, 77, 87, 143, 148, 211, 230, 231]. The ethanol percentage of the vesicles can reach up to 30% [63, 148, 230, 232]. The combination of these components causes transethosomes to mimic some of the properties of both transferosomes and ethosomes [148, 233, 234].

2.2.6.2. Efficacy of transethosomes

Transethosomes have shown improved skin permeation compared to both ethosomes and transferosomes, due to their irregular spherical shape and high vesicle elasticity, as well as their smaller particle size [63, 148, 235]. Due to the presence of both an edge activator, as well as ethanol, transethosomes are more deformable that the counterparts they are combined from; this causes higher skin permeability [63, 77, 148, 202]. A reason for the higher skin permeability could be because the edge activator and ethanol may cause a rearrangement of the lipid bilayer of these vesicles, leading to better vesicle malleability [63, 77, 148, 202, 230, 234]. Transethosomes can entrap a wide range of molecules, depending on their weights, and retention of drug within the skin was reported to be more effective when compared to the vesicles discussed previously [63, 202, 230, 233, 234, 236, 237].

2.2.6.2.1. Mechanism through which transethosomes are transported into the skin

Due to the combination of the structures of transferosomes and ethosomes, it is suggested that the mechanism of skin penetration is a fusion of both [63, 148, 230]. Bagde *et al.* [230] investigated the mechanism of skin permeation of transethosomes and suggested that, although the mechanism through which ethosomes penetrate the skin is still unclear, the mechanism of penetration of transferosomes used by transethosomes is the generating of an osmotic gradient due to evaporation of water from the skin surface, causing a greater concentration of vesicle on

the skin [45]. In light of transethosomes' efficacy and mechanism of transportation, it is possible that these lipid nanocarriers possess excellent drug delivery potential [143].

2.2.6.3. Clinical applications of transethosomes

It is suggested that transethosomes possess high biocompatibility and superior delivery into the skin, therefore, it could be successfully applied as topical drug carrier, as done by Garg *et al.* [233] in the form of a gel [143].

2.2.6.4. Advantages of transethosomes

Although little information is available on transethosomes, since they are still a relatively new subject, the composition of transethosomes suggests they possess advantages of both transferosomes and ethosomes, as previously discussed in this chapter [63, 232, 234, 237].

2.2.6.5. Challenges concerning transethosomes

Since transethosomes contain components of both ethosomes and transferosomes, they may be similar to challenges affecting them. Since their discovery in 2012, little information is available concerning the disadvantages of transethosomes. There is not much information on the toxicological profile of transethosomes, for toxicology studies are currently being performed [235].

2.2.7. Challenges concerning lipid nanocarriers

Although lipid nanocarriers possess numerous advantages, they are bound to have some disadvantages as well. The most general limitations occur during the design and characterization of lipid nanocarriers [37, 238]. The ability to reproduce a specific shape of the carrier has proven to be a limiting factor in the design of these carriers [238, 239]. Other difficulties during design include loss of function, when applied to skin, due to enzymatic activity and changes in isothermal phases causing segregation from a vesicle [37, 238, 240]. These problems are all as a consequence of the biological and physical instability of lipid nanocarriers, especially liposomes [41]. High manufacturing costs and impure phospholipid content may also affect the use of lipid nanocarriers [41, 47, 241].

2.3. Stability of lipid nanocarriers

Stability may play a large role in the use of drug carrier systems. According to Verma and Utreja [242] liposomes showed improved stability and would therefore be an ideal topical carrier [243]. Ethosomes, however, have a tendency to leak hydrophilic or ionized drugs when compared to liposomes, which may lead to problems when a formulation had to be stored for an extensive period of time [242]. Transferosomes and transethosomes have shown better stability when compared to ethosomes [242, 244]. It is, however, suggested that stability of lipid vesicles is improved when the membrane is more rigid [245, 246].

Stability of lipid nanocarriers can be determined by evaluating the size, as well as the structures thereof over time, which are the most common methods [87, 197, 205, 247]. It was determined that storage at lower temperatures causes the lipid nanocarriers to be stable for longer periods of time [247]. Another method for determining stability for lipid nanocarriers, is the assessment of entrapment efficiency [181]. Entrapment efficiency is not applicable in this study, due to the study focusing on the biocompatibility of lipid nanocarriers in the skin, but it is still an important aspect of stability.

A significant physical parameter for predicting a vesicles' stability, is the use of Zeta potential [63, 143]. A high negative charge of the surface of vesicles is an indicator of better stability of these lipid nanocarriers, by preventing agglomeration [87, 143].

When a formulation is unstable, it may have an effect on the pH of said formulation, after which a change in pH may cause even further degradation of the sample. Therefore, pH buffering can be used to keep a steady pH, even when the formulation becomes unstable, to halt further degradation because of pH changes [248]. Some lipid nanocarriers show instability at a low pH, or even after long term storage in a buffer, therefore having an effect on the biocompatibility [249].

2.4. Biocompatibility

According to Kohane and Langer [250] biocompatibility is defined as "an expression of the benignity of the relation between a substance and its biological environment." A substance can be inappropriate, even if it has a mostly benign reaction with biological tissue. The cell's ability to endure in the presence of substances can roughly be determined by *in vitro* studies [250].

2.4.1. Development of biocompatibility testing

There are different guidelines published to aid in biocompatibility testing. These include the International Standard – Biological Evaluation of Medical Devices [251], OECD (Organization for Economic Co-operation and Development) Guidelines for the Testing of Chemicals [132], FDA (US Food and Drug Administration) Clinical Trials Guidance Documents and ECVAM (European Centre for the Validation of Alternative Methods) Guidelines (European Union Reference Laboratory for alternatives to animal testing).

The relationship between the lipid nanocarriers' physiochemical properties and the interaction the carriers have on biological environments, is complex [252]. Lipid nanocarriers resemble cell organelles considering their dimensions [252, 253]. They mainly consist of lipids and surfactants [252, 253]. Thus, the resemblance in size can cause the carriers to interfere with the cell's vital functions, which in turn can result in potential cytotoxicity [252, 253]. Lipid nanocarriers are furthermore formulated using excipients that are generally recognized as safe (GRAS), however

the effects of nanotoxicity have not yet been researched satisfactorily and nanotoxicity is not yet predictable when utilizing current toxicological methods. This area of research is nonetheless a rapidly developing and emerging subject in the nanomedicine research field [252]. Different factors of lipid nanocarriers may affect their toxicity, such as their shape, size, surface area and ability to create reactive oxygen species (ROS); toxicity studies, such as oxidative stress, should be performed to establish whether these factors are causing toxicity [252, 254]. Using novel pharmaceutical nanotechnology, these grey areas can be investigated and the desired outcomes can be achieved, because highly adaptable complex lipid nanocarriers require impartiality during testing and application design [37, 41].

2.4.2. In vitro skin toxicity

The field of molecular biology has experienced the expansion of new technology in determining mechanisms for cellular toxicity [255]. Together with this technological breakthroughs, predictive toxicology has become much more prevalent and popular in determining the safety of drugs and drug development [256]. Novel tools used in toxicology and human risk assessment are adverse outcome pathways (AOPs) [256, 257]. AOPs use existing knowledge and provide a mechanistic representation of toxicological effects in cells, by linking molecular initiation events (MIE) with key events [256-259]

2.4.2.1. End points

The evaluation of safety is a prerequisite when new substances or carriers are introduced into the market [30]. Toxic effects are usually related to the concentration of drug *in vitro*; in this case the concentration of the colloidal carrier [31]. There are three ways by which the free concentration of colloidal carriers can affect the cell in a toxicological manner, namely: a concentration dependent impairment of mitochondrial function; cell membrane integrity is affected by lipid nanocarriers, especially liposomes, which induce the release of LDH (lactate dehydrogenase); by affecting oxidative stress markers in cells [260]. Therefore, toxicological risk assessment or biocompatibility has become a key aspect during development of novel treatments.

Research is focusing on alternative methods, instead of animal testing. Emphasis is given to skin corrosion as a result of necrosis, because accurate data cannot be obtained from animal testing alone [31, 261]. In skin toxicity assays, the measurement of necrosis is classified as *in vitro* skin corrosion potential [115]. *In vitro* models with specific endpoints, based on clinical or histopathological endpoints, can determine general mechanisms of toxicity from drug delivery systems [31, 260, 262, 263]. Mitochondrial activity is one of these endpoints, because nanoparticles have a pronounced influence on these organelles [260, 264, 265].

The mitochondria plays an important role in apoptosis and necrosis of cells, and interference into their function can lead to impaired cellular energy as well as lipid metabolism that can lead to the

release of cell death mediators [266, 267]. The role of the mitochondria in cell death is depicted in Fig. 1.1. One of the main key events in necrosis or apoptosis of cells is the mitochondrial membrane permeability, which triggers most forms of cell death [267, 268]. Low mitochondrial membrane potential can signal mitochondrial dysfunction, which can ultimately lead to necrosis or apoptosis [260].

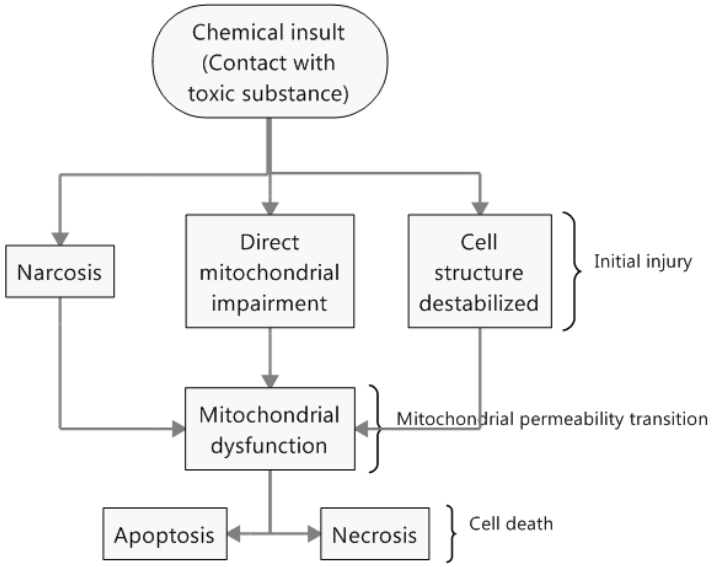


***Figure 1.1**: Outcome pathway from chemical insult to cell death. Initial injury because of contact with potential toxic substances can cause narcosis (damage to the cell's plasma membrane induced by chemicals [30, 269]), direct inhibition of the mitochondria or destabilization of the cell's structure. This causes a mitochondrial permeability transition, which will finally lead to apoptosis or, in the case of this study, necrosis.*

(Figure adapted from Vinken and Blaauboer [30])

2.4.2.2. Risk assessment

Predictive toxicology forms a significant part in the risk assessment of novel drugs, because the outcome of these predictions in preclinical studies will determine whether clinical development should or should not be pursued [257, 270-272]. The existing practice in toxicological risk assessment is based on the clinical and histopathological endpoints in animal studies. In the case of skin studies, skin sensitization potential of allergens, as well as skin corrosion potential, are assessed [28, 273].

The norm for human risk assessment testing was on animals for a great part in history. As described previously this resulted in an enormous amount of animals needed in laboratories. The high numbers of animals, as well as distress caused by the experiments caused immense debate on the ethical, scientific and financial feasibility of these testing procedures [28-30]. Another issue concerning animal studies is the fact that there is always a degree of uncertainty when testing, for humans and animals differ vastly in terms of kinetic and dynamics of drugs [31, 32]. In light

of these problems and uncertainties concerning animal testing, the three R's principle was introduced in 1959 by Russel and Burch, stating that the use of animals in laboratory testing should be reduced, refined and preferably replaced [28, 271, 273].

The first step in toxicological risk assessment is the selection of the cellular system. The cellular system should be carefully chosen according to what needs to be evaluated. In the case of mitochondrial damage evaluation, cell types that naturally endowed a high number of mitochondria should be selected. Secondly, the types of cytotoxic assays should be selected. The viability assay most commonly used is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, but there are a range of alternative assays that can be utilized, such as bioluminescent measurement of ATP (adenosine triphosphate) content, and accumulation of the neutral red dye [30, 274].

2.4.2.3. In vitro tests as alternative for animal testing

Successful novel formulations and alternatives to simple lotions and creams have been developed, yet there are still some concerns regarding toxicity in cosmetics and pharmaceutics. For a great part in history, human risk assessment has been tested in animal models. This resulted in an enormous amount of animals needed in laboratories. The great numbers of animals, as well as distress caused by the tests resulted in a great deal of debate on the ethical, scientific and financial feasibility of these testing procedures [28-30]. Another issue concerning animal studies is the fact that there is always a degree of uncertainty when testing, for humans and animals differ vastly in terms of kinetic and dynamics of drugs [31, 32]. Due to these reasons, the EU Cosmetics Regulation (EC 1223 / 2009) foresaw a ban on the use of animals for testing of cosmetic ingredients in 2013, and requires the use of alternative methods for toxicology analysis [33, 35, 36].

Since the introduction of the three R's (reduce, refine, replace) principle in 1959 by Russel and Burch, many countries have introduced legislation to aid in the reduction of utilizing laboratory animals [28, 271, 273]. Substantial efforts have been made to develop alternative methods for toxicity testing on animals, such as human cell-based *in vitro* assays [29, 275, 276]. Therefore, *in vitro* studies are being used for preclinical investigations, instead of using laboratory animals [36, 276]. *In vitro* models mostly rely on cell viability assays, and these models promise to attain mechanism derived information to assess risk [277-282]. This tactic reduced the number of animals used in preclinical toxicity testing [276, 283].

The alternatives to animal testing in this study are the following eukaryotic cells: the human malignant melanoma cell line, A375 (ATCC® CRL1619™), and normal human keratinocytes, HaCat (ATCC® number CRI-1619™). These cell lines are different cells that are part of the skin's epidermis and are of human origin. One cell type models need to have a high rate of

reproducibility to assess risks concerning drugs [284]. Keratinocytes form part of the epidermis, on its apical side, forming part of the main barrier for transdermal delivery [284, 285]. It forms a stratified epithelium under optimal conditions, and is therefore widely accepted and validated for use as skin models [284, 286]. Melanoma is a cancer type that develops from melanocytes, which produce the pigments of the skin [287]. It is important to add melanocytes into a full thickness skin model to increase the model's physiology properties [284, 288].

2.4.3. Challenges concerning in vitro toxicity screening of lipid nanocarriers

In vitro studies depict higher effect levels than animal studies, regarding mitochondrial dysfunction during toxicological responses, causing it to magnify the relevance of cell studies in preclinical testing, as well as predictive toxicological screenings [260]. A challenge of *in vitro* cytotoxicity studies is the accurate conversion of the test results into the correct perspective for risk assessment [30].

Other challenges of *in vitro* cytotoxicity studies include the deviation of data between *in vivo* and *in vitro* studies, which is caused by the limited amount of target sites of single cell cultures, as well as functionality; variability of assay setup and chemicals may interfere with target concentrations of carriers in the systems; differences in metabolic processes between *in vivo* and *in vitro* systems may cause problems due to the variability of clearance and metabolite formation [29, 31, 289-298]. Research has been focused more on the cellular mechanisms and what causes possible cytotoxic interactions with them [273]. The data obtained by these investigations will assist in developing the future research that aims to optimize *in vitro* biocompatibility studies, as well as enable its use in quantitative studies [273, 299].

2.5. Reference List

1. Bhushan, B., Dermal and transdermal delivery, in *Encyclopedia of Nanotechnology*, B. Bhushan, Editor. 2012, Springer Science & Business. p. 517-525.
2. El-Kattan, A., C.S. Asbill, and S. Haidar, Transdermal testing: Practical aspects and methods. *Pharmaceutical science & technology today*, 2000. **3**(12): p. 426-430.
3. Aulton, M.E. and K.M.G. Taylor, Aulton's pharmaceutics: The design and manufacture of medicines. 4th ed. 2013: Elsevier Ltd. 894.
4. Chen, H. and J. Fang, Therapeutic patents for topical and transdermal drug delivery systems*. Expert opinion on therapeutic patents*, 2005. **10**(7): p. 1035-1043.
5. Osborne, D.W. and A.H. Amann, Topical drug delivery formulations. *Drug and the pharmaceutical sciences: A series of textbooks and monographs*, ed. J. Swarbrick. Vol. 42. 1990, New York: Marcel Dekker Inc. 420.
6. Singh, D., N. Mital, and G. Kaur, Topical drug delivery systems: A patent review*. Expert opinion on therapeutic patents*, 2016. **26**(2): p. 213-228.
7. Cole, H., J. Hutton, and T. Sollmann, The clean inunction treatment of syphilis with mercury*. Journal of the American Medical Association*, 1924. **82**(3): p. 199-200.
8. Sartin, J.S. and H.O. Perry, From mercury to malaria to penicillin: The history of the treatment of syphilis at the mayo clinic - 1916-1955*. Journal of the American academy of dermatology*, 1995. **32**(2): p. 255-261.
9. Hadgraft, J. and M.E. Lane, Passive transdermal drug delivery systems: Recent considerations and advances*. American journal of drug delivery*, 2006. **4**(3): p. 153-160.
10. Nounou, M.*, et al.*, Liposomal formulation for dermal and transdermal drug delivery: Past, present and future*. Recent patents on drug delivery & formulation*, 2008. **2**(1): p. 9-18.
11. Toll, R.*, et al.*, Penetration profile of microspheres in follicular targeting of terminal hair follicles*. Journal of investigative dermatology*, 2004. **123**(1): p. 168-176.
12. Arora, A., M.R. Prausnitz, and S. Mitragotri, Micro-scale devices for transdermal drug delivery*. International journal of pharmaceutics*, 2008. **364**(2): p. 227-236.
13. Cevc, G., Transdermal drug delivery of insulin with ultradeformable carriers*. Clinical pharmacokinetics*, 2003. **41**(5): p. 461-474.
14. Thomas, B.J. and B.C. Finnin, The transdermal revolution*. Drug discovery today*, 2004. **9**(16): p. 697-703.
15. Hadgraft, J. and R.H. Guy, Transdermal drug delivery: Development issues and research initiatives. 1989: Marcel Dekker.
16. Alexander, A.*, et al.*, Approaches for breaking the barriers of drug permeation through transdermal drug delivery*. Journal of controlled release*, 2012. **164**(1): p. 26-40.
17. Jepps, O.G.*, et al.*, Modeling the human skin barrier- towards a better understanding of dermal absorption*. Advanced drug delivery reviews*, 2013. **65**(2): p. 152-168.

18. Joshi, A. and J. Raje, Sonicated transdermal drug transport. *Journal of controlled release*, 2002. **83**: p. 13-22.
19. Li, J.*, et al.*, The application of skin metabolomics in the context of transdermal drug delivery. *Pharmacology reports*, 2016. **69**(2): p. 252-259.
20. Mitragotri, S., Breaking the skin barrier. *Advanced drug delivery reviews*, 2004. **56**(5): p. 555-556.
21. Subedi, R.K.*, et al.*, Recent advances in transdermal drug delivery. *Archives of Pharmacal Research*, 2010. **33**(3): p. 339-351.
22. Cevc, G., A. Schäitzlein, and G. Blume, Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. *Journal of controlled release*, 1995. **36**: p. 3-16.
23. Langer, R., Transdermal drug delivery: Past progress, current status, and future prospects. *Advanced drug delivery reviews*, 2004. **56**(5): p. 557-558.
24. Rein, H., Experimental electroendosmotic studies on living human skin. *Zeitschrift Fur Biologie*, 1924. **81**: p. 125-140.
25. Scheuplein, R.J., Mechanism of percutaneous adsorption. *Journal of investigative dermatology*, 1965. **45**(5): p. 334-346.
26. Scheuplein, R.J., Permeability of the skin: A review of major concepts and some new developments. *Journal of investigative dermatology*, 1976. **67**(5): p. 672-676.
27. Bell, G.H.*, et al.*, Transdermal and oral dl-methylphenidate-ethanol interactions in c57bl/6j mice: Transesterification to ethylphenidate and elevation of d-methylphenidate concentrations. *Journal of pharmaceutical science*, 2011. **100**(7): p. 2966-2978.
28. Blaauboer, B.J., Biokinetic modeling and in vitro-in vivo extrapolations. *Journal of toxicology and environmental health, Part B, Critical Reviews*, 2010. **13**(2-4): p. 242-252.
29. Kramer, N.I.*, et al.*, Biokinetics in repeated-dosing in vitro drug toxicity studies. *Toxicology in vitro*, 2015. **30**(1 Part A): p. 217-224.
30. Vinken, M. and B.J. Blaauboer, In vitro testing of basal cytotoxicity: Establishment of an adverse outcome pathway from chemical insult to cell death. *Toxicology in vitro*, 2017(39): p. 104-110.
31. Blaauboer, B.J.*, et al.*, The use of biomarkers of toxicity for integrating in vitro hazard estimates into risk assessment for humans. *ALTEX*, 2012. **29**(4): p. 411-425.
32. Renwick, A.G. and N.R. Lazarus, Human variability and noncancer risk assessment — an analysis of the default uncertainty factor. *Regulatory toxicology and pharmacology*, 1998. **27**: p. 3-20.
33. Coecke, S.*, et al.*, Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicology in vitro*, 2013. **27**(5): p. 1570-1577.

34. European Parliament, C.o.t.E.U., Regulation (EC) no 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, O.J.o.t.E. Union, Editor. 2009. p. L 342.
35. Punt, A.*, et al.*, Evaluation of research activities and research needs to increase the impact and applicability of alternative testing strategies in risk assessment practice*. Regulatory toxicology and pharmacology*, 2011. **61**: p. 105–114.
36. Spielmann, H.*, et al.*, Successful validation of in vitro methods in toxicology by Zebet, the National Centre for Alternatives in Germany at the BFR (Federal Institute for Risk assessment)*. Experimental and toxicologic pathology*, 2008. **60**(2-3): p. 225-233.
37. Cevc, G., Lipid vesicles and other colloids as drug carriers on the skin*. Advanced drug delivery reviews*, 2004. **56**(5): p. 675-711.
38. Sharma, J.*, et al.*, Colloidal drug carriers*. The internet journal of family practice*, 2009. **9**(2).
39. Cevc, G. and U. Vierl, Nanotechnology and the transdermal route: A state of the art review and critical appraisal*. Journal of controlled release*, 2010. **141**(3): p. 277-299.
40. Alvarez-Roman, R.*, et al.*, Skin penetration and distribution of polymeric nanoparticles*. Journal of controlled release*, 2004. **99**(1): p. 53-62.
41. Alexander, A.*, et al.*, Recent expansion of pharmaceutical nanotechnologies and targeting strategies in the field of phytopharmaceuticals for the delivery of herbal extracts and bioactives*. Journal of controlled release*, 2016. **241**: p. 110-124.
42. Kumar, L.*, et al.*, Fatty acid vesicles acting as expanding horizon for transdermal delivery*. Artificial cells, nanomedicine, and biotechnology*, 2017. **45**(2): p. 251-260.
43. Neubert, R.H.H., Potentials of new nanocarriers for dermal and transdermal drug delivery*. European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
44. Honeywell-Nguyen, P.L. and J.A. Bouwstra, Vesicles as a tool for transdermal and dermal delivery*. Drug discovery today: Technologies*, 2005. **2**(1): p. 67-74.
45. Priyanka, R. and D. Shipra, Transferosomes - a novel carrier for transdermal drug delivery system*. International journal of pharmacy & technology*, 2012. **4**(1): p. 1854-1865.
46. Vinod, K.R.*, et al.*, Critical issues related to transferosomes – novel vesicular stystem*. Acta scientiarum polonorum - Technologia alimentaria*, 2012. **11**(1): p. 67-82.
47. Akhtar, N., Vesicles: A recently developed novel carrier for enhanced topical drug delivery*. Current drug delivery*, 2014. **11**(1): p. 87-97.
48. Jerajani, H.R.*, et al.*, Evaluation of clinical efficacy and safety of once daily topical administration of 1% oxiconazole cream and lotion in dermatophytosis: An open label, non-comparative multicentre study*.* Indian Journal of Dermatology, Venereology and Leprology, 2000. **66**(4): p. 188-192.
49. Goyal, P.*, et al.*, Liposomal drug delivery systems – clinical applications*. Acta Pharmaceutica*, 2005. **55**: p. 1–25.

50. Lauer, A.C., *et al.*, Transfollicular drug delivery. *Pharmaceutical research*, 1995. **12**(2): p. 179-186.
51. Niemiec, S.M., C. Ramachandran, and N.D. Weiner, Influence of nonionic liposomal composition on topical delivery of peptide drugs into pilosebaceous units: An in vivo study using the hamster ear model. *Pharmaceutical research*, 1995. **12**(8): p. 1184-1188.
52. Yu, W.H., *et al.*, Topical gene delivery to murine skin. *Journal of investigative dermatology*, 1999. **112**(3): p. 370-5.
53. Ghahary, A., *et al.*, Liposome associated interferon-alpha-2b functions as an anti-fibrogenic factor in dermal wounds in the guinea pig. *Molecular and cellular biochemistry*, 2000. **208**: p. 129–137.
54. Betz, G., R. Imboden, and G. Imanidis, Interaction of liposome formulations with human skin in vitro. *International journal of pharmaceutics*, 2001. **229**: p. 117–129.
55. Paul, A., G. Cevc, and B.K. Bachhawat, Transdermal immunization with large proteins by means of ultradeformable drug carriers. *European journal of immunology*, 1995. **25**(12): p. 3521-3524.
56. Cevc, G., *et al.*, The skin: A pathway for systemic treatment with patches and lipid-based agent carriers. *Advanced drug delivery reviews*, 1996. **18**: p. 346-378.
57. Cevc, G., Drug delivery across the skin. *Expert opinion on investigating drugs*, 1997. **6**(12): p. 1887-937.
58. Cevc, G., G. Blume, and A. Schäitzlein, Transfersomes-mediated transepidermal delivery improves the regio-specificity and biological activity of corticosteroids in vivo. *Journal of controlled release*, 1997. **45**: p. 211–226.
59. Cevc, G., *et al.*, Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochimica et Biophysica Acta*, 1998. **1368**: p. 201–215.
60. Cevc, G. and G. Blume, New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, transfersomes. *Biochimica et Biophysica Acta*, 2001. **1514**: p. 191-205.
61. Lauer, A.C., *et al.*, Targeted delivery to the pilosebaceous unit via liposomes. *Advanced drug delivery reviews*, 1996. **18**: p. 311-324.
62. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Can drug-bearing liposomes penetrate intact skin? *Journal of pharmacy and pharmacology*, 2006. **58**(4): p. 415-429.
63. Ascenso, A., *et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
64. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. *Journal of pharmacy and pharmacology*, 2001. **53**: p. 1069-1077.

65. Romero, E.L. and M.J. Morilla, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2013. **8**: p. 3171-3186.
66. Benson, H.A.E., Transdermal drug delivery- penetration enhancement techniques. *Current drug delivery*, 2005. **2**(1): p. 23-33.
67. Benson, H.A., Transfersomes for transdermal drug delivery. *Expert opinion in drug delivery*, 2006. **3**(6): p. 727-737.
68. Ghanbarzadeh, S. and S. Arami, Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. *BioMed Research International*, 2013. **2013**: p. 1-7.
69. Manosroi, A.*, et al.*, Novel elastic nanovesicles for cosmeceutical and pharmaceutical applications. *Chiang Mai journal of science*, 2009. **36**(2): p. 168-178.
70. Cortesi, R.*, et al.*, Liposomes- and ethosomes-associated distamycins: A comparative study. *Journal of liposome research*, 2010. **20**(4): p. 277-285.
71. Vermuri, S. and C.T. Rhodes, Preparation and characterization of liposomes as therapeutic delivery systems: A review. *Pharmaceutica Acta Helvetiae*, 1995. **70**: p. 95-111.
72. Mezei, M. and V. Gulasekharam, Liposomes - a selective drug delivery system for the topical route of administration. I. Lotion dosage form. *Life sciences*, 1980. **26**: p. 1473-1477.
73. Honeywell-Nguyen, P.L.*, et al.*, The in vivo and in vitro interactions of elastic and rigid vesicles with human skin. *Biochimica et Biophysica Acta*, 2002. **1573**: p. 130-140.
74. Cevc, G.*, et al.*, Overcoming semipermeable barriers, such as the skin, with ultradeformable mixed lipid vesicles, transfersomes, liposomes, or mixed lipid micelles. Langmuir, 2003. **19**: p. 10753-10763.
75. Touitou, E., B. Godin, and C. Weiss, Enhanced delivery of drugs into and across the skin by ethosomal carriers. *Drug development research*, 2000. **40**: p. 406-415.
76. Mezei, M. and V. Gulasekharam, Liposomes-a selective drug delivery system for the topical route of administration: Gel dosage form. *Journal of pharmacy and pharmacology*, 1982. **34**: p. 473-474.
77. Menezes, A.C.*, et al.*, Development and characterization of novel 1-(1-naphthyl)piperazine-loaded lipid vesicles for prevention of uv-induced skin inflammation. *European journal of pharmaceutical B*, 2016. **104**: p. 101-109.
78. Cosco, D.*, et al.*, Ultradeformable liposomes as multidrug carrier of resveratrol and 5-fluorouracil for their topical delivery. *International journal of pharmaceutics*, 2015. **489**(1-2): p. 1-10.
79. Maurer, N., D.B. Fenske, and P.R. Cullis, Developments in liposomal drug delivery systems. *Expert opinion on biological therapy*, 2001. **1**(6): p. 923-947.

80. Richards, R.L., *et al.*, Liposomes, lipid a, and aluminum hydroxide enhance the immune response to a synthetic malaria sporozoite antigen. *Infection and immunity*, 1988. **56**(3): p. 682-686.
81. Cevc, G., Material transport across permeability barriers by means of lipid vesicles, in *Handbook of biological physics*, R. Lipowsky and E. Sackmann, Editors. 1995, Elsevier Science B.V. p. 465-490.
82. Barry, B.W., Novel mechanisms and devices to enable successful transdermal drug delivery. *European journal of pharmaceutical science*, 2001. **14**: p. 101-114.
83. Varun, T., A. Sonia, and P. Bharat, Niosomes and liposomes - vesicular approach towards transdermal drug delivery. *International journal of pharmaceutical and chemical sciences*, 2012. **1**(3): p. 632-644.
84. Agarwal, R., O.P. Katare, and S.P. Vyas, Preparation and in vitro evaluation of liposomalniosomal delivery systems for antipsoriatic drug dithranol. *International journal of pharmaceutics*, 2001. **228**: p. 43-52.
85. Bangham, A.D., M.M. Standish, and J.C. Watkins, Diffusion of univalent ions accross the lamellae of swollen phospholipids. *Journal of molecular biology*, 1965. **13**: p. 238-252.
86. Pierre, M.B., M. Dos Santos, and I. Costa, Liposomal systems as drug delivery vehicles for dermal and transdermal applications. *Archives of dermatological research*, 2011. **303**(9): p. 607-621.
87. Abdellatif, A.A. and H.M. Tawfeek, Transfersomal nanoparticles for enhanced transdermal delivery of clindamycin. *AAPS PharmSciTech*, 2016. **17**(5): p. 1067-1074.
88. Schaller, M. and H.C. Korting, Interaction of liposomes with human skin the role of the stratum corneum. *Advanced drug delivery reviews*, 1996. **18**: p. 303-309.
89. Verma, D.D., *et al.*, Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: A skin penetration and confocal laser scanning microscopy study. *European journal of pharmaceutics and biopharmaceutics*, 2003. **55**(3): p. 271-277.
90. Manconi, M., *et al.*, Development and characterization of liposomes containing glycols as carriers for diclofenac. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2009. **342**(1-3): p. 53-58.
91. Neubert, R.H., Potentials of new nanocarriers for dermal and transdermal drug delivery. *European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
92. Lakshmi, P. and D. Prasanthi, Vesicles - a mechanism of transdermal permeation: A review. *Asian journal of pharmaceutical and clinical research*, 2012. **5**(1): p. 18-25.
93. De Leeuw, J., *et al.*, Liposomes in dermatology today. *Journal of the European academy of dermatology and venereology*, 2009. **23**(5): p. 505-516.
94. Lian, T. and R.J.Y. Ho, Trends and developments in liposome drug delivery systems. *Journal of pharmaceutical science*s, 2001. **90**(6): p. 66-680.

95. Sharma, A. and U.S. Sharma, Liposomes in drug delivery: Progress and limitations. *International journal of pharmaceutics*, 1997. **154**: p. 123-140.
96. Zamboni, W.C., Liposomal, nanoparticle, and conjugated formulations of anticancer agents. *Clinical cancer research*, 2005. **11**(23): p. 8230-8234.
97. Lauer, A.C., *et al.*, Targeted delivery of the pilosebaceous unit via liposomes. *Advanced drug delivery reviews*, 1996. **18**: p. 311-324.
98. Fang, Y.P., *et al.*, Comparison of 5-aminolevulinic acid-encapsulated liposome versus ethosome for skin delivery for photodynamic therapy. *International journal of pharmaceutics*, 2008. **356**(1-2): p. 144-152.
99. Katahira, N., *et al.*, Enhancement of topical delivery of a lipophilic drug from charged multilamellar liposomes. *Journal of Drug Targeting*, 1999. **6**(6): p. 405-414.
100. Touitou, E., *et al.*, Liposomes as carriers for topical and transdermal delivery. *Journal of pharmaceutical science*s, 1994. **83**(9): p. 1189-1203.
101. Elsayed, M.M., *et al.*, Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. *International journal of pharmaceutics*, 2007. **332**(1-2): p. 1-16.
102. Zellmer, S., W. Pfeil, and J. Lasch, Interaction of phosphatidylcholine liposomes with the human stratum corneum. *Biochimica et Biophysica Acta*, 1995. **1237**: p. 176-182.
103. Li, L. and R.M. Hoffman, Topical liposome delivery of molecules to hair follicles in mice. *Journal of dermatological science*, 1997. **17**: p. 101-108.
104. Du Plessis, J., *et al.*, The influence of particle size of liposomes on the deposition of drug into skin. *International journal of pharmaceutics*, 1994. **103**: p. 277-282.
105. Vanic, Z., *et al.*, Characterization of various deformable liposomes with metronidazole. *Drug development and industrial pharmacy*, 2013. **39**(3): p. 481-488.
106. Jain, S., *et al.*, Formulation and evaluation of ethosomes for transdermal delivery of lamivudine. *AAPS PharmSciTech*, 2007. **8**(4): p. E1-E9.
107. Schreier, H. and J. Bouwstra, Liposomes and niosomes as topical drug carriers dermal and transdermal drug delivery. *Journal of controlled release*, 1994. **30**: p. 1-15.
108. El Zaafarany, G.M., *et al.*, Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. *International journal of pharmaceutics*, 2010. **397**(1-2): p. 164-172.
109. Manosroi, A., P. Jantrawut, and J. Manosroi, Anti-inflammatory activity of gel containing novel elastic niosomes entrapped with diclofenac diethylammonium. *International journal of pharmaceutics*, 2008. **360**(1-2): p. 156-163.
110. Duangjit, S., *et al.*, Evaluation of meloxicam-loaded cationic transfersomes as transdermal drug delivery carriers. *AAPS PharmSciTech*, 2013. **14**(1): p. 133-140.
111. Lasic, D.D., *et al.*, Gelation of liposome interior: A novel method for drug encapsulation. *Federation of European Bioclaemical Societies Letters*, 1992. **312**(2, 3): p. 255-258.

112. Saraf, S.*, et al.*, Advancements and avenues in nanophytomedicines for better pharmacological responses. *Journal of nanoscience and nanotechnology*, 2015. **15**(6): p. 4070-4079.
113. Holley, F.O. and C. Van Steennis, Postoperative analgesia with fentanyl: Pharmacokinetics and pharmacodynamics of constant-rate IV and transdermal delivery. British Journal of Anaesthesia, 1988. **60**: p. 608-613.
114. Park, Y.H.*, et al.*, Assessment of dermal toxicity of nanosilica using cultured keratinocytes, a human skin equivalent model and an in vivo model. Toxicology, 2010. **267**(1-3): p. 178-181.
115. Hulzebos, E.*, et al.*, Use of structural alerts to develop rules for identifying chemical substances with skin irritation or skin corrosion potential. *QSAR & combinatorial science*, 2005. **24**(3): p. 332-342.
116. El Maghraby, G.M., B.W. Barry, and A.C. Williams, Liposomes and skin: From drug delivery to model membranes. *European journal of pharmaceutical science*s, 2008. **34**(4-5): p. 203-222.
117. Lieb, L.M.*, et al.*, Topical delivery enhancement with multilamellar liposomes into pilosebaceous units: I. In vitro evaluation using fluorescent techniques with the hamster ear model. *Journal of investigative dermatology*, 1992. **99**(1): p. 108-113.
118. Bernard, E., J.L. Dubois, and J. Wepierre, Importance of sebaceous glands in cutaneous penetration of an antiandrogen: Target effect of liposomes. *Journal of pharmaceutical science*s, 1997. **86**(5): p. 573-578.
119. Ganesan, M.G.*, et al.*, Influence of liposomal drug entrapment on percutaneous absorption. *International journal of pharmaceutics*, 1984. **20**: p. 139-154.
120. Jayaraman, S.C., C. Ramachandran, and N.D. Weiner, Topical delivery of erythromycin from various formulations: An in vivo hairless mouse study. *Journal of pharmaceutical science*s, 1996. **85**(10): p. 1082-1084.
121. Dhamecha, D.*, et al.*, Drug vehicle based approaches of penetration enhancement. *International journal of pharmacy and pharmaceutical sciences*, 2009. **1**(1): p. 24-46.
122. Kirjavainen, M.*, et al.*, Interaction of liposomes with human skin in vitro –the influence of lipid composition and structure. *Biochimica et Biophysica Acta*, 1996. **1304**: p. 179-189.
123. Cevc, G. and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochimica et Biophysica Acta*, 1992. **1104**: p. 226-232.
124. Megrab, N.A., A.C. Williams, and B.W. Barry, Oestradiol permeation across human skin, silastic and snake skin membranes: The effects of ethanol / water co-solvent systems. *International journal of pharmaceutics*, 1995. **116**: p. 101-112.
125. Lautenschläger, H., Liposomes in dermatological preparations. *Journal of applied cosmetology*, 1990. **8**: p. 1-9.

126. Verma, D.D. and A. Fahr, Synergistic penetration enhancement effect of ethanol and phospholipids on the topical delivery of cyclosporin A. *Journal of controlled release*, 2004. **97**(1): p. 55-66.
127. Magdassi, S., Delivery systems in cosmetics. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 1997. **123-124**: p. 671-679.
128. Naeff, R., Feasibility of topical liposome drugs produced on an industrial scale. *Advanced drug delivery reviews*, 1996. **18**: p. 343-347.
129. Vinod, K.R., *et al.*, Critical issues related to transfersomes - novel vesicular system. Acta Scientiarum Polonorum Technologia Alimentaria, 2012. **11**(1): p. 67-82.
130. Verma, D.D., *et al.*, Particle size of liposomes influences dermal delivery of substances into skin. *International journal of pharmaceutics*, 2003. **258**(1-2): p. 141-151.
131. Korting, H.C., *et al.*, Liposome encapsulation improves efficacy of betamethasone dipropionate in atopic eczema but not in psoriasis vulgaris. *European journal of clinical pharmacology*, 1990. **39**: p. 349-351.
132. Planas, M.E., *et al.*, Noninvasive percutaneous induction of topical analgesia by a new type of drug carrier, and prolongation of local pain insensitivity by anesthetic liposomes. *Anesthesia and analgesia*, 1992. **75**: p. 615-621.
133. Gesztes, A. and M. Mezei, Topical anesthesia of the skin by liposome-encapsulated tetracaine. *Anesthesia and analgesia*, 1988. **67**: p. 1079-1081.
134. Barenholz, Y., Liposome application: Problems and prospects. *Current opinion in colloid & interface science*, 2001. **6**: p. 66-77.
135. Manosroi, A., K. Podjanasoonthon, and J. Manosroi, Development of novel topical tranexamic acid liposome formulations. *International journal of pharmaceutics*, 2002. **235**: p. 61-70.
136. Date, A.A., B. Naik, and M.S. Nagarsenker, Novel drug delivery systems: Potential in improving topical delivery of antiacne agents. *Skin pharmacology and physiology*, 2006. **19**(1): p. 2-16.
137. Touitou, E., *et al.*, Intracellular delivery mediated by an ethosomal carrier. *Biomaterials*, 2001. **22**: p. 3053-3059.
138. Pierre, M.B.R., *et al.*, Stratum corneum lipids liposomes for the topical delivery of 5-aminolevulinic acid in photodynamic therapy of skin cancer- preparation and in vitro permeation study. *BMC Dermatology*, 2001. **1**: p. 5-10.
139. Kim, M.K., *et al.*, Targeted and sustained delivery of hydrocortisone to normal and stratum corneum-removed skin without enhanced skin absorption using a liposome gel. *Journal of controlled release*, 1997. **46**: p. 243-251.
140. Schmid, M.H. and H.C. Korting, Therapeutic progress with topical liposome drugs for skin disease. *Advanced drug delivery reviews*, 1996. **18**: p. 335-342.

141. Lopez-Berestein, G.*, et al.*, Liposome-encapsulated amphotericin b for treatment of disseminated candidiasis in neutropenic mice*. The journal of infectious diseases*, 1984. **150**(2): p. 278-283.
142. Simoes, M.C., J.J. Sousa, and A.A. Pais, Skin cancer and new treatment perspectives: A review*. Cancer letters*, 2015. **357**(1): p. 8-42.
143. Chen, Z.X.*, et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers*. European journal of pharmaceutical science*s, 2017. **99**: p. 240-245.
144. Foradada, M.*, et al.*, Chemical degradation of liposomes by serum components detected by NMR*. Chemistry and physics of lipids*, 2000. **104**: p. 133–148.
145. Krickau, D.P., R.H. Mueller, and J. Thomsen, Degradation kinetics of hydrolytically susceptible drugs in o/w emulsions-effects of interfacial area and lecithin*. International journal of pharmaceutics*, 2007. **342**(1-2): p. 62-71.
146. Watabe, N.*, et al.*, Oxidation decomposition of unsaturated fatty acids by singlet oxygen in phospholipid bilayer membranes. *Journal of Oleo science*, 2007. **56**(2): p. 73-80.
147. Touitou, E.*, et al.*, Ethosomes — novel vesicular carriers for enhanced delivery characterization and skin penetration properties*. Journal of controlled release*, 2000. **65**: p. 403-418.
148. Song, C.K.*, et al.*, A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation*. Colloids and Surfaces B: Biointerfaces*, 2012. **92**: p. 299-304.
149. Honeywell-Nguyen, P.L.*, et al.*, Transdermal delivery of pergolide from surfactant-based elastic and rigid vesicles: Characterization and in vitro transport studies*. Pharmaceutical research*, 2002. **19**(7): p. 991-997.
150. Guo, J.*, et al.*, Lecithin vesicular carriers for transdermal delivery of cyclosporin A*. International journal of pharmaceutics*, 2000. **194**: p. 200-207.
151. Bouwstra, J.A. and P.L. Honeywell-Nguyen, Skin structure and mode of action of vesicles*. Advanced drug delivery reviews*, 2002. **54**(1): p. S41–S55.
152. Caddeo, C.*, et al.*, Effect of quercetin and resveratrol co-incorporated in liposomes against inflammatory/oxidative response associated with skin cancer*. International journal of pharmaceutics*, 2016. **513**(1-2): p. 153-163.
153. Ascenso, A.*, et al.*, In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. *European journal of pharmaceutics and biopharmaceutics*, 2014. **88**(1): p. 48-55.
154. Duangjit, S.*, et al.*, Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes*. Journal of drug delivery*, 2011. **2011**: p. 1-9.
155. El Zaafarany, G.M.*, et al.*, Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery*. International journal of pharmaceutics*, 2010. **397**(1-2): p. 164-172.

156. Zheng, W.S., *et al.*, Preparation and quality assessment of itraconazole transfersomes. *International journal of pharmaceutics*, 2012. **436**(1-2): p. 291-298.
157. Bahia, A.P., *et al.*, New insights into the mode of action of ultradeformable vesicles using calcein as hydrophilic fluorescent marker. *European journal of pharmaceutical science*s, 2010. **39**(1-3): p. 90-96.
158. Honeywell-Nguyen, P.L. and J.A. Bouwstra, Vesicles as a tool for transdermal and dermal delivery. Drug Discov Today Technol, 2005. **2**(1): p. 67-74.
159. Cevc, G. and U. Vierl, NSAID formulations, based on highly adaptable aggregates, for improved transport through barriers and topical drug delivery. 2009, IdeaAG: United States.
160. Cevc, G. and U. Vierl, Aggregates with increased deformability, comprising at least three amphpats, for improved transport through semi-permeable barriers and for the non-invasive drug application in vivo, especially through the skin, in *United States Patent Application Publication*. 2012: United States.
161. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Interactions of surfactants (edge activators) and skin penetration enhancers with liposomes. *International journal of pharmaceutics*, 2004. **276**(1-2): p. 143-161.
162. Paul, A., G. Cevc, and B.K. Bachhawat, Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. *Vaccine*, 1998. **16**(213): p. 188-195.
163. Trotta, M., *et al.*, Deformable liposomes for dermal administration of methotrexate. *International journal of pharmaceutics*, 2004. **270**(1-2): p. 119-125.
164. Jain, S., *et al.*, Ultra deformable liposome: A recent tool for effective transdermal drug delivery. *Indian journal of pharmaceutical sciences*, 2003. **65**(3): p. 223-231.
165. Nandure, H.P., *et al.*, Ethosome: A novel drug carrier. *International journal of pharmaceutical research & allied sciences*, 2013. **2**(3): p. 18-30.
166. Cevc, G. and D. Gebauer, Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. *Biophysical Journal*, 2003. **84**: p. 1010-1024.
167. Cevc, G. and G. Blume, Biological activity and characteristics of triamcinolone-acetonide formulated with the self-regulating drug carriers, transfersomes®. *Biochimica et Biophysica Acta* (BBA) - Biomembranes, 2003. **1614**(2): p. 156-164.
168. Cevc, G. and G. Blume, Hydrocortisone and dexamethasone in very deformable drug carriers have increased biological potency, prolonged effect, and reduced therapeutic dosage. *Biochimica et Biophysica Acta*, 2004. **1663**(1-2): p. 61-73.
169. Honeywell-Nguyen, P.L., *et al.*, Transdermal delivery of pergolide from surfactant-based elastic and rigid vesicles characterization and in vitro transport studies. *Pharmaceutical research*, 2002. **19**(7): p. 991-997.

170. Honeywell-Nguyen, P.L. and J.A. Bouwstra, The in vitro transport of pergolide from surfactant-based elastic vesicles through human skin: A suggested mechanism of action. *Journal of controlled release*, 2003. **86**: p. 145-156.
171. Schätzlein, A. and G. Cevc, Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: A high-resolution confocal laser scanning microscopy study using highly deformable vesicles (transfersomes). *British journal of dermatology*, 1998. **138**(4): p. 583-592.
172. Honeywell-Nguyen, P.L., S. Arenja, and J.A. Bouwstra, Skin penetration and mechanisms of action in the delivery of the d2-agonist rotigotine from surfactant-based elastic vesicle formulations. *Pharmaceutical research*, 2003. **20**(10): p. 1619-1625.
173. Trotta, M.*, et al*., Elastic liposomes for skin delivery of dipotassium glycyrrhizinate. *International journal of pharmaceutics*, 2002. **241**: p. 319-327.
174. Malakar, J.*, et al.*, Formulation, optimization and evaluation of transferosomal gel for transdermal insulin delivery. *Saudi pharmaceutical journal*, 2012. **20**(4): p. 355-363.
175. Boncheva, M.*, et al.*, Depth profiling of stratum corneum hydration in vivo: A comparison between conductance and confocal raman spectroscopic measurements. *Experimental dermatology*, 2009. **18**(10): p. 870-876.
176. Elsayed, M.M.*, et al.*, Deformable liposomes and ethosomes: Mechanism of enhanced skin delivery. *International journal of pharmaceutics*, 2006. **322**(1-2): p. 60-66.
177. Cevc, G., A. Schätzlein, and H. Richardsen, Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label clsm experiments and direct size measurements. *Biochimica et Biophysica Acta*, 2002. **1564**: p. 21-30.
178. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Skin delivery of oestradiol from lipid vesicles: Importance of liposome structure. *International journal of pharmaceutics*, 2000. **204**: p. 159-169.
179. Boinpally, R.R.*, et al.*, Lecithin vesicles for topical delivery of diclofenac. *European journal of pharmaceutics and biopharmaceutics*, 2003. **56**(3): p. 389-392.
180. Rozman, B.*, et al.*, Dual influence of colloidal silica on skin deposition of vitamins c and e simultaneously incorporated in topical microemulsions. *Drug development and industrial pharmacy*, 2010.
181. Hiruta, Y.*, et al.*, Novel ultra-deformable vesicles entrapped with bleomycin and enhanced to penetrate rat skin. *Journal of controlled release*, 2006. **113**(2): p. 146-154.
182. Cevc, G.*, et al.*, Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochimica et Biophysica Acta*, 1998. **1368**: p. 201-215.
183. Jain, S., S. Jain, and N.K. Jain, Transfersomes for enhanced delivery of dexamethasone, in *28th Conference of CRS*. 2001: USA.

184. Garg, T.*, et al.*, Elastic liposomal formulation for sustained delivery of antimigraine drug: In vitro characterization and biological evaluation*. Drug development and industrial pharmacy*, 2008. **34**(10): p. 1100-1110.
185. Guo, J.*, et al.*, Lecithin vesicular carriers for transdermal delivery of cyclosporin A*. International journal of pharmaceutics*, 2000. **194**: p. 201-207.
186. Cevc, G., U. Vierl, and S. Mazgareanu, Functional characterisation of novel analgesic product based on self-regulating drug carriers*. International journal of pharmaceutics*, 2008. **360**(1-2): p. 18-28.
187. Dubey, V.*, et al.*, *T*ransdermal delivery of a pineal hormone: Melatonin via elastic liposomes*. Biomaterials*, 2006. **27**(18): p. 3491-3496.
188. Thiele, J.J. and S. Ekanayake-Mudiyanselage, Vitamin E in human skin: Organ-specific physiology and considerations for its use in dermatology*. Molecular aspects of medicine*, 2007. **28**(5-6): p. 646-667.
189. Simoes, S.I.*, et al.*, Developments in the rat adjuvant arthritis model and its use in therapeutic evaluation of novel non-invasive treatment by SOD in transfersomes*. Journal of controlled release*, 2005. **103**(2): p. 419-434.
190. Vorauer-Uhl, K.*, et al.*, Topically applied liposome encapsulated superoxide dismutase reduces postburn wound size and edema formation*. European journal of pharmaceutical science*s, 2001. **14**(1): p. 63-67.
191. Fesq, H.*, et al.*, Improved risk– benefit ratio for a topical triamcinolone acetonide intransfersome in comparison to equipotent cream and ointment: A randomized controlled trial*. British journal of dermatology*, 2003. **149**: p. 611-319.
192. Ita, K.B.*, et al.*, Dermal delivery of selected hydrophilic drugs from elastic liposomes: Effect of phospholipid formulation and surfactants*. Journal of pharmacy and pharmacology*, 2007. **59**(9): p. 1215-1222.
193. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Skin delivery of oestradiol from deformable and traditional liposomes: Mechanistic studies*. Journal of pharmacy and pharmacology*, 1999. **51**: p. 1123-1134.
194. Touitou, E.*, et al.*, Ethosomes — novel vesicular carriers for enhanced delivery: Characterization and skin penetration properties*. Journal of controlled release*, 2000. **65**(3): p. 403-418.
195. Touitou, E., B. Godin, and C. Weiss, Enhanced delivery of drugs into and across the skin by ethosomal carriers*. Drug development research*, 2000. **50**: p. 406-415.
196. Verma, P. and K. Pathak, Therapeutic and cosmeceutical potential of ethosomes: An overview. *Journal of advanced pharmaceutical technology & research*, 2010. **1**(3): p. 274-282.
197. Thadanki, M. and A.K. Babu, Review on ethosomes: A novel approach of liposomes*. International journal of pharmacy & life sciences*, 2015. **6**(1): p. 4171-4176.

198. Rakesh, R. and K.R. Anoop, Ethosomes for transdermal and topical drug delivery. *International journal of pharmacy & pharmaceutical sciences*, 2012. **4**(3): p. 17-24.
199. Paolino, D.*, et al.*, Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers. *Journal of controlled release*, 2005. **106**(1-2): p. 99-110.
200. Jain, S.*, et al.*, Ethosomes: A novel vesicular carrier for enhanced transdermal delivery of an anti-HIV agent. *Indian journal of pharmaceutical sciences*, 2004. **66**(1): p. 72-81.
201. Marto, J.*, et al.*, Ethosomes for enhanced skin delivery of griseofulvin. *Colloids and Surfaces B: Biointerfaces*, 2016. **146**: p. 616-23.
202. Abdulbaqi, I.M.*, et al.*, Ethosomal nanocarriers: The impact of constituents and formulation techniques on ethosomal properties, in vivo studies, and clinical trials. *International journal of nanomedicine*, 2016. **11**: p. 2279-2304.
203. Bendas, E.R. and M.I. Tadros, Enhanced transdermal delivery of salbutamol sulfate via ethosomes. *AAPS PharmSciTech*, 2007. **8**(4): p. E1-E8.
204. Ainbinder, D. and E. Touitou, Testosterone ethosomes for enhanced transdermal delivery. *Drug delivery*, 2005. **12**(5): p. 297-303.
205. Dayan, N. and E. Touitou, Carriers for skin delivery of trihexyphenidyl HCl: Ethosomes vs. Liposomes. *Biomaterials*, 2000. **21**: p. 1879-1885.
206. Nandure, H.P.*, et al.*, Ethosome a novel drug carrier. *International journal of pharmaceutical research & allied sciences*, 2013. **2**(3): p. 18-30.
207. Cevc, G., Optimised preparations of highly adaptable aggregates, U.S.P.A. Publication, Editor. 2014: United States. p. 1-32.
208. He, R., D. Cui, and F. Gao, Preparation of fluorescence ethosomes based on quantum dots and their skin scar penetration properties. *Materials letters*, 2009. **63**(20): p. 1662-1664.
209. Lopez-Pinto, J.M., M.L. Gonzalez-Rodriguez, and A.M. Rabasco, Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *International journal of pharmaceutics*, 2005. **298**(1): p. 1-12.
210. Lopez-Pinto, J.M., M.L. Gonzalez-Rodriguez, and A.M. Rabasco, Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *International journal of pharmaceutics*, 2005. **298**(1): p. 1-12.
211. Shaji, J. and S. Garude, Ultradeformable vesicles for enhanced transdermal delivery. *Indo American journal of pharmaceutical research*, 2014. **4**(8): p. 3588-3596.
212. Megrab, N.A., A.C. Williams, and B.W. Barry, Oestradiol permeation across human skin, silastic and snake skin membranes: The effects of ethanol or water co-solvent systems. *International journal of pharmaceutics*, 1995. **116**: p. 101-112.

213. Chen, M., X. Liu, and A. Fahr, Skin penetration and deposition of carboxyfluorescein and temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application. *International journal of pharmaceutics*, 2011. **408**(1-2): p. 223-34.
214. Dubey, V., D. Mishra, and N.K. Jain, Melatonin loaded ethanolic liposomes: Physicochemical characterization and enhanced transdermal delivery. *European journal of pharmaceutics and biopharmaceutics*, 2007. **67**(2): p. 398-405.
215. Panchagnula, R., *et al.*, Transdermal delivery of naloxone- effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin. *International journal of pharmaceutics*, 2001. **219**: p. 95-105.
216. Zhang, J.P., *et al.*, Ethosomes, binary ethosomes and transfersomes of terbinafine hydrochloride: A comparative study. *Archives of Pharmacal Research*, 2012. **35**(1): p. 109-117.
217. Elsayed, M.M., *et al*., Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. *Pharmazie,* 2007. **62**: p. 133-137.
218. Lodzki, M., *et al.*, Cannabidiol—transdermal delivery and anti-inflammatory effect in a murine model. *Journal of controlled release*, 2003. **93**(3): p. 377-387.
219. Verma, P. and K. Pathak, Nanosized ethanolic vesicles loaded with econazole nitrate for the treatment of deep fungal infections through topical gel formulation. *Nanomedicine*, 2012. **8**(4): p. 489-496.
220. Scognamiglio, I., *et al.*, Nanocarriers for topical administration of resveratrol: A comparative study. *International journal of pharmaceutics*, 2013. **440**(2): p. 179-187.
221. Limsuwan, T. and T. Amnuaikit, Development of ethosomes containing mycophenolic acid. *Procedia chemistry*, 2012. **4**: p. 328-335.
222. Dubey, V., *et al.*, Enhanced transdermal delivery of an anti-HIV agent via ethanolic liposomes. *Nanomedicine*, 2010. **6**(4): p. 590-596.
223. Xu, D.H., *et al.*, Synergistic effects of ethosomes and chemical enhancers on enhancement of naloxone permeation through human skin. *Pharmazie,* 2007. **62**(4): p. 316-318.
224. Dubey, V., *et al.*, Dermal and transdermal delivery of an anti-psoriatic agent via ethanolic liposomes. *Journal of controlled release*, 2007. **123**(2): p. 148-154.
225. Esposito, E., E. Menegatti, and R. Cortesi, Ethosomes and liposomes as topical vehicles for azelaic acid: A preformulation study. *International journal of cosmetic science*, 2004. **26**(5): p. 270-271.
226. Mishra, D., *et al.*, Systemic and mucosal immune response induced by transcutaneous immunization using hepatitis B surface antigen-loaded modified liposomes. *European journal of pharmaceutical science*s, 2008. **33**(4-5): p. 424-433.
227. Touitou, E., Compositions of applying active substance to or through the skin, U.S. Patent, Editor. 1996. p. 1-11.

228. Manosroi, A., P. Jantrawut, and J. Manosroi, Anti-inflammatory activity of gel containing novel elastic niosomes entrapped with diclofenac diethylammonium. *International journal of pharmaceutics*, 2008. **360**(1-2): p. 156-163.
229. Cevc, G., Transfersomes, liposomes and other lipid suspensions on the skin: Permeation enhancement, vesicle penetration, and transdermal drug delivery. *Therapeutic drug carrier systems*, 1996. **13**(3-4): p. 257-388.
230. Bagde, A., A. Mondal, and M. Singh, Drug delivery strategies for chemoprevention of uvb-induced skin cancer: A review. *Photodermatology, photoimmunology & photomedicine*, 2018. **34**(1): p. 60-68.
231. Shaji, J. and S. Garude, Development and characterisation of ketorolac tromethamine encapsulated transethosomes for enhanced transdermal development. *World journal of pharmacy and pharmaceutical sciences*, 2014. **3**(9): p. 1213-1227.
232. Kumar, L.*, et al.*, Ethanol based vesicular carriers in transdermal drug delivery: Nanoethosomes and transethosomes in focus. *Nanoworld journal*, 2016. **2**(3): p. 41-51.
233. Garg, V.*, et al.*, Systematic development of transethosomal gel system of piroxicam: Formulation optimization, in vitro evaluation, and ex vivo assessment. *AAPS PharmSciTech*, 2017. **18**(1): p. 58-71.
234. Mingzhu, L.*, et al.*, Dual drug encapsulation in a novel nano-vesicular carrier for the treatment of cutaneous melanoma: Characterization and in vitro/in vivo evaluation. *RSC Advances*, 2015. **5**(26): p. 20467-20478.
235. Chen, Z.X.*, et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers. *European journal of pharmaceutical science*, 2017. **99**: p. 240-245.
236. Chen, M.*, et al.*, Topical delivery of hyaluronic acid into skin using space-peptide carriers. *Journal of controlled release*, 2014. **173**: p. 67-74.
237. Ma, M.*, et al.*, Development of nanovesicular systems for dermal imiquimod delivery: Physicochemical characterization and in vitro/in vivo evaluation. *Journal of materials science: Materials in medicine*, 2015. **26**(6): p. 191.
238. Prow, T.W.*, et al.*, Nanoparticles and microparticles for skin drug delivery. *Advanced drug delivery reviews*, 2011. **63**(6): p. 470-491.
239. Caldorera-Moore, M.*, et al.*, Designer nanoparticles: Incorporating size, shape and triggered release into nanoscale drug carriers. *Expert opinion on drug delivery*, 2010. **7**(4): p. 479-495.
240. Bower, C.K.*, et al.*, Activity losses among T4 lysozyme charge variants after adsorption to colloidal silica. *Biotechnology and bioengineering*, 1999. **64**(3): p. 373-376.
241. Wang, X.*, et al.*, Advances of cancer therapy by nanotechnology. *Cancer research and treatment*, 2009. **41**(1): p. 1-11.

242. Verma, S. and P. Utreja, Vesicular nanocarrier based treatment of skin fungal infections: Potential and emerging trends in nanoscale pharmacotherapy. *Asian Journal of pharmaceutical sciences*, 2018.
243. Bozzuto, G. and A. Molinari, Liposomes as nanomedical devices. *International journal of nanomedicine*, 2014. **10**(1): p. 975-999.
244. Amnuaikit, T.*, et al.*, Vesicular carriers containing phenylethyl resorcinol for topical delivery system: Liposomes, transfersomes and invasomes. *Asian Journal of pharmaceutical sciences*, 2018.
245. Scholtz, J.C., Preparation, stability and in vitro evaluation of liposomes containing amodiaquine, in *Pharmaceutics*. 2010, North-West University: Potchefstroom. p. 113.
246. Tartau, L.*, et al.*, Biocompatibility investigation and in vivo evaluation of ketoprofen entrapped in lipid vesicles. *Annals of RSCB*, 2010. **XV**(2): p. 347-352.
247. Nooli, M.*, et al.*, Solid lipid nanoparticles as vesicles for oral delivery of olmesartan medoxomil: Formulation, optimization and in vivo evaluation. *Drug development and industrial pharmacy*, 2017. **43**(4): p. 611-617.
248. Nieuwoudt, S., Preparation, stability and in vitro evaluation of liposomes containing chloroquine, in *Pharmaceutics*. 2010, North-West University: Potchefstroom. p. 182.
249. Luccardini, C.*, et al.*, Size, charge, and interactions with giant lipid vesicles of quantum dots coated with an amphiphilic macromolecule. Langmuir, 2006. **22**(5): p. 2304-2310.
250. Kohane, D.S. and R. Langer, Biocompatibility and drug delivery systems. *The royal society of chemistry*, 2010. **1**: p. 441–446.
251. ISO10993, Biological evaluation of medical devices - part 5*: Tests for in vitro cytotoxicity.* ISO 10993, 2009. **5**.
252. Zhang, X.Q.*, et al.*, Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine. *Advanced drug delivery reviews*, 2012. **64**(13): p. 1363-1384.
253. Shvedova, A.A., V.E. Kagan, and B. Fadeel, Close encounters of the small kind: Adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. *Annual review of pharmacology and toxicology*, 2010. **50**: p. 63-88.
254. Xia, T., N. Li, and A.E. Nel, Potential health impact of nanoparticles. *Annual review of public health*, 2009. **30**: p. 137-150.
255. Vinken, M., T. Vanhaecke, and V. Rogiers, *P*rimary hepatocyte cultures as in vitro tools for toxicity testing: Quo vadis? *Toxicology in vitro*, 2012. **26**(3): p. 541-4.
256. Vinken, M., Adverse outcome pathways and drug-induced liver injury testing. *Chemical research in toxicology*, 2015. **28**(7): p. 1391-7.
257. Vinken, M., The adverse outcome pathway concept: A pragmatic tool in toxicology. *Toxicology*, 2013. **312**: p. 158-165.

258. Ankley, G.T.*, et al.*, Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment*. Environmental toxicology and chemistry*, 2010. **29**(3): p. 730-41.
259. Villeneuve, D.L.*, et al.*, Adverse outcome pathway (AOP) development i: Strategies and principles*. Toxicological sciences*, 2014. **142**(2): p. 312-20.
260. Roursgaard, M.*, et al.*, In vitro toxicity of cationic micelles and liposomes in cultured human hepatocyte (HepG2) and lung epithelial (A549) cell lines*. Toxicology in vitro*, 2016. **36**: p. 164-171.
261. McKim, J.M., Jr., D.J. Keller, 3rd, and J.R. Gorski, An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing*. Cutaneous and ocular toxicology*, 2012. **31**(4): p. 292-305.
262. Nel, A.*, et al.*, A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment*. Perspectives - American chemical society*, 2013. **7**(8): p. 6422–6433.
263. Nel, A.*, et al.*, Nanomaterial toxicity testing in the 21st century: Use of a predictive toxicological approach and high-throughput screening. *Accounts of chemical research*, 2013. **46**(3): p. 607-621.
264. Hall, A.*, et al.*, Polyethylenimine architecture-dependent metabolic imprints and perturbation of cellular redox homeostasis*. Biochimica et Biophysica Acta*, 2015. **1847**(3): p. 328-342.
265. Jantzen, K.*, et al.*, Oxidative damage to DNA by diesel exhaust particle exposure in co-cultures of human lung epithelial cells and macrophages*. Mutagenesis*, 2012. **27**(6): p. 693-701.
266. Jones, D.P.*, et al.*, Mechanisms of pathogenesis in drug hepatotoxicity: Putting the stress on mitochondria. *Molecular interventions*, 2010. **10**(2): p. 98-111.
267. Tsujimoto, Y. and S. Shimizu, Role of the mitochondrial membrane permeability transition in cell death*. Apoptosis*, 2007. **12**(5): p. 835-840.
268. Pessayre, D.*, et al.*, Central role of mitochondria in drug-induced liver injury*. Drug metabolism reviews*, 2012. **44**(1): p. 34-87.
269. Escher, B., J.L. Hermens, and R. Schwarzenbach, Internal exposure: Linking bioavailability to effects. *International workshop, 27 august 2004, monte verità, switzerland.* Environmental Science and Pollution Research, 2005. **12**(1): p. 57-60.
270. Bass, A.S.*, et al.*, Exploratory drug safety: A discovery strategy to reduce attrition in development*. Journal of pharmacological and toxicological methods*, 2009. **60**(1): p. 69-78.

271. Fraczek, J.*, et al.*, Primary hepatocyte cultures for pharmaco-toxicological studies: At the busy crossroad of various anti-dedifferentiation strategies*. Archives of toxicology*, 2013. **87**(4): p. 577-610.
272. Pritchard, J.F.*, et al.*, Making better drugs: Decision gates in non-clinical drug development*. Nature reviews drug discovery*, 2003. **2**(7): p. 542-553.
273. Wong, C.L.*, et al.*, In vitro methods for hazard assessment of industrial chemicals - opportunities and challenges*. Frontiers in pharmacology*, 2015. **6**: p. 94.
274. Fotakis, G. and J.A. Timbrell, In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride*. Toxicology letters*, 2006. **160**(2): p. 171-177.
275. Lilienblum, W.*, et al.*, Alternative methods to safety studies in experimental animals: Role in the risk assessment of chemicals under the new european chemicals legislation (REACH)*. Archives of toxicology*, 2008. **82**(4): p. 211-236.
276. Vieira, M.S.*, et al.*, In vitro basal cytotoxicity assay applied to estimate acute oral systemic toxicity of grandisin and its major metabolite*. Experimental and toxicologic pathology*, 2011. **63**(5): p. 505-510.
277. Bandele, O.J.*, et al.*, In vitro toxicity screening of chemical mixtures using HEPG2/C3A cells*. Food and chemical toxicology*, 2012. **50**(5): p. 1653-1659.
278. Barile, F.A., P.J. Dierickx, and U. Kristen, In vitro cytotoxicity testing for prediction of acute human toxicity*. Cell biology and toxicology*, 1994. **10**(3): p. 155-162.
279. Eisenbrand, G.*, et al.*, Methods of in vitro toxicology*. Food and chemical toxicology,* 2002. **40**: p. 193–236.
280. Sali, N.*, et al.*, Multiparametric luminescent cell viability assay in toxicology models: A critical evaluation*. Journal of pharmacological and toxicological methods*, 2016. **79**: p. 45-54.
281. Weyermann, J., D. Lochmann, and A. Zimmer, A practical note on the use of cytotoxicity assays*. International journal of pharmaceutics*, 2005. **288**(2): p. 369-376.
282. Yang, A., D.L. Cardona, and F.A. Barile, In vitro cytotoxicity testing with fluorescence-based assays in cultured human lung and dermal cells*. Cell biology and toxicology*, 2002. **18**: p. 97-108.
283. Creppy, E.E.*, et al.*, Synergistic effects of fumonisin b1 and ochratoxin a: Are in vitro cytotoxicity data predictive of in vivo acute toxicity? *Toxicology*, 2004. **201**(1-3): p. 115-123.
284. Mathes, S.H., H. Ruffner, and U. Graf-Hausner, The use of skin models in drug development. *Advanced drug delivery reviews*, 2014. **69-70**: p. 81-102.
285. Matsui, T. and M. Amagai, Dissecting the formation, structure and barrier function of the stratum corneum*. International immunology*, 2015. **27**(6): p. 269-280.

286. Abhishek, S. and S.P. Krishnan, Epidermal differentiation complex: A review on its epigenetic regulation and potential drug targets*. Cell journal*, 2016. **18**(1): p. 1-6.
287. Clark, W.H.*, et al.*, The histogenesis and biologic behavior of primary human malignant melanomas of the skin*. Cancer research*, 1969. **29**: p. 705-726.
288. Abd, E.*, et al.*, Skin models for the testing of transdermal drugs*. Clinical pharmacology*, 2016. **8**: p. 163-176.
289. Armitage, J.M., F. Wania, and J.A. Arnot, Application of mass balance models and the chemical activity concept to facilitate the use of in vitro toxicity data for risk assessment*. Environmental science & technology*, 2014. **48**(16): p. 9770-9779.
290. Astashkina, A., B. Mann, and D.W. Grainger, A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity*. Pharmacology & therapeutics*, 2012. **134**(1): p. 82-106.
291. Groothuis, F.A.*, et al.*, Dose metric considerations in in vitro assays to improve quantitative in vitro-in vivo dose extrapolations*. Toxicology*, 2015. **332**: p. 30-40.
292. Gubbels-van Hal, W.M.*, et al.*, An alternative approach for the safety evaluation of new and existing chemicals, an exercise in integrated testing*. Regulatory toxicology and pharmacology*, 2005. **42**(3): p. 284-295.
293. Guillouzo, A., Liver cell models in in vitro toxicology*. Environmental health perspectives*, 1998. **106**(2): p. 511-532.
294. Hartung, T., Food for thought … on alternative methods for chemical safety testing*. ALTEX*, 2010: p. 3-14.
295. Kramer, N.I.*, et al.*, Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays*. Chemical research in toxicology*, 2012. **25**(2): p. 436-445.
296. Lin, Z. and Y. Will, Evaluation of drugs with specific organ toxicities in organ-specific cell lines*. Toxicological sciences*, 2012. **126**(1): p. 114-127.
297. Schirmer, K., Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish*. Toxicology*, 2006. **224**(3): p. 163-183.
298. Wilkening, S., F. Stahl, and A. Bader, Comparison of primary human hepatocytes and hepatoma cell line HEPG2 with regard to their biotransformation properties*. Drug metabolism and disposition*, 2003. **31**(8): p. 1035-1042.
299. Kimber, I.*, et al.*, Chemical allergy: Translating biology into hazard characterization*. Toxicological sciences*, 2011. **120 Supplementary 1**: p. S238-S268.

Chapter 3 – Image analysis of particle shape for characterizing of lipid colloidal carriers

Manuscript as to be submitted for the *International journal of pharmaceutics*.

This article is written according to the journal instructions that can be found at https://www.elsevier.com/journals/international-journal-of-pharmaceutics/0378-5173?generatepdf=true . The reference style followed was a numeric style and the language was American English as specified by the journal. This reference style and language was also kept constant throughout the dissertation to enable enhanced reading. There was not a strict adherence to word count for the purposes of the examination of the dissertation, however the manuscript will be condensed for publication purposes.

Title page

Image analysis of particle shape for characterizing of lipid colloidal carriers

Michelle Volkwyn*, Joe M Viljoen, Sonette du Preez, Lissinda H du Plessis

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Private bag x6001, Potchefstroom 2520, South Africa

*corresponding author Michellevolkwyn93@gmail.com

Abstract

Liposomes, transferosomes, ethosomes and transethosomes are lipid nanocarriers (LN) that have been developed as alternatives to traditional transdermal products. Characterization of these systems is an important first step in dosage form development. One of the standardized ways to characterize the LN is with dynamic light scattering (vesicle diameter, PDI and zeta potential), Quantitative image analyses, using specific size and shape variables have not been established for LN. In this study, the Malvern Morphologi G3 was used to establish size and shape parameters for the characterization of LN. The intensity mean of the LN was grayscale, indicative of the fluid filled vesicles. The LN were fairly solid, showed low elongation and high convexity, with varying levels of circularity. It was concluded that elongation, convexity and circularity were parameters that could be used complementary to dynamic light scattering.

Total word count: 136

Key words

Lipid nanocarrier; characterization; Image analysis, Malvern Morphologi G3, elongation, convexity, circularity

1. Introduction

Over the past ten years, novel colloidal carriers in the nanometer range have been proven successful in various fields utilizing nanotechnology. Colloidal carriers are defined as "particulate or vesicular dosage forms that serve as superficial drug reservoirs". Lipid colloidal carriers are colloidal carriers that have a particle or droplet size as small as 500 nm, and have the ability to deliver various drugs in effective concentrations [1-5]. They are water filled vesicles with a lipid membrane [6-8], and are divided into the following categories: liposomes, ultra-deformable vesicles (UDV) and solid lipid nanoparticles (SLN). The use of lipid nanocarriers (LN) in the skin has received increased attention in the field of drug-delivery research, since these LN have the capacity for controlled release by creating a localized depot in the skin [2, 3]. To ensure that drugs have controlled and reliable release for improved delivery into the skin, several lipid nanocarriers have been developed as an alternative to the traditional transdermal products [9, 10]. These systems include liposomes, transferosomes, ethosomes and transethosomes [9].

In 1964 Alec Bangham first described liposomes as "closed" vesicles, consisting of phospholipids, formed in an excess of a watery phase" [11]. This description was adjusted in later years, improving the definition of liposomes as "colloidal particles capable of encapsulating drugs, which are formed as concentric bio-molecular layers" [12-20]. Liposomes proved to be limited in being thermodynamically unstable, with poor permeability through the skin, and are mostly engaged for topical drug delivery, therefore improved formulations were developed [9, 15, 16, 19, 21, 22]. UDVs can be described as new novel lipid nanocarriers that demonstrate more deformability, which enhances their ability to deliver drugs transdermally compared to liposomes [15, 16, 23, 24]. They have the advantage over liposomes by minimizing the risk of vesicle rupture during skin penetration, as well as not compromising the structure of the skin [4, 8, 25, 26].

Transferosomes were introduced by Cevc and Blume [24] as the first generation of elastic lipid nanocarriers and are trademarked by IDEA AG in Munich, Germany. These ultradeformable vesicles, are artificial phospholipid-based elastic nanovesicles that contain edge activators such as sodium cholate, and are most commonly used in drug delivery technology [8, 11, 15, 16, 25, 27, 28]. Touitou *et al.* [21] introduced elastic lipid vesicles called ethosomes. Due to

facilitating non-invasive delivery of drugs within the skin's layers, these carriers were developed especially for transdermal delivery [9, 15, 29-36]. Only more recently, in 2012, did Song *et al.* [22] introduce the UDV, transethosomes, which comprised of the components of both transferosomes and ethosomes [15, 37-39]. This was an attempt to combine the advantageous characteristics of both transferosomes and ethosomes [40], causing them to be more deformable, due to their ethanol and surfactant content [15, 22].

Characterization of LN improves the understanding of synthesis and application of these nanoparticles, and could in turn be used for regulatory purposes [41]. In the design of UDV, similar to liposomes, the size and morphology of the particles remains essential as it may influence the stability, kinetics of drug release and drug distribution in the blood [42-44]. In addition, more emphasis has been placed on the shape of nanoparticles as it influences the drug release rate, cell uptake rate, stability, pharmacokinetic profiles, biodistribution, cytotoxicity, as well as therapeutic efficacy [42, 43, 45]. Particle surface properties is another important factor, as it has been linked to uptake mechanisms, the drug release rate, stability, pharmacokinetic profiles, and biodistribution [42, 43, 45].

One of the preferred methods for analysis of particle size is dynamic light scattering (DLS). DLS determines the size of dispersed particles by the intensity of the light scattered from the particles [46, 47]. Particle size is used as a means to compare different particles with each other, whether they be liquid, solid or gas. Particles larger than 600 nm in diameter are not able to cross the skin barrier, but will be able to deliver drugs topically [48]. Polydispersity index (PDI), also known as dispersity, is defined as a means to characterize the distribution of sizes of particles in a mixture [48, 49]. PDI is a dimensionless index where a higher index, such as 0.7, is indicative of a broad particle size distribution and a lower index, such as 0.05, is indicative of a significantly narrow particle size distribution [48]. Currently a PDI of 0.3 and lower is accepted for use as lipid-based carriers [48]. Conversely, zeta potential also known as electrokinetic potential, is defined as a charge that develops at the border between a solid surface and a liquid medium, therefore acting as a natural parameter to describe electrochemical properties [50]. It has been suggested that zeta potential is indicative of physical stability of emulsions, and is measured using laser Doppler micro-electrophoresis [47, 51]. Morphology of the particles are usually measured by SEM/TEM, however drying of the sample during analysis may change the properties of the vesicular systems [43, 52]. Stability may play a large role in the use of drug carrier systems, especially the stability of lipid nanocarriers. The most common methods for determining stability of lipid nanocarriers are by evaluating the size and the structure of the vesicles over time [32, 53-55]. A significant physical parameter for predicting a vesicles' stability, is the use of zeta potential, where a high

negative surface charge will be an indication that the vesicles would be stable for longer and would not agglomerate [15, 53, 56]. An unstable formulation might have an effect on the formulation's pH, which can lead to further degradation.

In addition to surface characteristics and size offered by SEM/TEM, image analysis software has enabled the additional measurement of various particle shape properties. These properties are measured in two dimensional (2D) projections of three dimensional (3D) images, contrasting the one dimensional (1D) offered by TEM/SEM. The Malvern Morphologi G3 (Malvern Instruments Ltd., Malvern, UK) is a particle characterization instrument for the measurement of particle size and shape by employing image analysis in segments to determine the exact shape of particles [57]. Particle size is given by the length, width and CE diameter. The particle form is provided by the aspect ratio and elongation, whereas the mean intensity (measure of transparency) and solidity provide an indication of the surface morphology and the internal microstructure of the particle. The shape of particles is measured by means of circularity, convexity and elongation and solidity can additionally be utilized to establish subtle differences between particles [57]. Figure 3.1 illustrates the parameters of image analysis.

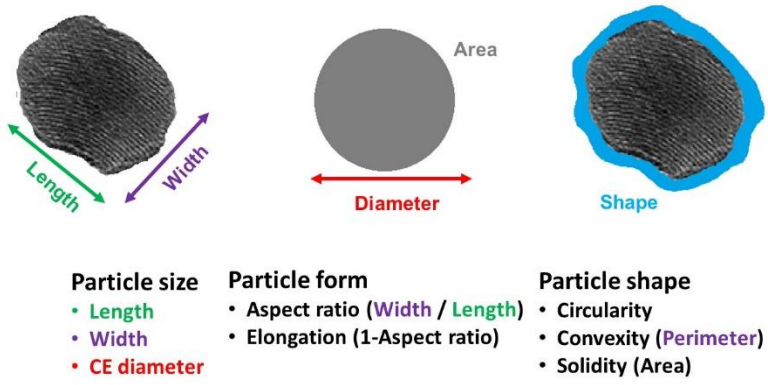


***Figure 3.1**: Illustration of the parameters of image analysis*

(Illustration adapted from Duffy [58])

To date, little to no studies have been performed to determine lipid nanocarrier stability using the Malvern Morphologi G3. In this study we demonstrated a systematic approach to characterize ultradeformable vesicles with optimization of the UDV formulas. We have compared our results to those obtained for liposomes, which we have included as a control, as numerous studies have been conducted on liposomes to characterize these vesicles. Liposomes could be perceived as the 'father'-vesicle. We aimed to quantify the characterization of the morphology, utilizing image analysis, of UDVs as a complementary

technique to DLS, comparing the image analysis results to results obtained from previous studies on powder particles. We have attempted to determine the image analysis parameters that best fit these lipid nanocarriers to improve characterization of similar carriers in the future. We included stability experiments to determine the effects of storage time on the image analysis parameters, as well as DLS parameters of the UDV with liposomes as the control.

2. Materials and methods

2.1. Materials

Chloroform (biotech grade, ≥99.8%), ethyl alcohol (pure, for molecular biology), L-α-phosphatidylcholine (from egg yolk, PC), methanol (suitable for protein sequencing), phosphate buffered saline (for cell cultures, PBS) and Span 80® were purchased from Sigma-Aldrich®, Merck (Pty) Ltd. (Darmstadt, Germany).

2.2. Formulation of lipid nanocarriers

The optimal composition for the liposomes, transferosomes, ethosomes and transethosomes was determined beforehand to enable vesicles suitable for standardization of morphological parameters (Supplementary Material). Formulation was performed at room temperature that was controlled at 25ºC at all times. It was established that only a 1000 µg/ml concentration of LN would be prepared each time, after which it would be diluted to the desired concentration.

The method for formulation of liposomes and UDV was adapted from Ascenso *et al.* [15] and the composition of the different LN is summarized in Table 3.1. PC was dissolved in a 2:1 chloroform:methanol mixture, after which the solution was heated to 40ºC. The beaker was rotated constantly until a film was visible on the bottom of the beaker. The aqueous phase (PBS for cell cultures) was subsequently added and the mixture homogenized at 6000 rpm for 1 min using the IKA ULTRA-TURRAX® tube disperser workstation system (IKA®-Werke GmbH & Co. KG, Staufen, Germany). When UDV was prepared, the Span 80® and ethanol was added to the PC before heating of the samples commenced. The vesicle suspension was cooled to 4ºC for a minimum of 2 h prior to characterization.

***Table 3.1**: Final composition of the different LN*

Vesicle type	Ingredient	Concentration needed (%)
Liposomes	L-α-phosphatidylcholine	100
Transferosomes	L-α-phosphatidylcholine	85
	Span 80®	15
Ethosomes	L-α-phosphatidylcholine	13.79
	Ethanol	86.21
Transethosomes	L-α-phosphatidylcholine	11.72
	Span 80®	2.07
	Ethanol	86.21

L-α-phosphatidylcholine from egg yolk is a phospholipid that has excellent biocompatibility and amphiphilicity, and was included as the lipid for the LN due to these characteristics [59]. Span 80® is a surfactant utilized for its edge activating properties, causing destabilization of the lipid bilayer and lowering the interfacial tension, and therefore leading to higher elasticity [60]. Ethanol, also known as ethyl alcohol, is a modulator of the lipid bilayer, causing it to be more elastic [60].

2.3. Particle size and zeta potential

Post system formulation and storage at 2-4ºC, the samples were prepared for analysis. Liposomes were used as a control to which the UDV were compared. A wet dispersion was created by dropping a single drop of prepared LN suspension in 5 ml PBS, after which the dispersion was transferred to a disposable Zetasizer cell. Analysis commenced on the Malvern Zetasizer at 25ºC (Malvern Instruments Ltd, Malvern, Worcestershire, UK), which utilizes DLS and the Doppler micro-electrophoresis. To determine the size distribution the data was analyzed by the Malvern Zetasizer software (Malvern Instruments Ltd, Malvern, Worcestershire, UK) that yields the LN size in two parameters, i.e. the intensity (z-average) or the number average. For the purpose of this study the z-average was used. Results were presented in percentile parameter including the D10, D50 and D90, where D50 is an indication that 50% of the particles were smaller than this value. PDI was also determined employing the same software, and utilizing the D50 values of the number-based data, providing an indication of the particle size distribution.

2.4. pH at room temperature

A Mettler Toledo pH meter (Mettler Toledo, Columbus, OH, USA) was implemented to determine the pH of the LN at a specific temperature of 25 ºC.

2.5. Morphology

After the samples had been formulated and stored in the fridge, they were prepared for analysis. The Malvern Morphologi G3 (Malvern Instruments Ltd., Malvern, UK) with its associated software was employed to analyze all the vesicle samples. Each sample was injected into the wet dispersion cell composed of two sheets of glass, which are separated by a spacer that holds the sample in between the glass sheets. After the injection into the wet cell, the sample plate was placed onto the sample carrier where the images of individual vesicles were captured and analyzed by automated scanning. All samples were analyzed in triplicate. A 5x objective with a depth of field (DOF) of 24.44 µm, and a size range of 6.5 µm to 420 µm were selected to conduct the vesicle imaging and light intensity calibration was automatically done before each sample was analyzed [61] (Supplementary material). This objective was selected due to the optimization study's results concerning the vesicles' diameters (between ± 60 d.nm and ± 500 d.nm). The bottom light (diascopic light) was selected to illuminate the samples, according to the SOP for wet samples. All the samples were photographed, after which the individual vesicles could be viewed with the software program. Span was additionally calculated using the following equation, where a higher span value is indicative of a wider particle distribution:

$$Span = \frac{D90 - D10}{D50} \quad [1]$$

The span of the DLS and image analysis was compared, as well as vesicle diameter. The D50 values of the image analysis were used, however, as stated values were recorded for D10, D50 and D90 of the different parameters. These values were analyzed with the aim of creating quantifiable parameters. In addition the influence of storage time on the UDV as a measure of stability was also studied, with the aim to determine whether the parameters changed during storage.

Usually where small sizes of particles need to be detected, a number-based resolution system is implemented, whereas larger particles can be detected using a volume-based system, rendering it more time efficient [57]. When utilizing number-based resolution, each particle contributes to the results to the same extent, whereas the particles in volume-based resolution will contribute only according to their volume [57]. Therefore, based on the small size of the vesicles, number-based resolution was employed.

CE diameter (diameter of an equivalent circle) is defined as the particle diameter of a sphere of equivalent volume [57, 62]. Number distribution, conversely, is the number of particles

within each size class of the total population. According to the Malvern Morphologi G3 user manual [57], the intensity mean is “the average of the pixel greyscale levels in the object.” Number distribution is the number proportion of particles in each size class. The Malvern Morphologi G3 user manual furthermore describes solidity as “the object area divided by the area enclosed by the convex hull” [57]. Elongation levels of zero indicate no elongation is present, whereas levels closer to 1 are indicative of elongated particles [57]. Convexity is used as a measure of the surface roughness of a particle [57], with a value of 1 depicting smoothness and a value closer to 0 depicting an irregular object. According to the Malvern Morphologi G3 user manual, circularity quantifies how close a particle is shaped to a perfect circle or sphere, therefore circularity is a measure to describe deviation from a perfect circle [57]. Lastly, aspect ratio is defined as the ratio of the width to length of the particle, and in the range of 0 to 1, where a more circular particle would have a larger aspect ratio [57].

3. Results

Characterization of LN / UDV with DLS

A summary of the characterization data of the various LN is described in Table 3.2. The D50 values were used during the characterization through the Malvern Zetasizer. The mean vesicle diameter of the liposomes and ethosomes was measured as 930.64 ± 163.50 d.nm and 1002.56 ± 122.70 d.nm respectively. The transferosomes and transethosomes, however, are smaller than 600 nm in diameter, as these mean vesicle diameters were measured as 557.97 ± 51.10 d.nm and 530.33 ± 64.67 d.nm respectively. Larger vesicle sizes were preferred in this study in order to be able to observe the morphology of these vesicles better.

***Table 3.2**: Summary of the characterization data (Mean ± SD) of the different lipid nanocarriers, (n=3)*

Vesicle type	Diameter (d.nm)	PDI	Zeta potential (mV)	pH
Liposomes	930.64±163.50	0.69±0.07	-13.76±4.12	7.52±0.04
Transferosomes	557.97±51.11	0.66±0.16	-10.15±3.15	7.51 ± 8.882^{-16}
Ethosomes	1002.56±122.70	0.72±0.07	-11.89±1.96	7.52±0.03
Transethosomes	530.33±64.67	0.55±0.06	-14.00±1.86	7.52±0.01

The mean PDI of all of the lipid nanocarriers was larger than 0.3, where transethosomes presented with the lowest mean PDI (0.55 ± 0.06) and the ethosomes showed the highest mean value (0.72 ± 0.07). Due to the PDI values being higher than 0.3, it was indicative that all the LN showed a wide particle size distribution, which is undesired. Furthermore, all the

vesicles displayed a negative charge, where liposomes and transethosomes depicted a larger negative charge. Additionally, pH was determined as it may have an influence on zeta potential. The entire LN had portrayed a relatively neutral pH due to the use of PBS as the aqueous phase.

Figure 3.2 illustrates the effects of storage time on the DLS parameters. From Figure 3.2.A (effects of storage on vesicle diameter) it is clear that the size of the liposomes and ethosomes increased over 90 days, whereas the vesicle size of the transferosomes and transethosomes did not change over the course of time. Performing One-way ANOVA analysis, using the Dunnett multiple comparison test, established that the transferosomes and transethosomes differed statistically significantly in vesicle size, when compared to both liposomes and ethosomes ($p \leq 0.05$). Conversely, the PDI of the UDV, as well as the liposomes remained relatively stable over the course of 90 days, as shown in Figure 3.2.B. Again, One-way ANOVA analysis, utilizing Dunnett's multiple comparison test, indicated that statistically significant differences in PDI could be observed when the transferosomes and transethosomes were compared to the liposomes, as well as when the PDI of the transethosomes was compared to that of the ethosomes.

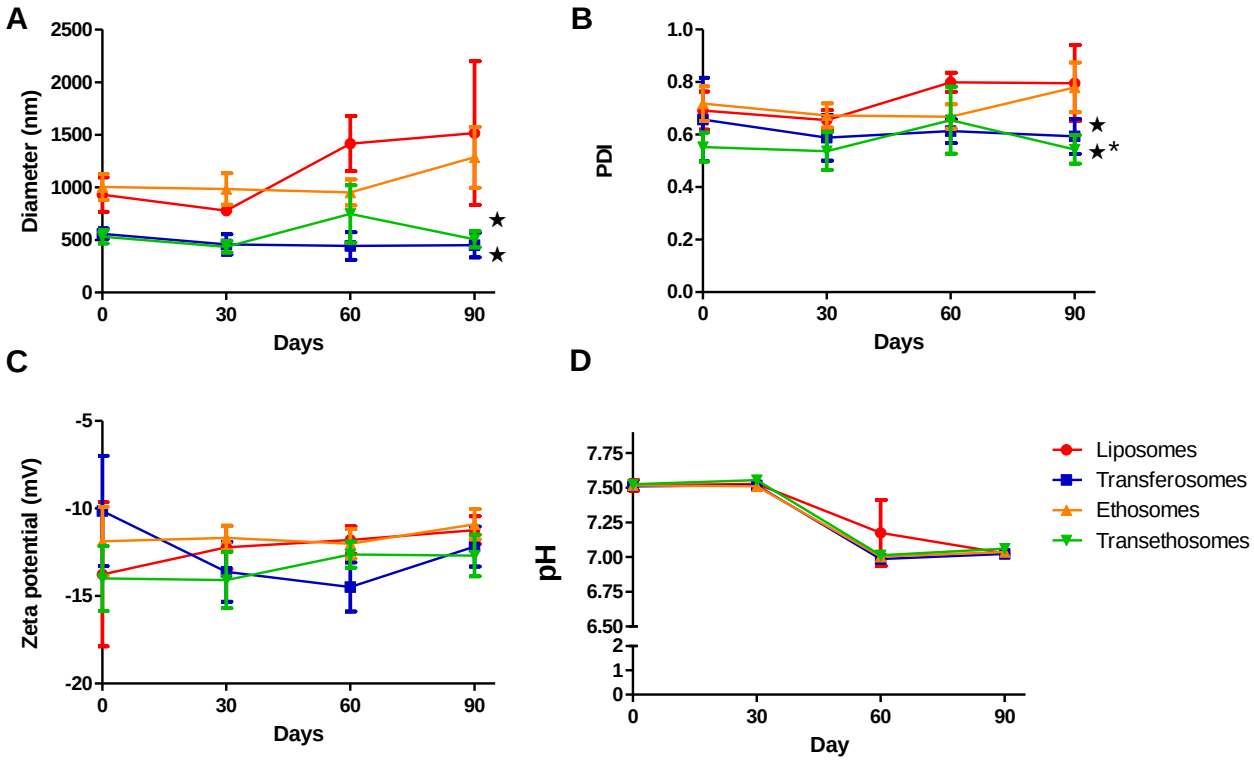


***Figure 3.2**: Stability of the different LN over 90 days by means of comparison of the DLS parameters; A. Diameter (d.nm ± SD), B. Polydispersity index (PDI ± SD), C. Zeta potential (mV ± SD), D. pH ± SD; using the Malvern Zetasizer and Mettler Toledo pH meter; n=3*

Considering the zeta potential of the LN (Figure 3.2.C), it is clear that the zeta potential of the liposomes did not vary notably and remained stable. Only a slight increase over time could be observed. In contrast, the zeta potential of the transferosomes decreased over the course of 60 days, indicating that they became more stable during that time. However, post 60 days, the zeta potential began to increase once more, signifying the vesicles were no longer stable. The zeta potential of the ethosomes and transethosomes followed the same trend as that of the liposomes, where the transethosomes depicted a more desirable zeta potential at the end of the experiment (after 90 days). No statistically significant results were obtained.

In view of pH determination at 25ºC, over 90 days (Figure 3.2.D), it was expected that the pH values would remain stable at approximately 7.5 as the LN were buffered using PBS, so that the LN would not be irritating to the skin. The pH of liposomes remained stable for the first 30 days, after which a steady decrease occurred. However, it never decreased below a value of 7.0, indicating that the PBS buffer was able to maintain a pH in the physiological pH range. All the UDVs portrayed the same trend as the liposomes concerning the pH. The lowest pH value recorded for the UDV, was that of the transferosomes at pH 6.98. No statistically significant differences between the pH values of the LN could be established.

Characterization and stability study using the Malvern Morphologi G3

3.1. Visual examination

Through visual examination of the LN as shown in Figure 3.3, it is clear that all the vesicles had been formed correctly, as they were spherical in shape and contained the aqueous phase (PBS). Results obtained are similar to that of Ascenso *et al.* [15], whom concluded that their transferosomes, ethosomes and transethosomes were fairly similar in terms of size and form. All the vesicles displayed a greyish color, in contrast to the study conducted by Ulusoy and Kursun [63] who observed talc particles. Additionally, during the 90 day study the vesicles remained visually stable, not differing from Figures 3.6, 3.7 and 3.8. (See Supplementary Material). No agglomeration of the vesicles was observed, throughout the course of the study, and no contaminant particles could be detected.

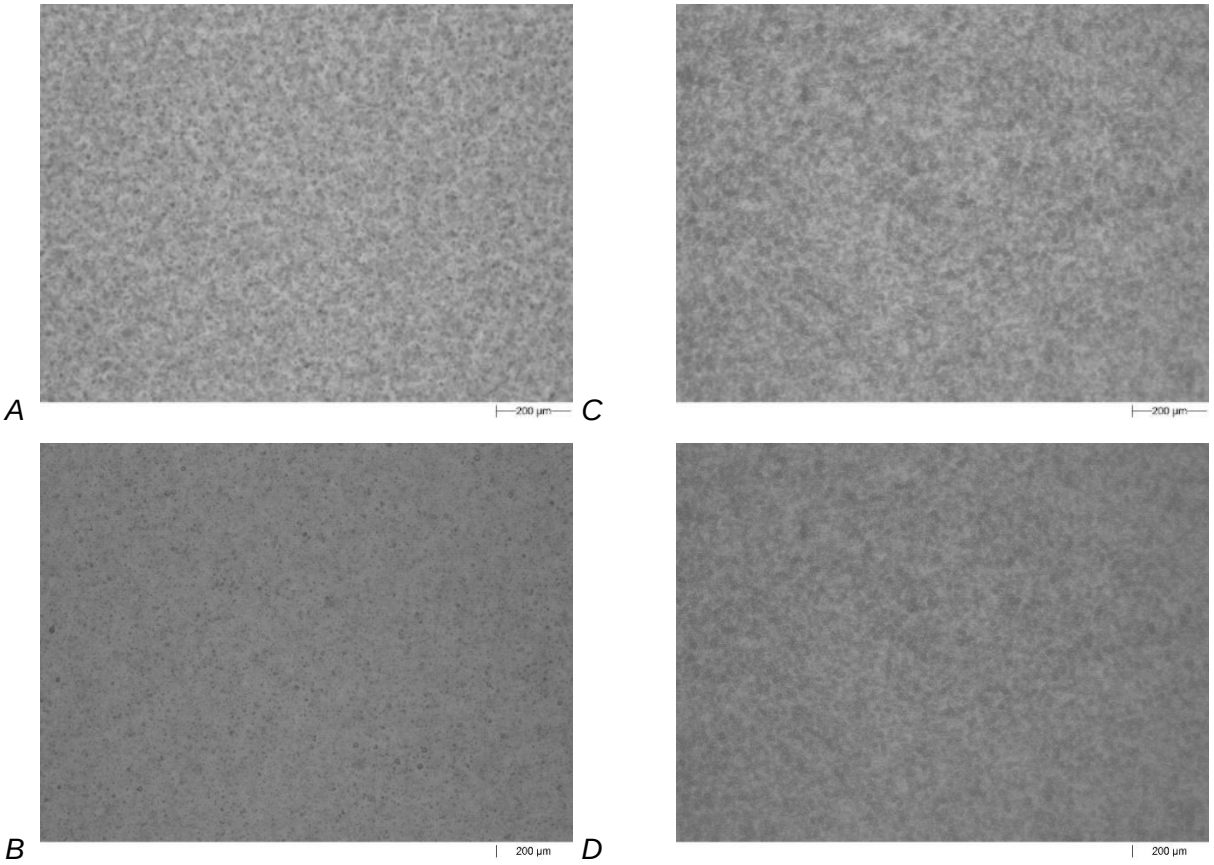


***Figure 3.3**: Visual inspection of formulated lipid nanocarriers using the Malvern Morphologi G3 for investigation of morphology during characterization, portraying that spherical vesicles were formed, where: A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes, n=3*

3.2. Image analysis parameters

As listed in Table 3.3, the CE diameters of the UDVs compared to the CE diameter of the liposomes are relatively similar, excluding the CE diameter of the ethosomes, as this value is slightly higher, indicating a marginally higher size distribution. However, no statistically significant differences could be concluded. Comparing the CE diameter (number distribution) to the particle diameter as determined by the Zetasizer, it is clear that the vesicles were significantly larger than determined by the Zetasizer. Regarding the span of the LN, it was determined that the liposomes, transferosomes, ethosomes and transethosomes depicted span-values of 1.49, 1.01, 2.06 and 1.15 respectively, when analyzed with image analysis, indicating a noteworthy wide particle size distribution. The span of the transferosomes and transethosomes decreased over time, suggesting that the vesicles' size distribution was more favorable, due to the size of the vesicle suspension being more conformed. Over the course of 90 days the vesicles remained stable with no statistical significant increases in CE diameter.

The entire LN had mean intensity values of below the greyscale level, demonstrating that a light grey color could be observed. The liposomes depicted a slightly higher intensity mean, signifying slightly higher transparency. From Table 3.3, it could be established that the UDVs overall showed a slightly lower intensity mean compared to the liposomes. Moreover, the UDVs exhibited extremely similar distribution, and no statistically significant differences could be concluded. All the vesicles had similar intensity means, towards a grey color. The intensity mean of the vesicles remained the same over the course of 90 days, which corresponds with the visual inspection (Supplementary material).

As depicted in Table 3.3, the distribution of solidity among these vesicles was narrow, at a high solidity value. These vesicles are filled with a liquid phase, therefore causing them to be more 'solid' as they are not just empty vesicles. Statistical analysis of these datasets showed no significant differences between the individual distributions of solidity vales, even after the course of the 90 day stability study. Low levels of elongation are observed throughout all the vesicles (≤0.51º± 0.04) as depicted in Table 3.3, which denotes that a higher level of convexity should be present. Elongation of the vesicles decreased over the testing period. Additionally, high levels of convexity were observed (≥0.76 ± 0.07), which is consistent with the lower elongation levels as mentioned previously. High convexity levels also suggest that circularity levels should be high, signifying the vesicles are close to a perfect circular shape. The convexity of the vesicles remained stable over 90 days. Conversely, circularity of the vesicles was varied, with liposomes presenting the lowest circularity number distribution. The ethosomes compare the closest to the liposomes with lower circularity levels, whereas the transferosomes and transethosomes were closely related to each other with higher circularity levels, suggesting they were close to a circular shape. Over the course of 90 days the circularity of the vesicles increased, indicating they became more spherical as time passed. The liposomes and ethosomes revealed the lowest aspect ratio, signifying that these vesicles showed a more elongated shape, whereas the transferosomes and transethosomes exhibited higher aspect ratio, indicating these vesicles were more spherical. The aspect ratio of the LN all remained relatively stable over the course of 90 days (see supplementary material).

***Table 3.3**: Comparing the different parameters of image analysis for the different lipid nanocarriers during characterization; Intensity mean, Greyscale: black = 0; mid-grey = 128; white = 255; CE diameter (µm); Solidity, solid = 1; Elongation, elongated = 1; Convexity, perfectly smooth = 1; Circularity, perfect circle = 1, using the Malvern Morphologi G3, n=3*

	Liposomes	***Transferosomes***	***Ethosomes***	***Transethosomes***
CE Diameter D(n, 0.5) (µm)	3.67±0.59	3.17±0.21	4.04±0.96	3.41±0.48
Mean intensity D[n, 0.5]	102.33±0.47	101.33±1.63	101.33±0.47	101.33±0.47
Solidity D[n, 0.5]	0.87±0.05	0.92±0.00	0.88±0.01	0.91±0.01
Elongation D[n, 0.5]	0.32±0.04	0.25±0.01	0.32±0.02	0.27±0.01
Convexity D[n, 0.5]	0.92±0.03	0.96±0.01	0.93±0.02	0.95±0.01
Circularity D[n, 0.5]	0.51±0.14	0.73±0.03	0.57±0.04	0.68±0.05
Aspect ratio D[n, 0.5]	0.68±0.04	0.74±0.01	0.59±0.04	0.72±0.01

4. Discussion

Liposomes and the selected UDVs were firstly prepared and optimized, after which the physical and morphological characteristics of the transferosomes, ethosomes and transethosomes were compared to the liposomes. Characterization of the different LN was conducted by means of DLS. The liposomes were found to be within the literature described range of between 20 nm to as large as a few micrometers [53, 64-66]. Additionally, the transferosomes and ethosomes presented with mean diameter values of 1002.56 nm (±122.70) and 557.97 nm (±51.11), respectively, which indicated these LN results were in concurrence with literature [15, 18, 21, 33, 67]. However, the transethosomes (530.33 nm ±64.67) obtained during this study may be considered relatively larger when related to previous studies, as literature indicated the size of transethosomes to vary between 40 and 200 nm [68]. Particles larger than 600 nm in diameter are normally not able to cross the skin barrier, therefore signifying that the prepared ethosomes and liposomes were more likely in the micro range, and would probably not be functional for transdermal drug delivery [48]. Nonetheless, the transferosomes and transethosomes will probably be able to penetrate the skin for transdermal drug delivery. In addition, the PDI range for the all the vesicles was measured as between 0.55 ± 0.06 and 0.72 ± 0.07, with transferosomes portraying a PDI-value closest to that of liposomes. This PDI-range suggests that all the vesicles possess a relatively wide size distribution, declaring the LN vesicles as having non-uniform vesicle sizes.

During stability testing it became evident the liposomes varied in size and were thus regarded as unstable over the course of the testing period as an increase in the PDI could be observed. Overall, although the PDI of the transferosomes indicated a wide size distribution, these vesicles could be considered stable as no statistical significant differences could be seen for both PDI and vesicle size over time. The ethosomes displayed relative stability up until 60 days whereafter the PDI and diameter values increased notably. Transethosomes which depicted the smallest mean diameter post preparation, proved the most stable overall during stability testing as no significant change in size occurred and the PDI remained within the same starting range. In general, when comparing the results derived from this study to other research where different DLS methods, such as the NanoBrook 90 plus PALS (Brookhaven Instruments Corporation, Long Island, NY, USA) and Malvern Nano-S (Malvern Instruments, Malvern, UK), were utilized to measure vesicle size and PDI, it is clear the liposomes, transferosomes and ethosomes from this study portrayed remarkably larger vesicle sizes and PDI-values [36, 69-71]. Conversely, transethosomes exhibited a similar vesicle diameter, but a slightly elevated PDI when compared to a study conducted by Rady *et al.* [72] in 2018.

When the zeta potential of the liposomes was determined, it remained stable, with only a slight increase over the 90 day timeframe, suggesting that liposomes are a suitable choice when a topical product needs a longer shelf life. In comparison to liposomes, transferosomes were initially unstable, with regard to zeta potential, but they stabilized more over time. Only after 60 days did the small increase in zeta potential show that the transferosomes become more unstable over time. The ethosomes, as well as the transethosomes, behaved most like the liposomes over the 90 day study, whereas the transethosomes exhibited a more desirable zeta potential than both the liposomes and the ethosomes. A varied range of the zeta potential of liposomes was observed when compared to other studies, depending on the exact composition of the liposomes, however, when compared to PC only liposomes, they tended to have a more unfavorable zeta potential [73-76]. The zeta potential of the UDV also showed more favorable in other studies with instruments such as the Malvern Nano-S and Malvern Nano ZS (Malvern Instruments, Malvern, UK) [36, 70-72]. The pH of the LN systems was buffered with PBS, thus ensuring a stable neutral pH throughout the study, due to the use of these carriers on the human skin.

Following characterization by means of DLS, image analysis utilizing the Malvern Morphologi G3 instrument, was subsequently employed to characterize the LN further. The general use of the Morphologi G3 has previously been focused more on powder particle shape and characterization, than suspensions and emulsions. The Malvern Morphologi G3 previously focused more on powder particles such as cement [77] or talc [63] which are irregular in shape,

with high intensity mean values, varied elongation, circularity and convexity. In contrast, this study focused more on the different parameters in characterizing lipid nanocarriers, which are a nanoemulsion, more than a suspension.

Through visual inspection, it was concluded that all of the lipid nanocarriers formed spherical vesicles that contained an aqueous phase (PBS). Throughout the study it was observed that the vesicles still had the spherical form and that it did not degrade after the 90 days stability testing period. No agglomeration occurred throughout the study. Visual inspection by means of the micrograph images, however, only proved successful in determining whether the vesicles were formed, and whether agglomeration transpired. A possible reason for this could be that the focus of the study was more on the parameters rather than the visual imaging.

After visual inspection, the lipid nanocarriers were inspected to determine their intensity mean, CE diameter, solidity, elongation, convexity and circularity throughout the 90 days. The intensity mean of the liposomes showed a slight decrease over time, indicating the liposomes had darkened slightly. Moreover, the intensity mean of the UDVs could be related to that of the liposomes, over the course of the 90 day study, where the transethosomes displayed the lowest intensity mean, which is an indication of them being the darkest in color.

The CE diameter of the liposomes and UDVs remained stable over the course of the study, and the span-values obtained for all the LN indicated a wide particle size distribution for the liposomes, as well as the UDVs, although the transferosomes and transethosomes showed a size distribution that was narrower compared to the liposomes and ethosomes.

Overall, the liposomes and UDVs portrayed high solidity values, due to the vesicles being filled with the liquid phase. Over the course of the stability study, these values remained fairly stable, only showing a slight increase. When compared to powder particles, the UDVs and liposomes showed lower solidity, whereas the powder particles showed extremely high solidity values, due to them being solid [78].

Regarding the elongation values attained, it was clear that both the liposomes and UDVs displayed relatively low levels. Over the course of 90 days, the elongation levels fluctuated only slightly, which could be due to the membranes of the vesicles that are malleable and elastic. Comparing these values to previous powder characterization studies, it was established that the lipid nanocarrier values were lower than the values obtained for powder particles as studied previously by Ulusoy and Kursun [63] and Di Pretoro *et al.* [79]. Due to the low elongation levels, it is suggested that the vesicles should, therefore, show higher convexity levels. This was confirmed by observing high convexity for all the LN, and these

values remained relatively unchanged over the course of the study. Powder characterization by Ulusoy and Kursun [63] and Di Pretoro *et al.* [79] showed lower convexity levels than those of the LN.

Although it is suggested that, if the convexity of the vesicles are high, the circularity levels of the vesicles should also be high, this was not observed during the study. The liposomes and ethosomes compared similar to one another, as did the transferosomes and transethosomes, with the latter showing higher circularity than the first. Over the course of the 90 day study, the circularity of the vesicles remained stable. Powder characterization studies indicated varied results, depending on the material the powder consisted of [45, 63, 79]. The vesicles were observed to be more circular and less irregularly shaped than the powder particles.

The aspect ratio of the liposomes remained stable over the course of the study, and the UDV compared similar to the liposomes, with only slightly more elevated aspect ratio values. When compared to powder characterization studies, the powders showed lower aspect ratio values [63, 79].

Due to the Malvern Morphologi G3 using image analysis to characterize the lipid nanocarriers quantitatively, the data was compared to other methods of image analysis. Li *et al.* [80] used cryo-transmission electron microscopy (cryo-TEM) to analyze liposomes qualitatively. Li *et al.*'s study mainly focused on the drug within the vesicle and not the vesicle itself. A study conducted by Azzi *et al.* [81] used atomic force microscopy in order to analyze the rigidity of blank liposomes; they also observed the liposomes' shape and size remained stable after UV exposure. Caddeo *et al.* [70] performed a study in 2018 on anti-oxidant activity of transferosomes and similarly used cryo-TEM analysis to determine the shape and size of the transferosomes. In this study, it could be concluded that the transferosomes prepared were also spherical in shape, but the size of the vesicles differed remarkably. In this study, we focused on characterizing these vesicles by observing the shape through image analysis, and not on the vesicle size. Image analysis of the ethosomes, as well as transethosomes, were compared to other studies, by Xie *et al.* [82] and Chen *et al.* [56], respectively. Both research entities used transmission electron microscopy (TEM) to determine basic vesicle shape, size and dispersion. All the vesicles produced in both studies, were a spherical shape, and the vesicle sizes were larger than expected, although Xie *et al.*'s ethosomes were smaller than in our study.

It can therefore be concluded that the different UDVs depicted relative stability over the course of 90 days, when compared to liposomes; with ethosomes having the most similar results compared to the liposomes. The transferosomes and transethosomes, however, showed more pronounced stability over time.

Neither DLS nor image analysis provides a perfect solution for the characterization of UDVs, however, a combination of both resulted in greater comprehensive characterization. The use of the Malvern Morphologi G3 to characterize and determine the stability of vesicles is still a new field; it could, however, be used as a complementary means of stability and characterization determination, when parameters such as elongation, convexity and circularity are taken into account. These parameters offer a better understanding on the spherical shape of the vesicles, as well as the 'smoothness' of the surface of the vesicles. Conversely, intensity mean, CE diameter, and solidity do not contribute enough information to the characterization of the vesicles that the DLS methods do not already determine.

5. Acknowledgements

This work is based on the research supported by wholly/sin part by the National Research Foundation of South Africa (NRF Innovation Master's scholarship, Ref.: SFH160719179397); and opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research is that of the author(s) alone, and that the NRF accepts no liability whatsoever in this regard.

ORCID ID
https://orcid.org/0000-0002-3177-5904

6. References

1. Alexander, A., *et al.*, Recent expansion of pharmaceutical nanotechnologies and targeting strategies in the field of phytopharmaceuticals for the delivery of herbal extracts and bioactives. *Journal of controlled release*, 2016. **241**: p. 110-124.
2. Kumar, L., *et al.*, Fatty acid vesicles acting as expanding horizon for transdermal delivery. *Artificial cells, nanomedicine, and biotechnology*, 2017. **45**(2): p. 251-260.
3. Neubert, R.H.H., Potentials of new nanocarriers for dermal and transdermal drug delivery. *European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
4. Cevc, G., Lipid vesicles and other colloids as drug carriers on the skin. *Advanced drug delivery reviews*, 2004. **56**(5): p. 675-711.
5. Sharma, J., *et al.*, Colloidal drug carriers. *The internet journal of family practice*, 2009. **9**(2).
6. Honeywell-Nguyen, P.L. and J.A. Bouwstra, Vesicles as a tool for transdermal and dermal delivery. *Drug discovery today: Technologies*, 2005. **2**(1): p. 67-74.

7. Priyanka, R. and D. Shipra, Transferosomes - a novel carrier for transdermal drug delivery system. *International journal of pharmacy & technology*, 2012. **4**(1): p. 1854-1865.
8. Vinod, K.R., *et al.*, Critical issues related to transferosomes – novel vesicular system. *Acta scientiarum polonorum - Technologia alimentaria*, 2012. **11**(1): p. 67-82.
9. Akhtar, N., Vesicles: A recently developed novel carrier for enhanced topical drug delivery. *Current drug delivery*, 2014. **11**(1): p. 87-97.
10. Cevc, G. and U. Vierl, Nanotechnology and the transdermal route: A state of the art review and critical appraisal. *Journal of controlled release*, 2010. **141**(3): p. 277-299.
11. Romero, E.L. and M.J. Morilla, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2013. **8**: p. 3171-3186.
12. Benson, H.A.E., Transdermal drug delivery- penetration enhancement techniques. *Current drug delivery*, 2005. **2**(1): p. 23-33.
13. Benson, H.A., Transfersomes for transdermal drug delivery. *Expert opinion in drug delivery*, 2006. **3**(6): p. 727-737.
14. Ghanbarzadeh, S. and S. Arami, Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. *BioMed Research International*, 2013. **2013**: p. 1-7.
15. Ascenso, A., *et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
16. Manosroi, A., *et al.*, Novel elastic nanovesicles for cosmeceutical and pharmaceutical applications. *Chiang Mai journal of science*, 2009. **36**(2): p. 168-178.
17. Cevc, G., *et al.*, The skin: A pathway for systemic treatment with patches and lipid-based agent carriers. *Advanced drug delivery reviews*, 1996. **18**: p. 346-378.
18. Cortesi, R., *et al.*, Liposomes- and ethosomes-associated distamycins: A comparative study. *Journal of liposome research*, 2010. **20**(4): p. 277-285.
19. Vermuri, S. and C.T. Rhodes, Preparation and characterization of liposomes as therapeutic delivery systems: A review. *Pharmaceutica Acta Helvetiae*, 1995. **70**: p. 95-111.
20. Mezei, M. and V. Gulasekharam, Liposomes - a selective drug delivery system for the topical route of administration. I. Lotion dosage form. *Life sciences*, 1980. **26**: p. 1473-1477.
21. Touitou, E., *et al.*, Ethosomes — novel vesicular carriers for enhanced delivery characterization and skin penetration properties. *Journal of controlled release*, 2000. **65**: p. 403-418.

22. Song, C.K., *et al.*, A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation. *Colloids and Surfaces B: Biointerfaces*, 2012. **92**: p. 299-304.
23. Ascenso, A., *et al.*, In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. *European journal of pharmaceutics and biopharmaceutics*, 2014. **88**(1): p. 48-55.
24. Cevc, G. and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochimica et Biophysica Acta*, 1992. **1104**: p. 226-232.
25. Zheng, W.S., *et al.*, Preparation and quality assessment of itraconazole transfersomes. *International journal of pharmaceutics*, 2012. **436**(1-2): p. 291-298.
26. Cevc, G., Transdermal drug delivery of insulin with ultradeformable carriers. *Clinical pharmacokinetics*, 2003. **41**(5): p. 461-474.
27. Duangjit, S., *et al.*, Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes. *Journal of drug delivery*, 2011. **2011**: p. 1-9.
28. El Zaafarany, G.M., *et al.*, Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. *International journal of pharmaceutics*, 2010. **397**(1-2): p. 164-172.
29. Touitou, E., B. Godin, and C. Weiss, Enhanced delivery of drugs into and across the skin by ethosomal carriers. *Drug development research*, 2000. **50**: p. 406-415.
30. Elsayed, M.M., *et al.*, Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. *International journal of pharmaceutics*, 2007. **332**(1-2): p. 1-16.
31. Verma, P. and K. Pathak, Therapeutic and cosmeceutical potential of ethosomes: An overview. *Journal of advanced pharmaceutical technology & research*, 2010. **1**(3): p. 274-282.
32. Thadanki, M. and A.K. Babu, Review on ethosomes: A novel approach of liposomes. *International journal of pharmacy & life sciences*, 2015. **6**(1): p. 4171-4176.
33. Rakesh, R. and K.R. Anoop, Ethosomes for transdermal and topical drug delivery. *International journal of pharmacy & pharmaceutical sciences*, 2012. **4**(3): p. 17-24.
34. Paolino, D., *et al.*, Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers. *Journal of controlled release*, 2005. **106**(1-2): p. 99-110.
35. Jain, S., *et al.*, Ethosomes: A novel vesicular carrier for enhanced transdermal delivery of an anti-HIV agent. *Indian journal of pharmaceutical sciences*, 2004. **66**(1): p. 72-81.
36. Marto, J., *et al.*, Ethosomes for enhanced skin delivery of griseofulvin. *Colloids and Surfaces B: Biointerfaces*, 2016. **146**: p. 616-23.

37. Bagde, A., A. Mondal, and M. Singh, Drug delivery strategies for chemoprevention of uvb-induced skin cancer: A review*. Photodermatology, photoimmunology & photomedicine*, 2018. **34**(1): p. 60-68.
38. Shaji, J. and S. Garude, Ultradeformable vesicles for enhanced transdermal delivery*. Indo American journal of pharmaceutical research*, 2014. **4**(8): p. 3588-3596.
39. Shaji, J. and S. Garude, Development and characterisation of ketorolac tromethamine encapsulated transethosomes for enhanced transdermal development*. World journal of pharmacy and pharmaceutical sciences*, 2014. **3**(9): p. 1213-1227.
40. Abdulbaqi, I.M.*, et al.*, Ethosomal nanocarriers: The impact of constituents and formulation techniques on ethosomal properties, in vivo studies, and clinical trials*. International journal of nanomedicine*, 2016. **11**: p. 2279-2304.
41. Mandal, A., Characterization of nanoparticles, C.-G. A., Editor. 2018.
42. Jindal, A.B., The effect of particle shape on cellular interaction and drug delivery applications of micro- and nanoparticles*. International journal of pharmaceutics*, 2017. **532**(1): p. 450-465.
43. Peltonen, L., Practical guidelines for the characterization and quality control of pure drug nanoparticles and nano-cocrystals in the pharmaceutical industry*. Advanced drug delivery reviews*, 2018. **131**: p. 101-115.
44. Cardona, J.*, et al.*, Image analysis framework with focus evaluation for in situ characterisation of particle size and shape attributes*. Chemical engineering science*, 2018. **191**: p. 208-231.
45. Krupa, A.*, et al.*, Preparation of solid self-emulsifying drug delivery systems using magnesium aluminometasilicates and fluid-bed coating process*. Powder technology*, 2014. **266**: p. 329-339.
46. Berne, B. and R. Pecora, Dynamic light scattering*. 1976.* USA, New York, 2000.
47. Malvern Instruments Limited, Malvern zetasizer ZS DLS user manual, in *Size Theory*. 2013, Malvern Instruments Ltd.: Malvern, Worcestershire, UK.
48. Danaei, M.*, et al.*, Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems*. Pharmaceutics*, 2018. **10**(57).
49. Stepto, R.F.T., Dispersity in polymer science (IUPAC recommendations 2009)*. Pure and applied chemistry*, 2009. **81**(2): p. 351-353.
50. Kirby, B.J. and E.F. Hasselbrink, *Z*eta potential of microfluidic substrates: 1. Theory, experimental techniques, and effects on separations*. Electrophoresis*, 2004. **25**: p. 187-202.
51. Kong, M. and H.J. Park, Stability investigation of hyaluronic acid based nanoemulsion and its potential as transdermal carrier*. Carbohydrate polymers*, 2011. **83**(3): p. 1303-1310.

52. Malvern Instruments Limited, Discriminating between polymorphs of acetaminophen using morphology-driected raman spectroscopy (MDRS), M.I. Ltd, Editor. 2015: Malvern, Worcestershire, UK.
53. Abdellatif, A.A. and H.M. Tawfeek, Transfersomal nanoparticles for enhanced transdermal delivery of clindamycin*. AAPS PharmSciTech*, 2016. **17**(5): p. 1067-1074.
54. Dayan, N. and E. Touitou, Carriers for skin delivery of trihexyphenidyl HCl: Ethosomes vs. Liposomes*. Biomaterials*, 2000. **21**: p. 1879-1885.
55. Nooli, M.*, et al.*, Solid lipid nanoparticles as vesicles for oral delivery of olmesartan medoxomil: Formulation, optimization and in vivo evaluation*. Drug development and industrial pharmacy*, 2017. **43**(4): p. 611-617.
56. Chen, Z.X.*, et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers*. European journal of pharmaceutical science*s, 2017. **99**: p. 240-245.
57. Malvern Instruments Limited, *Morphologi G3 user manual*, M.I. Limited, Editor. 2015, Malvern Instruments Limited: Malvern, Worcestershire, UK.
58. Duffy, J. Analytical tools for characterizting metal powders for additive manufacturing. 2016 [cited 2018 2018/10/24]; Available from: https://www.materials-talks.com/blog/2016/11/24/analytical-tools-for-characterizing-metal-powders-for-additive-manufacturing/.
59. Li, J.*, et al.*, A review on phospholipids and their main applications in drug delivery systems*. Asian Journal of pharmaceutical sciences*, 2015. **10**(2): p. 81-98.
60. Singh, S.*, et al.*, The role of surfactants in the formulation of elastic liposomal gels containing a synthetic opioid analgesic*. International journal of nanomedicine*, 2016. **11**: p. 1475-82.
61. Gamble, J.F.*, et al.*, Application of imaging based tools for the characterisation of hollow spray dried amorphous dispersion particles*. International journal of pharmaceutics*, 2014. **465**(1-2): p. 210-217.
62. Bourcier, D.*, et al.*, Influence of particle size and shape properties on cake resistance and compressibility during pressure filtration*. Chemical engineering science*, 2016. **144**: p. 176-187.
63. Ulusoy, U. and I. Kursun, Comparison of different 2D image analysis measurement techniques for the shape of talc particles produced by different media milling*. Minerals engineering*, 2011. **24**(2): p. 91-97.
64. Bangham, A.D., M.M. Standish, and J.C. Watkins, Diffusion of univalent ions accross the lamellae of swollen phospholipids*. Journal of molecular biology*, 1965. **13**: p. 238-252.

65. Pierre, M.B., M. Dos Santos, and I. Costa, Liposomal systems as drug delivery vehicles for dermal and transdermal applications. *Archives of dermatological research*, 2011. **303**(9): p. 607-621.
66. Sharma, A. and U.S. Sharma, Liposomes in drug delivery: Progress and limitations. *International journal of pharmaceutics*, 1997. **154**: p. 123-140.
67. Chauhan, N., K. Kumar, and N.C. Pant, An updated review on transfersomes: A novel vesicular system for transdermal drug delivery. *Universal journal of pharmaceutical research*, 2017. **2**(4): p. 49-52.
68. Shaji, J. and R. Bajaj, Transethosomes: A new prospect for enhanced transdermal delivery. *International journal of pharmaceutical sciences and research*, 2018. **9**(7): p. 2681-2685.
69. Alvarez-Roman, R.*, et al.*, Skin penetration and distribution of polymeric nanoparticles. *Journal of controlled release*, 2004. **99**(1): p. 53-62.
70. Caddeo, C.*, et al.*, Tocopherol-loaded transfersomes: In vitro antioxidant activity and efficacy in skin regeneration. *International journal of pharmaceutics*, 2018. **551**(1-2): p. 34-41.
71. Joshi, A.*, et al.*, In-vitro and ex-vivo evaluation of raloxifene hydrochloride delivery using nano-transfersome based formulations. *Journal of drug delivery science and technology*, 2018. **45**: p. 151-158.
72. Rady, M.*, et al.*, Dermal delivery of fe-chlorophyllin via ultradeformable nanovesicles for photodynamic therapy in melanoma animal model. *International journal of pharmaceutics*, 2018. **548**(1): p. 480-490.
73. Zhou, F.*, et al.*, Chitosan-coated liposomes as delivery systems for improving the stability and oral bioavailability of acteoside. *Food hydrocolloids*, 2018. **83**: p. 17-24.
74. Lu, M.*, et al.*, Comparison of exosome-mimicking liposomes with conventional liposomes for intracellular delivery of sirna. *International journal of pharmaceutics*, 2018. **550**(1-2): p. 100-113.
75. Varenne, F.*, et al.*, Application of validated protocols to characterize size and zeta potential of dispersed materials using light scattering methods. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2018.
76. Alemán, A.*, et al.*, Encapsulation of antioxidant sea fennel (crithmum maritimum) aqueous and ethanolic extracts in freeze-dried soy phosphatidylcholine liposomes. *Food research international*, 2018.
77. Yanrong, Z.*, et al.*, Characterization of the mesostructural organization of cement particles in fresh cement paste. *Construction and building materials*, 2016. **124**: p. 1038-1050.

78. Liu, E.J., K.V. Cashman, and A.C. Rust, Optimising shape analysis to quantify volcanic ash morphology. *GeoResJ*, 2015. **8**: p. 14-30.
79. Di Pretoro, G.*, et al.*, Impact of needle-like crystals on wet and solid-lipid extrusion processes. *Powder technology*, 2015. **270**: p. 476-483.
80. Li, T.*, et al.*, Drug nanocrystallisation within liposomes. *Journal of controlled release*, 2018. **288**: p. 96-110.
81. Azzi, J.*, et al.*, Novel findings for quercetin encapsulation and preservation with cyclodextrins, liposomes, and drug-in-cyclodextrin-in-liposomes. *Food hydrocolloids*, 2018. **81**: p. 328-340.
82. Xie, J.*, et al.*, Hyaluronic acid-containing ethosomes as a potential carrier for transdermal drug delivery. *Colloids and Surfaces B: Biointerfaces*, 2018. **172**: p. 323-329.

Supplementary material

In this study, the formulation of the lipid nanocarriers (LN) was adapted and optimized, to enable a systematic approach for characterization of the carriers. A combined approach of dynamic light scattering (DLS) and image analysis was implemented. The aim was to obtain LN that complied with criteria for topical or transdermal delivery of drugs.

1.1. Optimization of lipid nanocarriers

Figure 3.4 shows an experimental outline explaining the formulation and characterization of the LN.

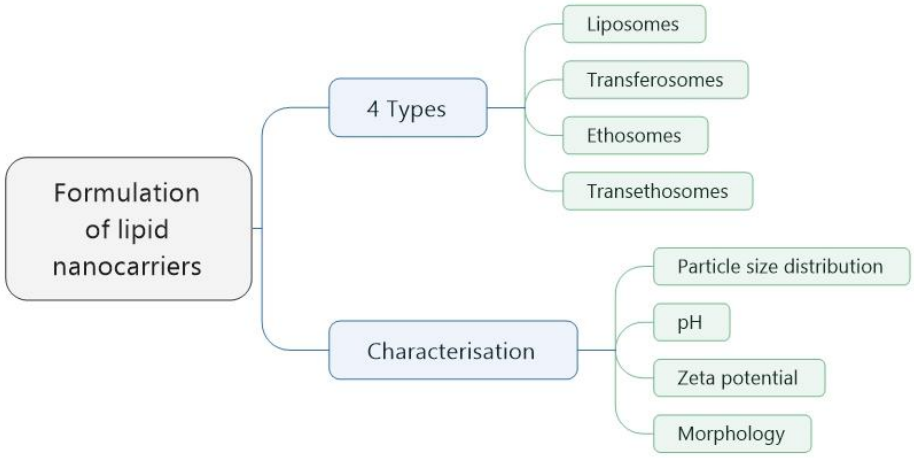


***Figure 3.4**: Experimental outline of formulation and characterization of LN*

1.1.1. Preparation of liposomes for initial characterization

Formulation was done at room temperature that was controlled at 25ºC at all times. The method for formulation of liposomes was adapted from Ascenso *et al.* [1]. L-α-phosphatidylcholine (PC) from egg yolk was used to create the vesicles with cholesterol from sheeps-wool that served as an edge activator for the liposomes. The PC and cholesterol was dissolved in a 2:1 chloroform:methanol mixture, after which the solution was heated to 40 ºC, using a heat plate. The beaker was rotated constantly until a film was left on the bottom of the beaker. The aqueous phase, distilled water, was then added. Glass beads were introduced to the beaker and the mixture was placed into an ultrasonic bath (Camlab Transsonic Bath TS540, Camlab, Cambridge, UK), where after the mixture was sonicated using the Hielscher UP200st Ultrasonic Processor ® (Hielscher, Teltow, Germany). The beaker was covered with Parafilm ® and placed in a fridge at 4 ºC for two hours.

The vesicles were formulated and characterized using DLS to determine particle size, polydispersity index and zeta potential using a Malvern Zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK). In addition, pH was determined as it could play an important role in the zeta potential of the LN. The basic characterization comprised of visual inspection of the vesicles under a light microscope (Zeiss Axiovert 25m, Carl Zeiss AG, Oberkochen, Germany), using the 10x objective, to determine whether emulsion formation had occurred.

1.1.2. Preparation of ultradeformable vesicles for initial characterization

The method described above was followed identically to formulate 20 ml the ultra-deformable vesicles, using Table 3.3 below for the composition of each lipid nanocarrier Other than the PC used, Span 80® and ethanol were used to prepare the UDV. Span 80® is a surfactant used for its edge activating properties, causing destabilization of the lipid bilayer and lowering the interfacial tension, and therefore leading to a higher elasticity [2]. Ethanol, also known as ethyl alcohol, is a modulator of the lipid bilayer, causing it to be more elastic. The Span 80® and ethanol required in the UDV, were added to the PC before it was heated

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) to determine whether statistically significant results were obtained.

1.1.3. Results

The vesicles were all inspected visually to assess whether the formation of vesicles occurred. The liposomes did not show vesicle formation, they showed clusters of crystals; it was speculated that the cholesterol was the cause of these crystals. It was hypothesized upon further inspection, that the liposomes would show undesirable results, therefore, the liposome formula should be adapted. Upon visual inspection of the UDV, it was determined that were formed successfully.

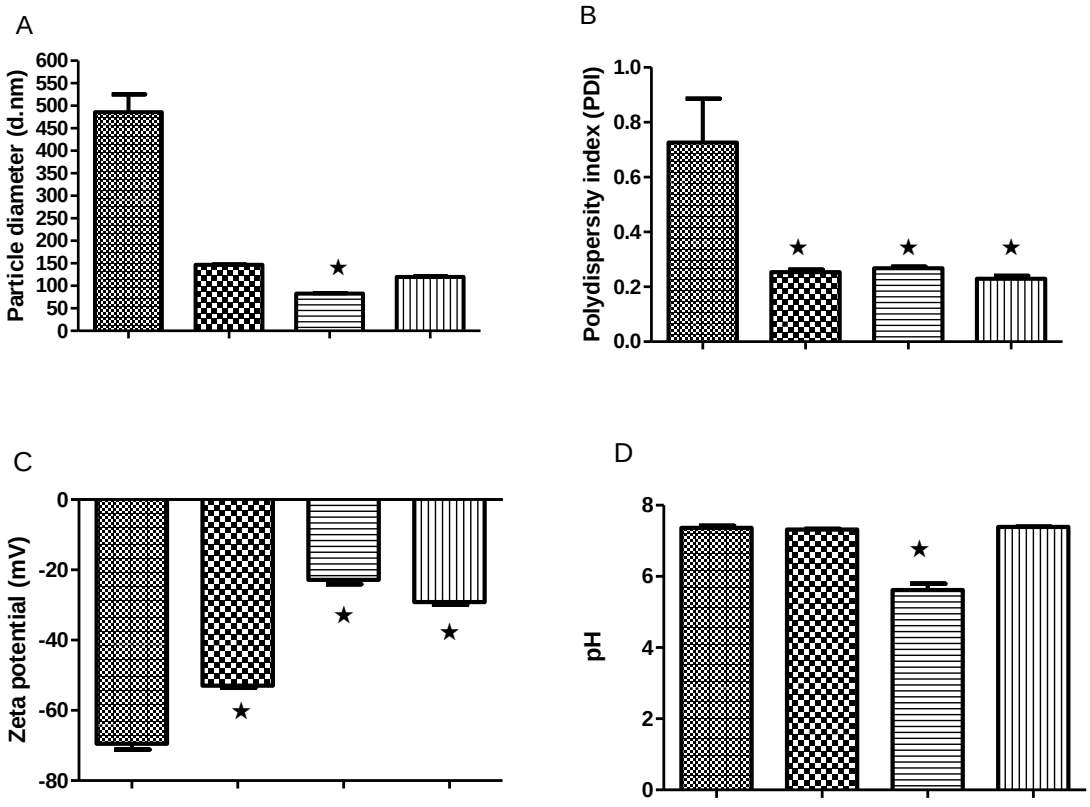
As shown in Figure 3.5.A the lipid nanocarriers were all smaller than 600 nm in diameter, indicating that they would be well suited for drug delivery into the skin. However, the much larger particle size of the liposomes could be attributed to the presence of the crystals in the formulation. The use of Span 80® and ethanol caused the UDV membranes to become more malleable. The ethosomes were statistically significantly smaller when compared to liposomes ($p \leq 0.05$, $n=3$).

The liposomes had the largest polydispersity index of 0.73 ± 0.15 as shown in Figure 3.5.B, indicating it had a wide particle size distribution. A reason for this could be the crystallization of some of the cholesterol in the formulation. It was therefore recommended that the formulation for liposomes be adjusted to prevent crystallization from occurring during the study. The transferosomes, ethosomes and transethosomes had PDIs of 0.254 ± 0.01, 0.278 ± 0.01 and

0.229 ± 0.01 respectively, lower than the limit set. The UDV differed statistically significant from the liposomes (p≤0.05, n=3).

The desired Zeta potential for emulsions for optimal stability is < -25 mV. The mean Zeta potential for the liposomes, transferosomes, ethosomes and transethosomes were -69.53 ± 7.40 mV, -52.97 ± 9.56 mV, -22.80 ± 5.71 mV, -29.17 ± 5.06 mV respectively, as shown in Figure 3.5.C. This indicates that the liposomes, transferosomes and transethosomes had acceptable stability. The ethosomes were less stable, but were in the acceptable range. The UDV differed statistically significantly from the liposomes (p≤0.05, n=3).

When optimization was conducted, the vesicles were prepared using distilled water as the aqueous phase. This caused the pH of the ethosomes to be lower than the desired physiological pH, as shown in Figure 3.5.D. The pH of the liposomes, ethosomes and transethosomes had a neutral pH. As these LN were formulated as transdermal carriers, a neutral pH was very important not to harm the skin cells, and phosphate buffered saline (PBS) was opted as the new aqueous phase, with a pH of 7.4. The pH of the ethosomes had statistically significant differences to the pH of the liposomes.



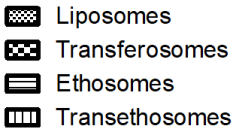


***Figure 3.5**: DLS parameters of the different LN during formulation optimization, **A**. Particle size (d.nm), **B**. Polydispersity index (PDI); **C**. Zeta potential (mV); **D**. pH at room temperature (25ºC) of the formulations characterized with Mettler Toledo pH meter, Results are presented as Mean ± SD (n=3)*

** Indicates a statistical significant difference from liposomes (p≤0.05)*

In conclusion, the liposomes were not acceptable (large PDI) and the formula needed improvement. In order to accurately determine the morphology and shape of the vesicles with image analysis, it was deemed important to have significantly larger particles.

1.2. Stability of the lipid nanocarriers by image analysis

1.2.1. Image analysis using the Malvern Morphologi G3

The Malvern Morphologi G3 has five objectives through which images could be acquired. As mentioned in the article, each objective has a depth of field (DOF), which is the depth at which the vesicles observed appeared sharp. Table 3.4 provides a guide through which the correct objective could be selected, depending on the particle size.

***Table 3.4**: Size ranges of each objective of the Malvern Morphologi G3 (Malvern Instruments Limited [3])*

Objective	Size range(μm)
50x	0.5-40
20x	1.8-100
10x	3.5-210
5x	6.5-420
2.5x	13-1000

1.2.1.1. Visual inspection

As mentioned in the article, the visual observation of the lipid nanocarriers remained mainly the same, suggesting that degradation had not occurred. As shown in Figure 3.6, the liposomes remained mainly the same, in terms of size range, on Day 30. The transferosomes, ethosomes and transethosomes also remained the same, indicating a relatively narrow size range with the transferosomes showing small sized vesicles.

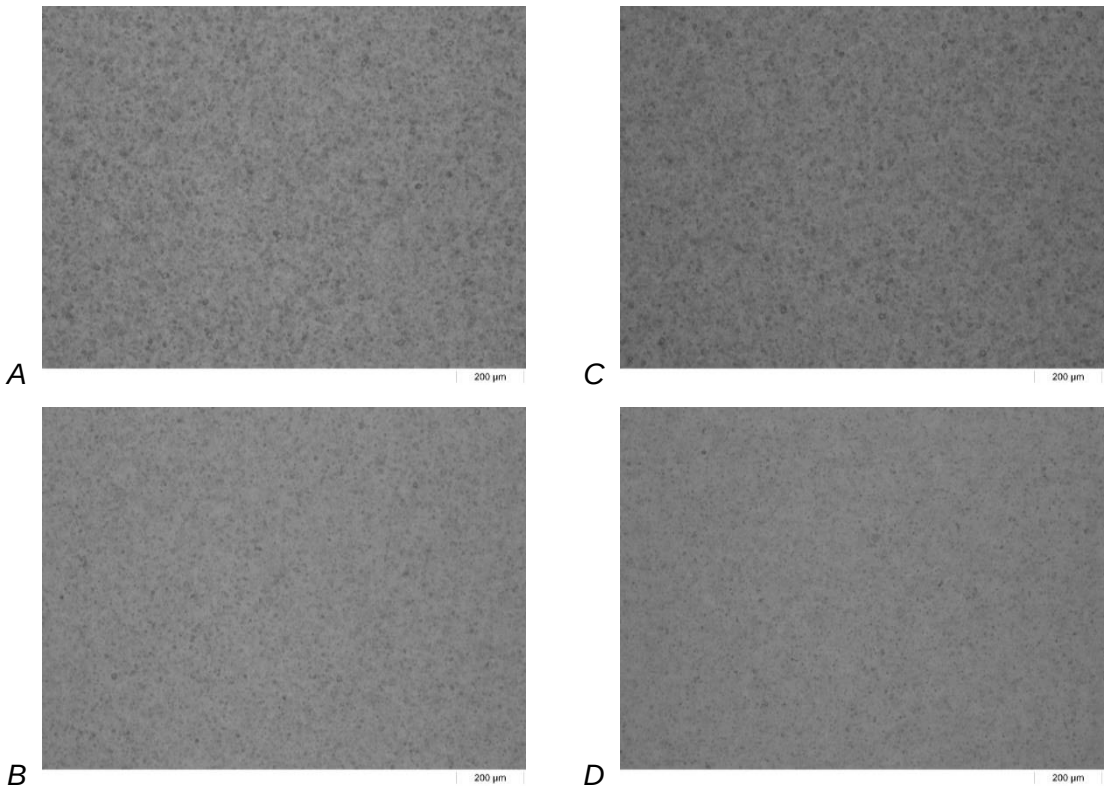


***Figure 3.6**: Visual inspection of formulated lipid nanocarriers for investigation of morphology on Day 30 of stability, A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes, n=3, using image analysis*

Liposomes remained stable in size and appearance by Day 60, as shown in Figure 3.7. The transferosomes seemed to have reduced in size, but no breakage of vesicles were observed. The ethosomes and transethosomes remained stable in size and appearance.

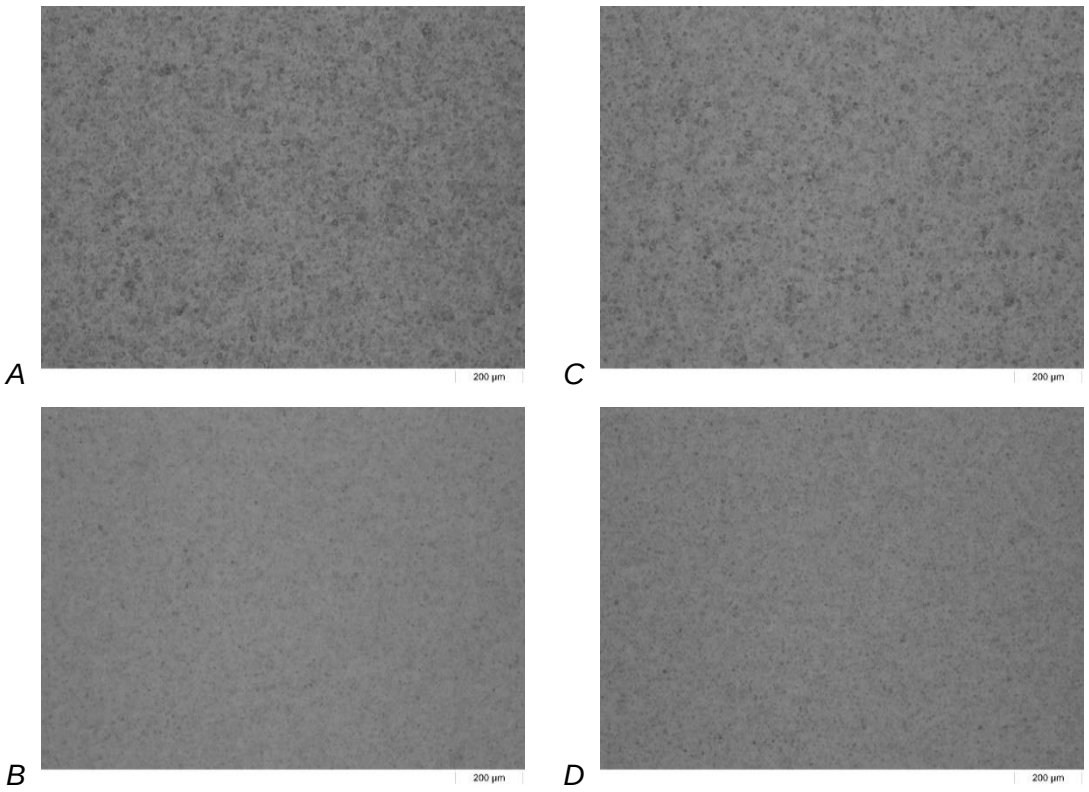


***Figure 3.7**: Visual inspection of formulated lipid nanocarriers for investigation of morphology on Day 60 of stability, A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes, n=3, using image analysis*

As depicted in Figure 3.8, the liposomes, transferosomes, ethosomes and transethosomes still seemed stable in size and appearance on Day 90.

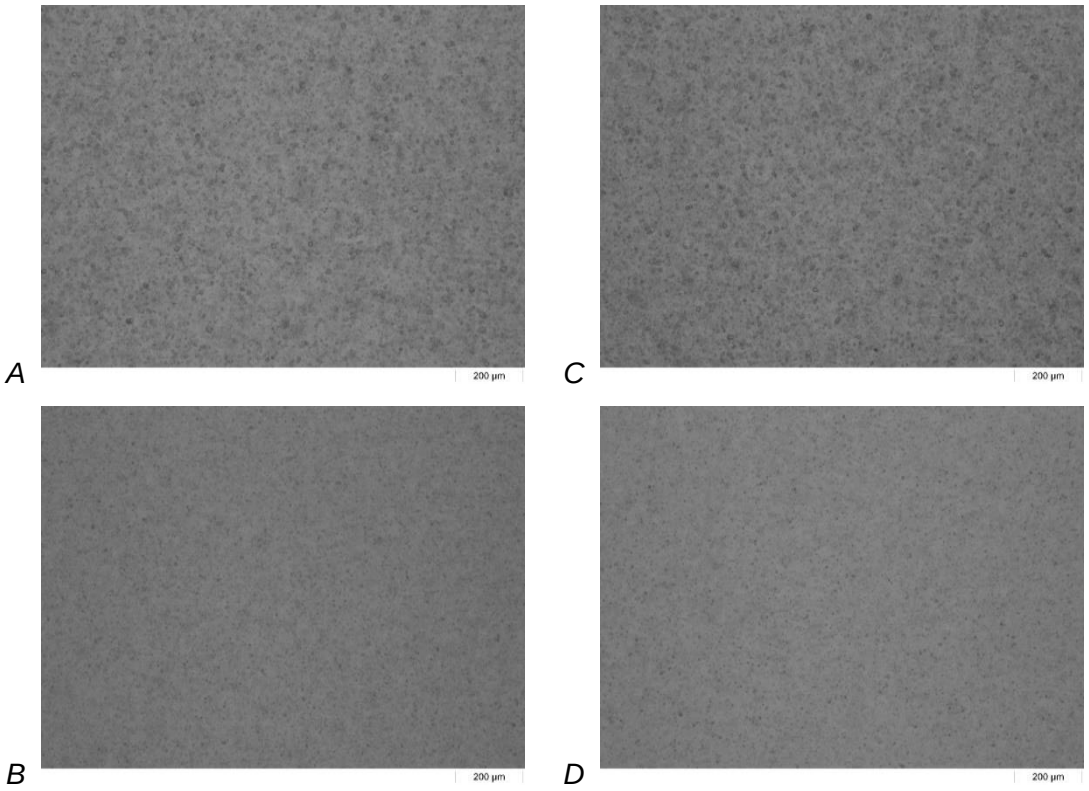


***Figure 3.8**: Visual inspection of formulated lipid nanocarriers for investigation of morphology*

on Day 90 of stability, A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes, n=3, using image analysis

The span of the particle size distribution was also determined, as shown in Table 3.5. It was observed that the particle size distribution was wide for the different LN, with liposomes and ethosomes portraying the widest particle size distribution. The transferosomes and transethosomes, however, showed statistically significant results when compared to the liposomes.

Table 3.5*: The span of the particle size distribution, as calculated from the D10, D50 and D90 values, p=0.0002, n=3*

	CE Diameter	***D[n, 0.1]***	***D[0.5]***	***D[n, 0.9]***	***Span***
Liposomes	*Day 30*	2.36	3.68	7.27	1.33
	Day 60	2.44	4.39	9.80	1.68
	Day 90	2.38	3.92	8.04	1.44
Transferosomes	*Day 30*	2.27	3.11	5.38	1.00
	Day 60	2.27	3.08	5.39	1.01
	Day 90	2.27	3.06	5.12	0.93
Ethosomes	*Day 30*	2.39	4.02	8.43	1.50
	Day 60	2.37	3.93	7.94	1.42
	Day 90	2.38	3.95	8.19	1.47
Transethosomes	*Day 30*	2.27	3.06	5.17	0.95
	Day 60	*2.30*	*3.32*	*5.8*	*1.05*
	Day 90	*2.26*	*3.13*	*5.36*	*0.99*

1.2.1.2. CE diameter

Table 3.6*: The CE diameter (number distribution) of the different lipid nanocarriers after 90 days, D[n, 0.5], Malvern Morphologi G3, n=3*

Day	**Liposomes**	**Transferosomes**	**Ethosomes**	**Transethosomes**
30	3.68±*0.25*	3.11±*0.26*	4.02±*0.405*	3.06±*0.15*
60	4.39±*0.46*	3.08±*0.19*	3.93±*0.495*	3.32±*0.19*
90	3.92±*0.21*	3.06±*0.38*	3.95±*0.14*	3.13±*0.05*

After 30 days the CE diameter (number distribution), exhibited an overall decrease when compared to Day 0. After 60 days, the liposomes and transethosomes showed an overall

increase in CE diameter (number distribution), while the transferosomes and ethosomes showed a decrease, as shown in Table 3.6. After 90 days, the liposomes, transferosomes and transethosomes indicated a decrease, while the ethosomes increased slightly. After statistical analysis was performed, the comparison of the data showed no statistical significant differences.

1.2.1.3. Intensity mean

Table 3.7*: Comparison of the intensity mean – number distribution (D[n, 0.5]) of the different lipid nanocarriers after 90 days, Greyscale: black = 0; mid-grey = 128; white = 255, using image analysis, n=3*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	102±0	101±1.633	102±0.00	100±0.00
60	101.333±0.471	102±0.00	101.33±0.47	101.67±0.47
90	102±0.00	102±0.00	101.67±0.47	101.33±0.47

After 30 days, the liposomes' intensity mean distribution showed a slight decrease overall, while the transferosomes and ethosomes displayed a slight increase, as shown in Table 3.7. The transethosomes showed a slight decrease in intensity mean distribution. The vesicles seemed to have darkened, although they still showed a grey color, over the course of 30 days. After statistical analysis was performed, the comparison of the data showed no statistical significant differences.

After 60 days, the liposomes and ethosomes displayed a decrease and the transferosomes and transethosomes a slight increase. The vesicles showed an overall darkening, except the transferosomes that showed slight lightening. The vesicles all still appeared as a grey color. After statistical analysis was performed, the comparison of the data indicated no statistical significant differences.

The intensity mean after 90 days, as depicted in Table 3.7, was as follows: The liposomes and ethosomes showed an increase, transferosomes remained stable, whereas the transethosomes indicated a slight decrease. The liposomes and ethosomes appeared to have lightened and the transferosomes and transethosomes appeared to have stayed mainly the same over the 90 day period; they all still portrayed a grey color. After statistical analysis, there was no statistical significant differences observed when the data of the different vesicles were compared.

1.2.1.4. Solidity

***Table 3.8**: Comparison of solidity – number distribution (D[n, 0.5]) of the different lipid nanocarriers after 90 days, solid = 1, using the image analysis, n=3*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	0.90±*0.00*	0.92±*0.00*	0.90±*0.01*	0.92±*0.01*
60	0.90±*0.01*	0.92±*0.00*	0.91±*0.01*	0.92±*0.00*
90	0.90±*0.00*	0.91±*0.01*	0.90±*0.00*	0.92±*0.01*

When compared to Day 0, liposomes, ethosomes and transethosomes showed an overall increase in solidity, while transferosomes indicated an overall decrease, as shown in Table 3.8. Statistical analysis determined that comparison of these datasets showed no statistically significant differences. After 30 days, the solidity of the liposomes decreased overall, while ethosomes and transethosomes displayed an overall increase; the transferosomes also displayed an increase. When compared to Day 60, the liposomes showed an overall increase in solidity (number distribution), after 90 days as seen in Table 3.8. The transferosomes, ethosomes and transethosomes all showed an overall decrease in solidity when compared to Day 60. Statistical analysis determined that comparison of these datasets indicated no statistically significant differences.

1.2.1.5. Elongation

***Table 3.9**: The elongation (number distribution) of the different lipid nanocarriers after 90 days, using image analysis, n=3, p=0.9990*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	0.29±*0.00*	0.26±*0.01*	0.29±*0.00*	0.27±*0.02*
60	0.30±*0.00*	0.26±*0.01*	0.28±*0.01*	0.25±*0.00*
90	0.29±*0.00*	0.28±*0.03*	0.28±0.00	0.26±*0.02*

When compared to Day 0, the elongation of the liposomes and ethosomes showed an overall decrease, suggesting they became more convex as time passed, as seen in Table 3.9, whereas the elongation of the transferosomes showed an overall increase, suggesting the decrease of convexity; the transethosomes displayed a slight decrease. The elongation of the liposomes increased overall after 60 days, while the transethosomes showed an overall decrease in elongation; the transferosomes remained stable, and decreased for ethosomes. After the total 90 days, liposomes yet again exhibited an overall decrease in elongation, therefore, suggesting they became more convex as time passed. The UDV showed an increase in elongation. After

statistical analysis was performed, the comparison of the data indicated no statistical significant differences.

1.2.1.6. Convexity

***Table 3.10**: The convexity (number distribution) of the different lipid nanocarriers after 90 days, D[n, 0.5], using image analysis, n=3, p=0.9787*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	*0.95±0.01*	*0.96±0.00*	*0.94±0.01*	0.96±*0.00*
60	*0.93±0.01*	*0.96±0.01*	0.95±*0.01*	0.96±*0.01*
90	*0.94±0.00*	0.96±*0.00*	0.94±*0.00*	0.96±*0.01*

The convexity (number distribution) of the lipid nanocarriers increased overall when compared to Day 0, as shown in Table 3.10. An overall decrease in convexity was observed for liposomes (as compared to Day 30), while the transethosomes showed an overall increase in convexity. The convexity of the transferosomes decreased, while the convexity of the ethosomes remained stable. After 90 days, the convexity of the liposomes, transferosomes and transethosomes exhibited an overall increase when compared to Day 60; the ethosomes, however, showed a decrease in convexity. After statistical analysis was performed, the comparison of the data indicated no statistical significant differences.

1.2.1.7. Circularity

***Table 3.11**: Circularity (number distribution) of the different lipid nanocarriers after 90 days, D[n, 0.5], using image analysis G3, n=3, p=0.9858*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	*0.64±0.01*	*0.71±0.01*	*0.63±0.03*	*0.72±0.02*
60	*0.60±0.03*	*0.70±0.03*	*0.65±0.02*	*0.72±0.01*
90	*0.63±0.01*	*0.70±0.02*	*0.65±0.00*	*0.72±0.05*

After 30 days, the circularity of the liposomes, ethosomes and transethosomes increased overall, when compared to Day 0, as shown in Table 3.11. However, the transferosomes showed an overall decrease in circularity over time, when compared to Day 0. The circularity of the liposomes decreased overall when compared to Day 30; the transferosomes exhibited a decrease, the ethosomes and transethosomes an increase. After 90 days, the circularity of the liposomes showed an overall increase, when compared to Day 60. The UDV showed a decrease in circularity. After statistical analysis was performed, the comparison of the data indicated no statistical significant differences.

1.2.1.8. Aspect ratio

***Table 3.12**: The aspect ratio (number distribution) of the different lipid nanocarriers after 90 days, D[n, 0.5], using image analysis, n=3, p= 0.9983*

Day	Liposomes	Transferosomes	Ethosomes	Transethosomes
30	*0.71±0.00*	*0.73±0.01*	*0.71±0.01*	*0.73±0.02*
60	*0.70±0.00*	*0.73±0.01*	*0.68±0.05*	*0.74±0.01*
90	*0.70±0.0*	*0.71±0.03*	*0.71±0.00*	*0.74±0.02*

After 30 days, the aspect ratio of the ethosomes increased, while the other vesicles remained stable, as depicted in Table 3.12. The vesicles all remained stable concerning aspect ratio throughout the remainder of the 90 days. After statistical analysis was performed, the comparison of the data indicated no statistical significant differences.

In conclusion, degradation due to stability could not be observed through visual inspection or the parameters of the image analysis, due to the liposomes and UDV being more stable. However, the parameters of image analysis of the Malvern Morphologi G3 could be used as a complementary means to have a comprehensive characterization study of the UDV.

1.3. References

1. Ascenso, A.*, et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes*. International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
2. Singh, S.*, et al.*, The role of surfactants in the formulation of elastic liposomal gels containing a synthetic opioid analgesic*. International journal of nanomedicine*, 2016. **11**: p. 1475-82.
3. Malvern Instruments Limited, *Morphologi G3 user manual*, M.I. Limited, Editor. 2015, Malvern Instruments Limited: Malvern, Worcestershire, UK.

Chapter 4 – *In vitro* toxicity of liposomes and ultradeformable vesicles in cultured melanoma and keratinocyte cell lines

Manuscript as to be submitted for the journal Toxicology *In Vitro*.

This article is written according the journal instructions that can be found at https://www.elsevier.com/journals/toxicology-in-vitro/0887-2333/guide-for-authors. The reference style that was followed was a numeric style and the language was American English as specified by the journal. This reference style and language was also kept constant throughout the dissertation to enable better reading. There was no strict adherence to word count for the purposes of the examination of the dissertation, however the manuscript will be condensed for publication purposes.

Title page

Michelle Volkwyn*, Lissinda H du Plessis, Joe M Viljoen

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Private bag x6001, Potchefstroom 2520, South Africa

*corresponding author Michellevolkwyn93@gmail.com

Abstract

Ultradeformable vesicles (UDV) are an alternative to liposomes containing lipid surfactant combinations that are highly favorable for skin penetration. Liposomes, transferosomes, ethosomes and transethosomes are lipid nanocarriers (LN) that have been investigated as an alternative to traditional transdermal products, and therefore, should not be toxic to the skin cells. Previous studies have suggested that lipids within liposomes cause inaccurate results when cell viability is determined by the Thiazolyl blue tetrazolium bromide (MTT) assay. The aim of this study was to verify whether the interference with the MTT assay could also be observed with ultradeformable vesicles. The effects of vesicle concentration, as well as treatment time on viability of human keratinocytes (HaCat) and human melanoma cells (A375) were investigated by means of the MTT and Trypan Blue dye exclusion assays. It was observed that the UDVs were non-toxic to the HaCat and A375 cells for short treatment periods, but low levels of toxicity were observed for HaCat cells treated for longer than 48 h. The MTT and Trypan Blue dye exclusion assay could be used to determine accurate cell viability, even though they utilize different mechanisms to determine cell viability.

Total word count: 189

Key words

Lipid nanocarrier; Liposomes, Ultradeformable vesicles, Cell viability; MTT; Trypan Blue, skin toxicity

1. Introduction

Successful transdermal formulations have been developed, yet concern still arise regarding toxicity in cosmetics and pharmaceutics. Colloidal carriers are defined as “particulate or vesicular dosage forms that serve as superficial drug reservoirs” [1, 2], and are novel formulations that have proven successful over the past 10 years in nanotechnology. Lipid nanocarriers, such as solid lipid nanoparticles (SLN) and polymeric nanoparticles, are nanoparticles that have been investigated for delivery of drugs to specific sites [3]. Lipid nanocarriers are colloidal systems that have a particle size as small as 500 nm, that also have the ability to deliver drugs in effective concentrations [4-6]. They are liquid filled vesicles with a lipid membrane [7-9], and are divided into liposomes, ultra-deformable vesicles (UDV) and SLN.

Liposomes spontaneously form concentric amphiphilic lipid bilayers that enclose a watery compartment when a lipid is brought into contact with an aqueous phase and become hydrated [10-25]. Liposomes are usually prepared from natural components of the cell membrane namely phospholipids [1, 21, 22, 26-32]. These phospholipids are relatively biocompatible, biodegradable and non-toxic [20, 33]. Ultradeformable vesicles are lipid nanocarriers which demonstrate more deformability than liposomes, thus penetrating skin more effectively for improved drug delivery [34-37]. They include transferosomes, ethosomes and transethosomes.

Transferosomes were introduced in 1992 by Cevc and Blume [36]. They are trademarked by IDEA AG in Munich, Germany, and are also known as ultra-deformable liposomes, and are mostly used in drug delivery technology as elastic lipid nanocarriers [9, 35, 37-39]. Transferosomes consist of phospholipids, as well as an edge activator, such as sodium cholate, to reach its high elasticity [9, 35, 37, 39-41]. Currently, toxicology studies of transferosomes have shown a lack of toxicity, which can be attributed to the natural lipids they consist of [8, 42, 43]. They also show good biocompatibility and biodegradability [9, 43].

Ethosomes, first introduced by Touitou *et al.* [44], are elastic lipid vesicles developed especially for non-invasive transdermal delivery of drugs, deep within the skin layers [11, 35,

45-52]. Ethosomes are known for their malleability, as well as their small size relative to liposomes [27, 46, 53, 54]. Extensive toxicological studies on the raw materials used to formulate ethosomes, have shown non-toxicity, and was safe to use in cell cultures, as well as pharmaceutical use [32, 47, 49, 50, 54-56].

Transethosomes were only introduced in 2012 by Song *et al.* [57]. It was attempted to combine the advantageous characteristics of transferosomes and ethosomes [58]. Transethosomes consist of the components of both ethosomes and transferosomes, causing them to be more deformable than both transferosomes and ethosomes [35, 59-61]. This is due to the vesicles containing ethanol, as well as a surfactant [35, 57]. Little information is available on transethosomes, it is suggested that, due to their composition, transethosomes possess advantages of both transferosomes and ethosomes [35, 62-64].

Biocompatibility is defined as “an expression of the benignity of the relation between a substance and its biological environment” [65]. A complex relationship exists between the lipid nanocarriers’ physiochemical properties and the interaction the carriers have on biological environments [66]. Lipid nanocarriers mainly consist of lipids and surfactants, and may resemble cell organelles with regard to their dimensions [66, 67]. This can cause interference with the cell’s vital functions, leading to potential cytotoxicity [66, 67].

Forms of toxicity in the skin are skin corrosion and skin irritation, which may occur in all layers of the skin, which is mostly caused by inorganic, as well as strong organic bases and acids [68]. Skin pigmentation is mainly regulated by two cell types, i.e. melanocytes and keratinocytes [69]. Melanocytes produce melanosomes, which in turn contain melanin, which is then received and distributed to the keratinocytes [69]. The keratinocytes within the epidermis are a possible site of toxicity in the skin, as they are sensitive to nanoparticles [70]. In skin toxicity assays, the measurement of necrosis is classified as *in vitro* skin corrosion potential [68]. *In vitro* models with specific endpoints can determine general mechanisms of toxicity from drug delivery systems [71-74], these endpoints are based on clinical or histopathological endpoints [71-74]. The mitochondria plays an important role in apoptosis and necrosis of cells [75, 76]. Due to the influence nanoparticles have on mitochondrial function, which can lead to impaired cellular energy as well as lipid metabolism that can lead to the release of cell death mediators, mitochondrial activity is considered one of these endpoints [74-78]. The colorimetric tetrazolium dye (MTT) assay measures cytotoxicity, proliferation or activation by detecting the living cells. The degree of activation of the cells is determined by the signal generated during this process [79, 80]. The MTT formazan is taken up by the cells, by means of endocytosis and is deposited into needle-like crystals, which will after time, dissolve again [81]. The MTT formazan undergoes reduction in the presence of

succinate, NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate) within mitochondria into a purple substance which accumulates in cytoplasmic granules [81, 82]. This process is frequently used to evaluate cytotoxicity potential of drugs that will be incorporated into liposomes [79].

The MTT assay is considered the standard assay for *in vitro* cytotoxicity [83]. However, various studies have proved interference of lipid and liposomes with the MTT assay related to colocalization of the formazan within lipid droplets [79, 82, 84]. Another study has suggested that the amount of MTT formazan, which is reduced by cells, is dependent on physiochemical mechanisms, such as electrostatic forces, rather than cytological processes [82]. In this study we demonstrate a systematic approach to determining the biocompatibility of the UDV in different concentrations and with different treatment times, in comparison with liposomes, as well as the influence of the different LN on the accuracy of the colorimetric Thiazolyl blue tetrazolium bromide (MTT) assay, which compares the cell response of the entire well of cells, in comparison to the Trypan Blue dye exclusion assay, where individual live cells are counted, as suggested by Angius and Floris [79] in 2015.

2. Materials and methods

2.1. Materials

The Thiazolyl blue tetrazolium bromide (MTT), chloroform (biotech grade, ≥99.8%), dimethyl sulphoxide (DMSO), ethyl alcohol (pure, for molecular biology), L-α-phosphatidylcholine (from egg yolk, PC), methanol (suitable for protein sequencing), phosphate buffered saline (for cell cultures, PBS), Triton X (for molecular biology), Trypan blue solution and Span 80® were purchased from Sigma-Aldrich®, Merck (Pty) Ltd. (Darmstadt, Germany). The A375 cell line (Catalogue no. CRI-1619), and the HaCat cell line (Catalogue no. PCS-200-011) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modification of Eagle's Medium (DMEM) / High glucose medium was purchased from Separations (Thermo Fisher Scientific, Waltham, MA, US). Fetal bovine serum (FBS), L-glutamine, non-essential amino acids (NEAA), trypsin-EDTA solution and 1% penicillin-streptomycin solution was purchased from Lonza (Basel, Switzerland).

2.2. Formulation of lipid nanocarriers

Formulas for the liposomes, transferosomes, ethosomes and transethosomes were determined by an optimization study to ensure that they would be suitable for use in cell cultures (see Supplementary Material). Formulation of the lipid nanocarriers was carried out

at a controlled temperature of 25ºC at all times, as well as under sterile conditions, in order to be used to treat cell lines, as adapted from Ascenso *et al.* [35]. The vesicles were prepared at a concentration of 1000 µg/ml and would be diluted according to the experimental design. The composition of the lipid nanocarriers are depicted in Table 4.1. L-α-phosphatidylcholine (PC) was dissolved in a 2:1 chloroform:methanol mixture (3 ml), and then heated to 40ºC. A film was created by constantly rotating the beaker while heating occurred, causing the methanol and chloroform to evaporate. The aqueous phase (PBS for cell cultures) was added and then homogenized using the IKA ULTRA-TURRAX® tube disperser workstation system (Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa). The vesicle suspension was placed in a fridge at 4ºC for two hours before use in experiments.

It was determined that only a 1000 µg/ml concentration of LN would be prepared each time, after which it would be diluted to the desired concentration (Supplementary material). The composition of the LN is showed in Table 4.1.

Table 4.1*: The composition of the different lipid nanocarriers*

Lipid nanocarrier	**Ingredient**	**Concentration (% w/v)**
Liposomes	L-α-phosphatidylcholine (PC)	100%
Transferosomes	L-α-phosphatidylcholine	85%
	Span 80®	15%
Ethosomes	L-α-phosphatidylcholine	13.79%
	Ethanol	86.21%
Transethosomes	L-α-phosphatidylcholine	11.72%
	Span 80®	2.07%
	Ethanol	86.21%

2.3. Cell cultures

The eukaryotic cells, A375 skin cells (ATCC® number CRl-1619™) and HaCat cells (ATCC® number PCS-200-011TM) were maintained in a sterile environment. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose that contained 4500 mg/L glucose and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% of a 4 mM L-glutamine and 1% non-essential amino acids (NEAA).

Cell cultures were initially incubated in a 25 cm^2 cell culture flask, at 37ºC, 5% CO_2 and >90% humidity, in a Thermo Scientific™ BBD 6220 CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). The growth media was renewed twice per week. When the cultures

reached 80 to 90% confluency within the 25 cm^2 flask, they were sub-cultured to 75 cm2 flasks, utilizing 0.25% trypsin-EDTA to detach the cells from the flask surface.

2.4. Vesicle concentration study

Before the vesicle concentration study commenced, a cell optimization study was performed to determine the optimal cell density to which the study will be performed (see supplementary material). The method used for the MTT assay was adapted from Angius and Floris [79] and ISO10993 [83], after which it was concluded that a cell density of 100 000 cells/ml would be used during the study. A375 and HaCat cells were seeded in 96-well plates and incubated at 37ºC (5% CO_2, >90% humidity) for 24 h (illustrated in detail in supplementary material). This allowed the cells to adhere to the walls of the plate. The cells were treated with the liposomes and UDV at concentrations of 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml, after which the treated cells were incubated at 37ºC (5% CO_2, >90% humidity) for a further 24 h. Following treatment the MTT assay was performed as follows: Cell medium was removed and cells were rinsed twice with PBS. An MTT solution was prepared beforehand with serum free media (5 mg/ml). The MTT solution was added to each well and diluted to 100 µl by means of serum free media to a MTT concentration of 0.5mg/ml. The cells were incubated for 3 h, after which the MTT was extracted and 100 µl DMSO was added into each well. The cells were incubated once more at 37ºC (5% CO_2, >90% humidity) for another hour to ensure that all MTT crystals were dissolved. The plate was analyzed in-house in a SpectraMax Paradigm® microplate reader (Molecular Devices, Sunnyvale, CA, USA), equipped with SoftMax® Pro Microplate Data Acquisition and Analysis software (Molecular Devices, Sunnyvale, CA, USA), at a wavelength of 560 nm, while background absorption was measured at 620 nm. These two values were subtracted and cell viability was expressed as a percentage relative to the control (set at 100% viable). This method was followed for both HaCat and A375 cell lines. This study was done in triplicate on three separate occasions.

2.5. Treatment time study

A375 and HaCat cells were again seeded in 96-well plates at a density of 100 000 cells/ml and incubated at 37ºC (5% CO_2, >90% humidity) for 24 h according to the needs of the assay that would be carried out (as illustrated in the supplementary material). As supported by Angius and Floris [79], a vesicle concentration of 100 µg/ml was used to treat the cells for treatment periods of 0 h, 24 h and 48 h, after which a MTT study as well as a Trypan Blue dye exclusion assay were carried out to determine a comparative cell viability.

Following treatment the Trypan Blue marked plates were rinsed twice with PBS, after which 40 µl trypsin-EDTA was added to each well and incubated (5 min for A375 cell line and 12 min for HaCat cell line). The trypsin-EDTA was then neutralized with 160 µl DMEM, and 10 µl of the cell suspension was taken and added into 2 ml disposable vials, each containing 10 µl of 0.4% Trypan Blue. The suspension and Trypan Blue were mixed thoroughly and 10 µl was placed on each side of a Reichert Bright-Line hemocytometer (Merck, Kenilworth, New Jersey, United States) and covered with a cover glass. The cells for each well were then photographed and the Trypan Blue stained (dead) cells counted, after which the following formula was used to determine the cell concentration (cells/ml), after which percentage cell viability was calculated relative to the control:

$$Cells/ml = \frac{Total\ cells\ counted\ \times Dilution\ factor}{No.of\ sqaures\ counted} \times 10000 \quad [1]$$

This study was performed in triplicate on three separate occasions

2.6. Statistical analysis

The data collected was analyzed statistically with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data was analyzed by means of three-way ANOVA with Dunnet's post-hoc comparison.

3. Results

3.1. Preparation of the liposomes and UDV

A formula optimization study was performed previously to determine the optimal formulations for the liposomes and UDV. After the optimization study, a comprehensive characterization study was carried out utilizing both dynamic light scattering (DLS) and image analysis. A detailed description of the preparation and characterization of the LN can be found in chapter 3. The average diameter of the liposomes was 930.64 ± 163.50 nm, with a wide vesicle size distribution, as confirmed by a PDI value of 0.69 ± 0.07. The transferosomes and transethosomes proved fairly similar in diameter, 557.97 ± 51.11 nm and 530.33 ± 64.67 nm respectively. However, the vesicle size distribution was not as wide for the transethosomes, supported by the PDI value of 0.55 ± 0.06, whereas the transferosomes had a wider size distribution, as supported by a PDI value of 0.66 ± 0.16. The ethosomes showed the greatest vesicle diameter and PDI values of the UDV, with an average diameter of 1002.56 ± 122.70

and PDI value of 0.72 ± 0.07. Therefore, it could be suggested that, of the UDV the ethosomes would behave most like the liposomes concerning size and PDI.

3.2. Vesicle concentration study

The linearity range of the MTT assay was established for both the A375 and the HaCat cells (Supplementary material). The MTT absorbance proved linear for up to 200 000 cells/ml. Figure 4.1 illustrates the MTT absorbance as a function of the lipid nanocarrier concentration (Mean ± SD) of both cell lines seeded at a cell density of 100 000 cells/ml, and incubated for 24 h. None of the LN caused significant decrease in the absorbance values when compared to the control for the A375 cells (Fig 4.1.A). The HaCat cells showed lower absorbance values when treated with 10 µg/ml and 10 µg/ml of the different vesicles, however, the viability was increased and relatively constant throughout the higher vesicle concentrations, as shown in Figure 4.1.B. Both treated cell lines showed higher absorbance values than the control cells, which confirms the findings of Angius and Floris [79], where they stated that a higher reduction of MTT to formazan takes place when cells are treated with lipids, such as liposomes, leading to absorbance values higher than the control values.

A correlation could be observed between the lipid content of the LN and the absorbance values for both cell lines, although the excipients of the UDV could also influence the absorbance values. At the vesicle concentration of 100 µg/ml, which is the LN concentration to be utilized for the next part of the study, the results obtained were varied. The ethosomes, which had the second lowest PC content of 13.79%, portrayed the highest absorbance value at 0.45 ± 0.03 nm. The transethosomes, containing the lowest PC percentage at 11.72%, had the second highest absorbance at 0.41 ± 0.01 nm, whereas the transferosomes, which had the second highest PC content of 85%, portrayed the second highest absorbance value at 0.41 ± 0.00 nm. The liposomes, which consist of only PC, however, had the lowest absorbance value at 0.37 ± 0.04 nm. The results obtained for the HaCat cell line differed from those obtained from the A375 cell line, where the liposomes, which have the highest PC, portrayed the highest absorbance values at 0.37 ± 0.07 nm. The ethosomes had the second highest absorbance at 0.37 ± 0.06 nm. The transferosomes had the third highest absorbance values at the same vesicle concentration, at 0.36 ± 0.06 nm, whereas, the transethosomes, containing the lowest percentage of PC, had the lowest absorbance at 0.33 ± 0.06 nm.

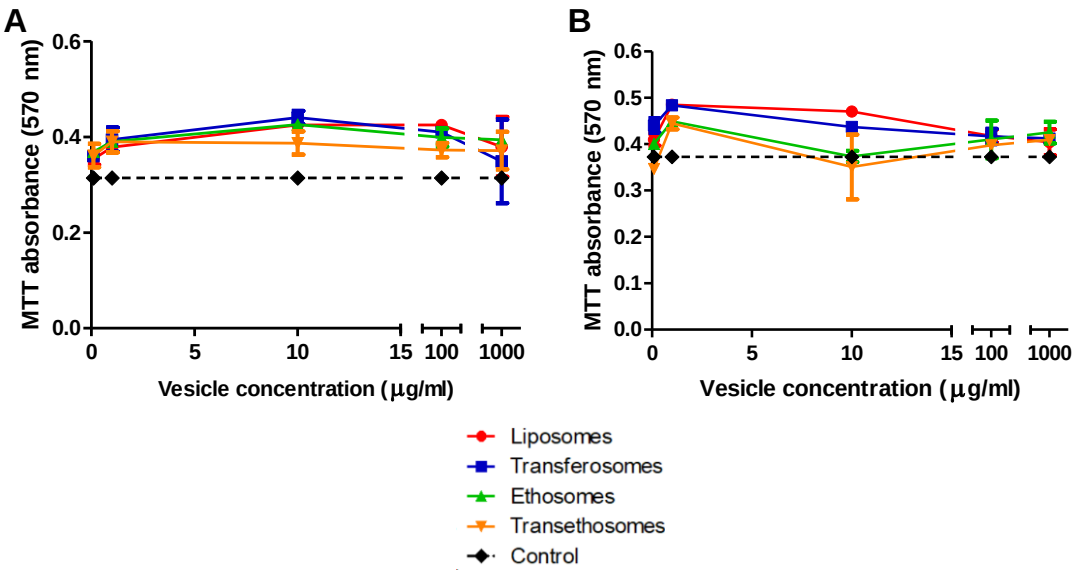


***Figure 4.1**: (MTT) absorbance as a function of the lipid nanocarrier concentration (Mean ± SD) of A. A375 cells and B. HaCat cells seeded at a cell density of 100 000 cells/ml, incubated for 24 h, (n=3)*

3.3. Treatment time study

Figure 4.2 illustrates the cell viability (%) of A375 cells treated with liposomes and the UDV for different treatment times (h), as determined by MTT and Trypan Blue dye exclusion assay. With microscopic observation of the Trypan Blue stained cells the control cells increased 2.8 fold after the first 24 h and after 48 h increased 4.1 fold compared to 0 h treatment time, indicating normal proliferation. A similar trend was observed with the MTT assay, although the cells only increased 0.9 fold after the first 24 h, with an overall increase after 48 h of 1.25 fold.

The cell viability of the A375 cells treated by liposomes, as determined via the Trypan Blue dye exclusion assay, increased at 24 h, and increased once again at 48 h treatment time, indicating a linear proliferation. The same linear proliferation could, however, not be observed for the MTT assay, where the cell viability seemed to create a plateau over the treatment period. The cell viability of all the LN treatments were higher than the control cells with both the MTT and Trypan Blue methods, where the cells treated with UDVs behaved comparatively to liposomes.

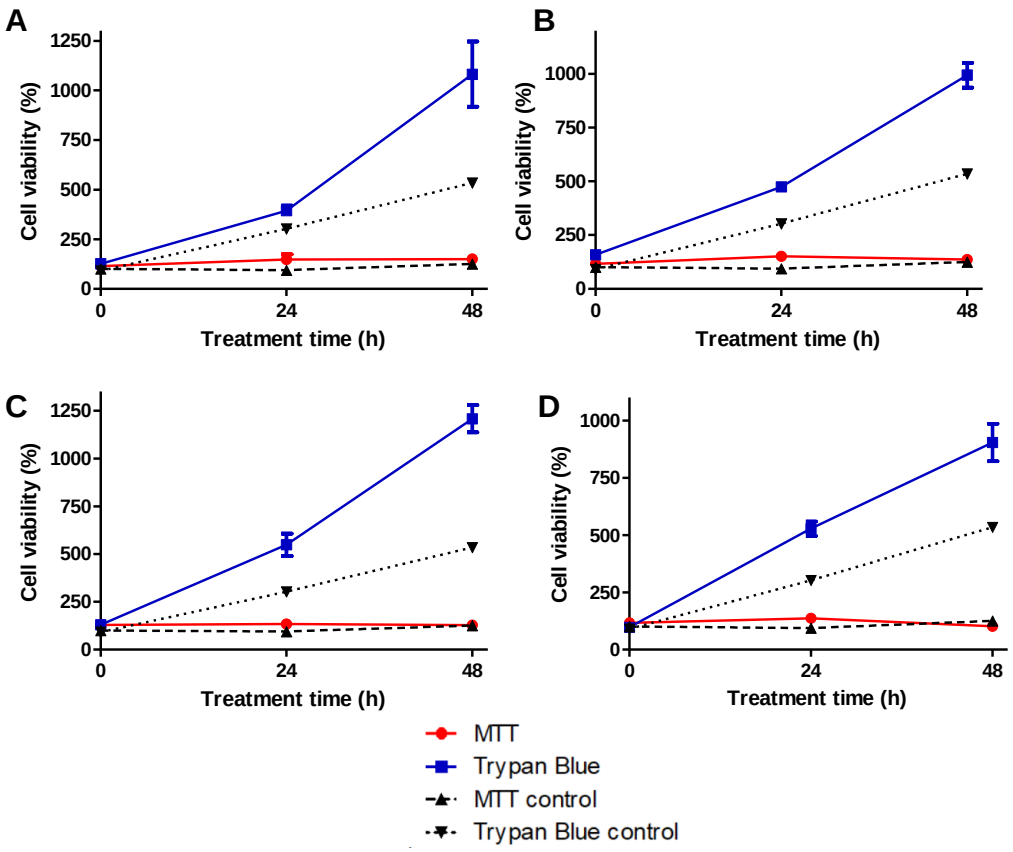


***Figure 4.2**: Cell viability (% ± SD) of A375 cells treated with A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes for different treatment times (h), using MTT and Trypan Blue dye exclusion assay, n=3*

With microscopic observation of the HaCat Trypan Blue stained cells the control cells doubled after the first 24 h and then doubled once again after 48 h, indicating normal proliferation. A similar trend was observed with the MTT assay, although the cells only increased 1.15 fold after the first 24 h, with an overall increase after 48 h of 1.5 fold.

The cell viability, as determined via the MTT assay, of the HaCat cells treated by liposomes increased at 24 h and decreased at 48 h treatment time, as shown in Figure 4.3.A. A similar trend was observed when the cells were observed microscopically with Trypan Blue exclusion. At 48 h, cell viability determined with the MTT assay decreased 1.5 times and with Trypan Blue exclusion 1.2 times. A similar trend was observed with the ethosomes and the transethosomes with both assays (Figure 4.3.C and 13.D). The transferosomes proved the least cytotoxic to HaCat cells as determined with the Trypan Blue method (Figure 4.3.B). The cell viability of the MTT HaCat cells decreased after 48 h of treatment with transferosomes, as depicted in Figure 4.3.B. The MTT cells showed a slightly higher viability after 24 h of treatment period, when compared to the control cells.

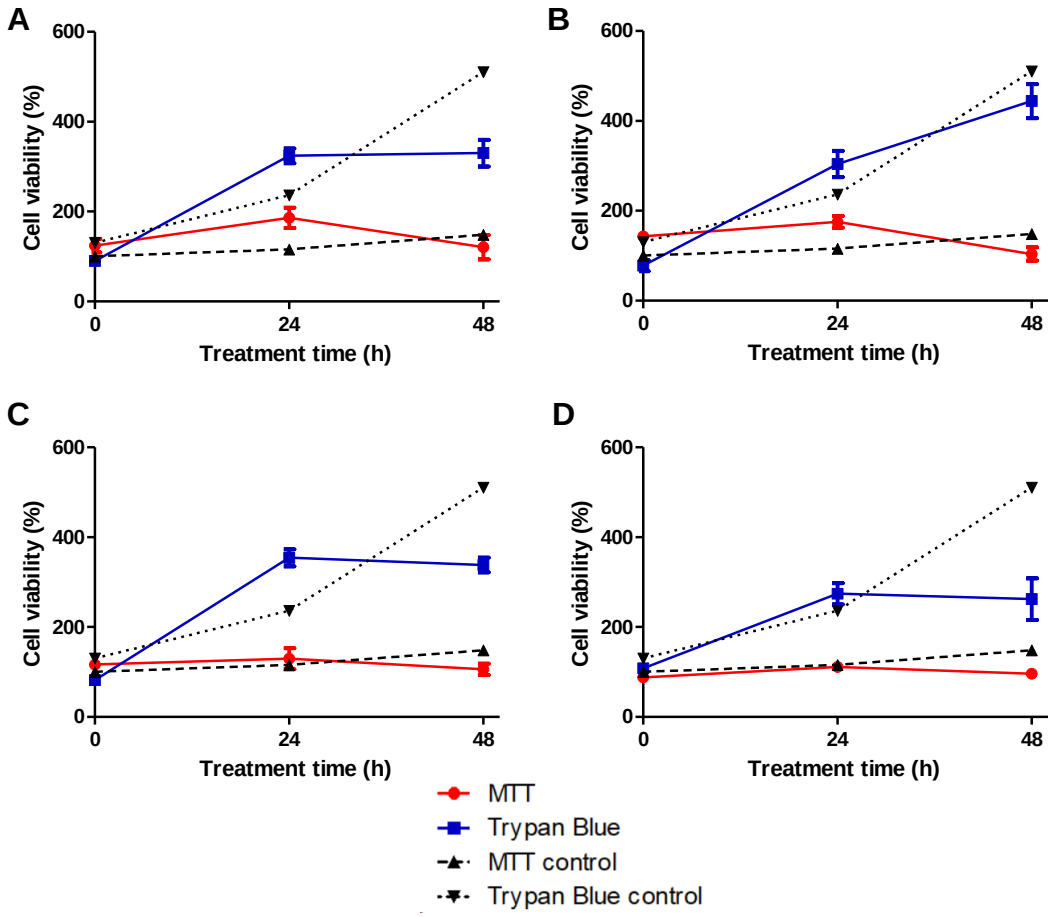


***Figure 4.3**: Cell viability of HaCat cells (%) treated with A. Liposomes, B. Transferosomes, C. Ethosomes, D. Transethosomes for different treatment times (h), using MTT and Trypan Blue dye exclusion, n=3*

4. Discussion

During the vesicle concentration study, it was clear that the PC within the liposomes, as well as the UDV, had an influence on the absorbance values during the MTT study in comparison with the control cells, therefore, confirming the study Angius and Floris [79]. This was observed for both the A375 and HaCat cell lines. Due to the absorbance values being higher than the control cells, it could be speculated that proliferation of cells had occurred after treatment with the vesicles, yet could not be definitively proven due to the formazan, which could concentrate within the lipid vesicles.

Previous toxicological studies have shown that liposomes, transferosomes and ethosomes are not toxic to cultured cells, but little information is available on the toxicity of transethosomes to cultured cells [8, 9, 32, 47, 49, 50, 54-56, 85, 86]. Both ethosomes and transethosomes showed extremely similar effects on both the A375 and HaCat cells, concluding that the

transethosomes may indeed possess the advantages of both transferosomes and ethosomes [35, 62-64]. All four lipid nanocarrier systems proved to be non-toxic to both the A375 and HaCat cells for shorter treatment periods, therefore confirming the biocompatibility of these carriers.

During the treatment time study, it was observed that proliferation of the A375 cells had occurred, even after the 48 h treatment period. For the Trypan Blue counted cells, exponential growth was observed, even more than that of the control cells, indicating that the LN could have caused higher proliferation than normal. A possible reason for this is, that the A375 cells are cancerous and their proliferation rate is higher than that of healthy cells. The same, however, could not be observed for the MTT cells, where a plateau was observed after which the cell viability started to decrease after 48 h treatment time.

Observation of the Trypan Blue counted HaCat cells showed that the cell viability decreased after 24 h, suggesting that proliferation was inhibited. Toxicity of MTT cells treated by transferosomes and transethosomes were observed after 48 h treatment time, due to the cell viability of these cells dropping below 100% (90.7 ± 11.1% and 97.4 ±13.2% respectively).

It was therefore concluded that the UDV affects the absorbance levels of the MTT study due to the MTT formazan, which concentrates within their lipid content, similarly to liposomes, as previously suggested by Angius and Floris [79]. It was also concluded that possible toxicity of the HaCat cells would occur when treatment time exceeds 48 h. The observation of the absorbance values of the LN only in comparison to that of the cells treated with the different systems is a recommendation for future studies, as well as the observation of the 48 h treatment study with different concentrations of vesicles. Another recommendation is to compare viability assays that utilizes the same mechanism to determine cell viability, due to the Trypan Blue dye exclusion assay and the MTT assay which utilize different mechanisms to deliver even more accurate results.

5. Declaration of interest

It is declared that no conflict of interest was present during this study.

6. Acknowledgements

This work is based on the research supported wholly/in part by the National Research Foundation of South Africa (NRF Innovation Master's scholarship, Ref.: SFH160719179397); and opinions, findings and conclusions or recommendations expressed in any publication

generated by the NRF supported research is that of the author(s) alone, and that the NRF accepts no liability whatsoever in this regard.

ORCID ID
https://orcid.org/0000-0002-3177-5904

7. References

1. Cevc, G., Lipid vesicles and other colloids as drug carriers on the skin. *Advanced drug delivery reviews*, 2004. **56**(5): p. 675-711.
2. Sharma, J., *et al.*, Colloidal drug carriers. *The internet journal of family practice*, 2009. **9**(2).
3. Alvarez-Roman, R., *et al.*, Skin penetration and distribution of polymeric nanoparticles. *Journal of controlled release*, 2004. **99**(1): p. 53-62.
4. Alexander, A., *et al.*, Recent expansion of pharmaceutical nanotechnologies and targeting strategies in the field of phytopharmaceuticals for the delivery of herbal extracts and bioactives. *Journal of controlled release*, 2016. **241**: p. 110-124.
5. Kumar, L., *et al.*, Fatty acid vesicles acting as expanding horizon for transdermal delivery. *Artificial cells, nanomedicine, and biotechnology*, 2017. **45**(2): p. 251-260.
6. Neubert, R.H.H., Potentials of new nanocarriers for dermal and transdermal drug delivery. *European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
7. Honeywell-Nguyen, P.L. and J.A. Bouwstra, Vesicles as a tool for transdermal and dermal delivery. *Drug discovery today: Technologies*, 2005. **2**(1): p. 67-74.
8. Priyanka, R. and D. Shipra, Transferosomes - a novel carrier for transdermal drug delivery system. *International journal of pharmacy & technology*, 2012. **4**(1): p. 1854-1865.
9. Vinod, K.R., *et al.*, Critical issues related to transferosomes – novel vesicular system. *Acta scientiarum polonorum - Technologia alimentaria*, 2012. **11**(1): p. 67-82.
10. Pierre, M.B., M. Dos Santos, and I. Costa, Liposomal systems as drug delivery vehicles for dermal and transdermal applications. *Archives of dermatological research*, 2011. **303**(9): p. 607-621.
11. Akhtar, N., Vesicles: A recently developed novel carrier for enhanced topical drug delivery. *Current drug delivery*, 2014. **11**(1): p. 87-97.

12. Vermuri, S. and C.T. Rhodes, Preparation and characterization of liposomes as therapeutic delivery systems: A review*. Pharmaceutica Acta Helvetiae*, 1995. **70**: p. 95-111.
13. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Can drug-bearing liposomes penetrate intact skin? *Journal of pharmacy and pharmacology*, 2006. **58**(4): p. 415-429.
14. Maurer, N., D.B. Fenske, and P.R. Cullis, Developments in liposomal drug delivery systems*. Expert opinion on biological therapy*, 2001. **1**(6): p. 923-947.
15. Cevc, G. and U. Vierl, Nanotechnology and the transdermal route: A state of the art review and critical appraisal*. Journal of controlled release*, 2010. **141**(3): p. 277-299.
16. Paul, A., G. Cevc, and B.K. Bachhawat, Transdermal immunization with large proteins by means of ultradeformable drug carriers*. European journal of immunology*, 1995. **25**(12): p. 3521-3524.
17. Richards, R.L.*, et al.*, Liposomes, lipid a, and aluminum hydroxide enhance the immune response to a synthetic malaria sporozoite antigen*. Infection and immunity*, 1988. **56**(3): p. 682-686.
18. Cevc, G., Material transport across permeability barriers by means of lipid vesicles, in *Handbook of biological physics*, R. Lipowsky and E. Sackmann, Editors. 1995, Elsevier Science B.V. p. 465-490.
19. Nounou, M.*, et al.*, Liposomal formulation for dermal and transdermal drug delivery: Past, present and future*. Recent patents on drug delivery & formulation*, 2008. **2**(1): p. 9-18.
20. Goyal, P.*, et al.*, Liposomal drug delivery systems – clinical applications*. Acta Pharmaceutica*, 2005. **55**: p. 1–25.
21. Barry, B.W., Novel mechanisms and devices to enable successful transdermal drug delivery*. European journal of pharmaceutical science*, 2001. **14**: p. 101-114.
22. Varun, T., A. Sonia, and P. Bharat, Niosomes and liposomes - vesicular approach towards transdermal drug delivery*. International journal of pharmaceutical and chemical sciences*, 2012. **1**(3): p. 632-644.
23. Ghahary, A.*, et al.*, Liposome associated interferon-alpha-2b functions as an anti-fibrogenic factor in dermal wounds in the guinea pig*. Molecular and cellular biochemistry*, 2000. **208**: p. 129–137.
24. Agarwal, R., O.P. Katare, and S.P. Vyas, Preparation and in vitro evaluation of liposomalniosomal delivery systems for antipsoriatic drug dithranol*. International journal of pharmaceutics*, 2001. **228**: p. 43-52.

25. Bangham, A.D., M.M. Standish, and J.C. Watkins, Diffusion of univalent ions accross the lamellae of swollen phospholipids. *Journal of molecular biology*, 1965. **13**: p. 238-252.
26. Abdellatif, A.A. and H.M. Tawfeek, Transfersomal nanoparticles for enhanced transdermal delivery of clindamycin. *AAPS PharmSciTech*, 2016. **17**(5): p. 1067-1074.
27. Verma, D.D., *et al.*, Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: A skin penetration and confocal laser scanning microscopy study. *European journal of pharmaceutics and biopharmaceutics*, 2003. **55**(3): p. 271-277.
28. Manconi, M., *et al.*, Development and characterization of liposomes containing glycols as carriers for diclofenac. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2009. **342**(1-3): p. 53-58.
29. Neubert, R.H., Potentials of new nanocarriers for dermal and transdermal drug delivery. *European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
30. Cevc, G., *et al.*, Overcoming semipermeable barriers, such as the skin, with ultradeformable mixed lipid vesicles, transfersomes, liposomes, or mixed lipid micelles. Langmuir, 2003. **19**: p. 10753-10763.
31. Lakshmi, P. and D. Prasanthi, Vesicles - a mechanism of transdermal permeation: A review. *Asian journal of pharmaceutical and clinical research*, 2012. **5**(1): p. 18-25.
32. Cortesi, R., *et al.*, Liposomes- and ethosomes-associated distamycins: A comparative study. *Journal of liposome research*, 2010. **20**(4): p. 277-285.
33. Sharma, A. and U.S. Sharma, Liposomes in drug delivery: Progress and limitations. *International journal of pharmaceutics*, 1997. **154**: p. 123-140.
34. Ascenso, A., *et al.*, In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. *European journal of pharmaceutics and biopharmaceutics*, 2014. **88**(1): p. 48-55.
35. Ascenso, A., *et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
36. Cevc, G. and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochimica et Biophysica Acta*, 1992. **1104**: p. 226-232.
37. Manosroi, A., *et al.*, Novel elastic nanovesicles for cosmeceutical and pharmaceutical applications. *Chiang Mai journal of science*, 2009. **36**(2): p. 168-178.
38. Duangjit, S., *et al.*, Characterization and in vitro skin permeation of meloxicam-loaded liposomes versus transfersomes. *Journal of drug delivery*, 2011. **2011**: p. 1-9.

39. El Zaafarany, G.M.*, et al.*, Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery*. International journal of pharmaceutics*, 2010. **397**(1-2): p. 164-172.
40. Romero, E.L. and M.J. Morilla, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes*. International journal of nanomedicine*, 2013. **8**: p. 3171-3186.
41. Zheng, W.S.*, et al.*, Preparation and quality assessment of itraconazole transfersomes*. International journal of pharmaceutics*, 2012. **436**(1-2): p. 291-298.
42. Vinod, K.R.*, et al.*, Critical issues related to transfersomes - novel vesicular system*.* Acta Scientiarum Polonorum Technologia Alimentaria, 2012. **11**(1): p. 67-82.
43. Jain, S.*, et al.*, Ultra deformable liposome: A recent tool for effective transdermal drug delivery*. Indian journal of pharmaceutical sciences*, 2003. **65**(3): p. 223-231.
44. Touitou, E.*, et al.*, *Ethosomes — novel vesicular carriers for enhanced delivery: Characterization and skin penetration properties. Journal of controlled release*, 2000. **65**(3): p. 403-418.
45. Touitou, E., B. Godin, and C. Weiss, Enhanced delivery of drugs into and across the skin by ethosomal carriers*. Drug development research*, 2000. **50**: p. 406-415.
46. Elsayed, M.M.*, et al.*, Lipid vesicles for skin delivery of drugs: Reviewing three decades of research*. International journal of pharmaceutics*, 2007. **332**(1-2): p. 1-16.
47. Verma, P. and K. Pathak, Therapeutic and cosmeceutical potential of ethosomes: An overview. *Journal of advanced pharmaceutical technology & research*, 2010. **1**(3): p. 274-282.
48. Thadanki, M. and A.K. Babu, Review on ethosomes: A novel approach of liposomes*. International journal of pharmacy & life sciences*, 2015. **6**(1): p. 4171-4176.
49. Rakesh, R. and K.R. Anoop, Ethosomes for transdermal and topical drug delivery*. International journal of pharmacy & pharmaceutical sciences*, 2012. **4**(3): p. 17-24.
50. Paolino, D.*, et al.*, Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers*. Journal of controlled release*, 2005. **106**(1-2): p. 99-110.
51. Jain, S.*, et al.*, Ethosomes: A novel vesicular carrier for enhanced transdermal delivery of an anti-HIV agent*. Indian journal of pharmaceutical sciences*, 2004. **66**(1): p. 72-81.
52. Marto, J.*, et al.*, Ethosomes for enhanced skin delivery of griseofulvin*. Colloids and Surfaces B: Biointerfaces*, 2016. **146**: p. 616-23.
53. Dayan, N. and E. Touitou, Carriers for skin delivery of trihexyphenidyl HCl: Ethosomes vs. Liposomes*. Biomaterials*, 2000. **21**: p. 1879-1885.
54. Nandure, H.P.*, et al.*, Ethosome a novel drug carrier. *International journal of pharmaceutical research & allied sciences*, 2013. **2**(3): p. 18-30.

55. Jain, S.*, et al.*, Formulation and evaluation of ethosomes for transdermal delivery of lamivudine*. AAPS PharmSciTech*, 2007. **8**(4): p. E1-E9.
56. Touitou, E.*, et al.*, Intracellular delivery mediated by an ethosomal carrier*. Biomaterials*, 2001. **22**: p. 3053-3059.
57. Song, C.K.*, et al.*, A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation*. Colloids and Surfaces B: Biointerfaces*, 2012. **92**: p. 299-304.
58. Abdulbaqi, I.M.*, et al.*, Ethosomal nanocarriers: The impact of constituents and formulation techniques on ethosomal properties, in vivo studies, and clinical trials*. International journal of nanomedicine*, 2016. **11**: p. 2279-2304.
59. Bagde, A., A. Mondal, and M. Singh, Drug delivery strategies for chemoprevention of uvb-induced skin cancer: A review*. Photodermatology, photoimmunology & photomedicine*, 2018. **34**(1): p. 60-68.
60. Shaji, J. and S. Garude, Ultradeformable vesicles for enhanced transdermal delivery*. Indo American journal of pharmaceutical research*, 2014. **4**(8): p. 3588-3596.
61. Shaji, J. and S. Garude, Development and characterisation of ketorolac tromethamine encapsulated transethosomes for enhanced transdermal development*. World journal of pharmacy and pharmaceutical sciences*, 2014. **3**(9): p. 1213-1227.
62. Mingzhu, L.*, et al.*, Dual drug encapsulation in a novel nano-vesicular carrier for the treatment of cutaneous melanoma: Characterization and in vitro/in vivo evaluation*. RSC Advances*, 2015. **5**(26): p. 20467-20478.
63. Ma, M.*, et al.*, Development of nanovesicular systems for dermal imiquimod delivery: Physicochemical characterization and in vitro/in vivo evaluation*. Journal of materials science: Materials in medicine*, 2015. **26**(6): p. 191.
64. Kumar, L.*, et al.*, Ethanol based vesicular carriers in transdermal drug delivery: Nanoethosomes and transethosomes in focus*. Nanoworld journal*, 2016. **2**(3): p. 41-51.
65. Kohane, D.S. and R. Langer, Biocompatibility and drug delivery systems*. The royal society of chemistry*, 2010. **1**: p. 441–446.
66. Zhang, X.Q.*, et al.*, Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine*. Advanced drug delivery reviews*, 2012. **64**(13): p. 1363-1384.
67. Shvedova, A.A., V.E. Kagan, and B. Fadeel, Close encounters of the small kind: Adverse effects of man-made materials interfacing with the nano-cosmos of biological systems*. Annual review of pharmacology and toxicology*, 2010. **50**: p. 63-88.

68. Hulzebos, E.*, et al.*, Use of structural alerts to develop rules for identifying chemical substances with skin irritation or skin corrosion potential*. QSAR & combinatorial science*, 2005. **24**(3): p. 332-342.
69. Yamaguchi, Y. and V.J. Hearing, Physiological factors that regulate skin pigmentation*. Biofactors*, 2009. **35**(2): p. 193-199.
70. Park, Y.H.*, et al.*, Assessment of dermal toxicity of nanosilica using cultured keratinocytes, a human skin equivalent model and an in vivo model*. Toxicology*, 2010. **267**(1-3): p. 178-181.
71. Blaauboer, B.J.*, et al.*, The use of biomarkers of toxicity for integrating in vitro hazard estimates into risk assessment for humans*. ALTEX*, 2012. **29**(4): p. 411-425.
72. Nel, A.*, et al.*, A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment*. Perspectives - American chemical society*, 2013. **7**(8): p. 6422–6433.
73. Nel, A.*, et al.*, Nanomaterial toxicity testing in the 21st century: Use of a predictive toxicological approach and high-throughput screening. *Accounts of chemical research*, 2013. **46**(3): p. 607-621.
74. Roursgaard, M.*, et al.*, In vitro toxicity of cationic micelles and liposomes in cultured human hepatocyte (HepG2) and lung epithelial (A549) cell lines*. Toxicology in vitro*, 2016. **36**: p. 164-171.
75. Jones, D.P.*, et al.*, Mechanisms of pathogenesis in drug hepatotoxicity: Putting the stress on mitochondria. *Molecular interventions*, 2010. **10**(2): p. 98-111.
76. Tsujimoto, Y. and S. Shimizu, Role of the mitochondrial membrane permeability transition in cell death*. Apoptosis*, 2007. **12**(5): p. 835-840.
77. Hall, A.*, et al.*, Polyethylenimine architecture-dependent metabolic imprints and perturbation of cellular redox homeostasis*. Biochimica et Biophysica Acta*, 2015. **1847**(3): p. 328-342.
78. Jantzen, K.*, et al.*, Oxidative damage to DNA by diesel exhaust particle exposure in co-cultures of human lung epithelial cells and macrophages*. Mutagenesis*, 2012. **27**(6): p. 693-701.
79. Angius, F. and A. Floris, Liposomes and MTT cell viability assay: An incompatible affair. *Toxicology in vitro*, 2015. **29**(2): p. 314-319.
80. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays*. Journal of immunological methods*, 1983. **65**: p. 55-63.
81. Liu, Y.*, et al.*, Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction*. Journal of neurochemistry*, 1997. **69**: p. 581-593.

82. Diaz, G.*, et al.*, Journal of neurochemistryLocalization of MTT formazan in lipid droplets. An alternative hypothesis about the nature of formazan granules and aggregates*. European journal of histochemistry*, 2007. **51**(3): p. 213-218.
83. ISO10993, Biological evaluation of medical devices - part 5*: Tests for in vitro cytotoxicity.* ISO 10993, 2009. **5**.
84. Soenen, S.J., A.R. Brisson, and M. De Cuyper, Addressing the problem of cationic lipid-mediated toxicity: The magnetoliposome model*. Biomaterials*, 2009. **30**(22): p. 3691-3701.
85. Chen, Z.X.*, et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers*. European journal of pharmaceutical science*s, 2017. **99**: p. 240-245.
86. Perez, A.P.*, et al.*, Topical amphotericin B in ultradeformable liposomes: Formulation, skin penetration study, antifungal and antileishmanial activity in vitro*. Colloids and Surfaces B: Biointerfaces*, 2016. **139**: p. 190-198.

Supplementary material

In the previous study (Chapter 3) the formulation of the lipid nanocarriers (LN) was adapted and optimized and these formulas were utilized in the cell viability study.

1.1. Preparation of liposomes and ultradeformable carriers

The LN that were prepared were diluted to the desired concentrations as listed in Table 4.2.

Table 4.2: Dilution guide for the dilution of LN

Concentration needed	Stock suspension	Volume of stock needed (µl)	Volume of PBS needed to make up to 20 ml (ml)
100 µg/ml	1000 µg/ml	2000	18
100 µg/ml	1000 µg/ml	200	19.8
10 µg/ml	100 µg/ml	2000	18
1 µg/ml	100 µg/ml	200	19.8
0.1 µg/ml	10 µg/ml	200	19.8

1.2. Cell optimization study

Before biocompatibility of the different vesicles could be determined, an optimal cell density was obtained to ensure that the cells would show a constant growth pattern. Therefore, in this section, the cell optimization study using MTT will be discussed.

1.2.1. Methodology

A375 and HaCat cells were seeded in a 96-well plate at a density of 1.6×10^6 cells/ml, 8.0×10^5 cells/ml, $4.0.\times10^5$ cells/ml, 2.0×10^5 cells/ml and 1.0×10^5 cells/ml (as shown in Figure 4.4) and will be incubated at 37ºC (5% CO_2, >90% humidity) for 24 h. This allowed the cells to adhere to the walls of the plate. The MTT method followed is described in the article.

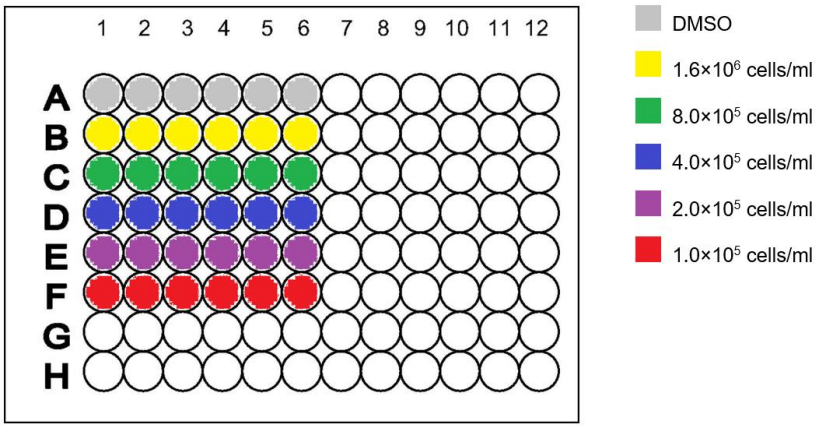


Figure 4.4: *Seeding of cells in 96-well plate for cell concentration optimization study*

1.2.2. Results and discussion

Exponential growth was observed for both the A375 and HaCat cell lines, as depicted in Figure 4.5, although a plateau was reached at a cell density of 500 000 cells/ml and higher. A reason for this could be because the cells are at a much higher confluency, causing the cells to grow at a slower pace, due to the cells not having enough space to grow, whereas Angius and Floris [1] obtained more linear growth due to the use of 12-well plates instead of 96-well plates. The cell viability (absorbance in nm) of both cell lines were extremely similar, with minor differences at the different cell densities. This conformity suggests that the method for seeding the cells, as well as determining their viability is valid. A linear regression was performed on the cell viability graphs to determine the point of best linearity, which would then be used to determine the optimal cell density (cells/ml) in which the cells would be seeded for the further experimentation. It was determined that an r^2-value of 0.0.0079 was obtained from linear regression of the A375 cell line (as shown in Fig.4.5. A and Fig. 4.5.C) and an r^2-value of 0.0.8580 was obtained from the HaCat cell line (as shown in Fig. 4.5.B and Fig. 4.5.D). These values suggested that a cell density of 100 000 cells/ml would be the optimal density for both cell lines for the further experiments that needed to be performed, as it suggests that constant growth can be expected from these cells.

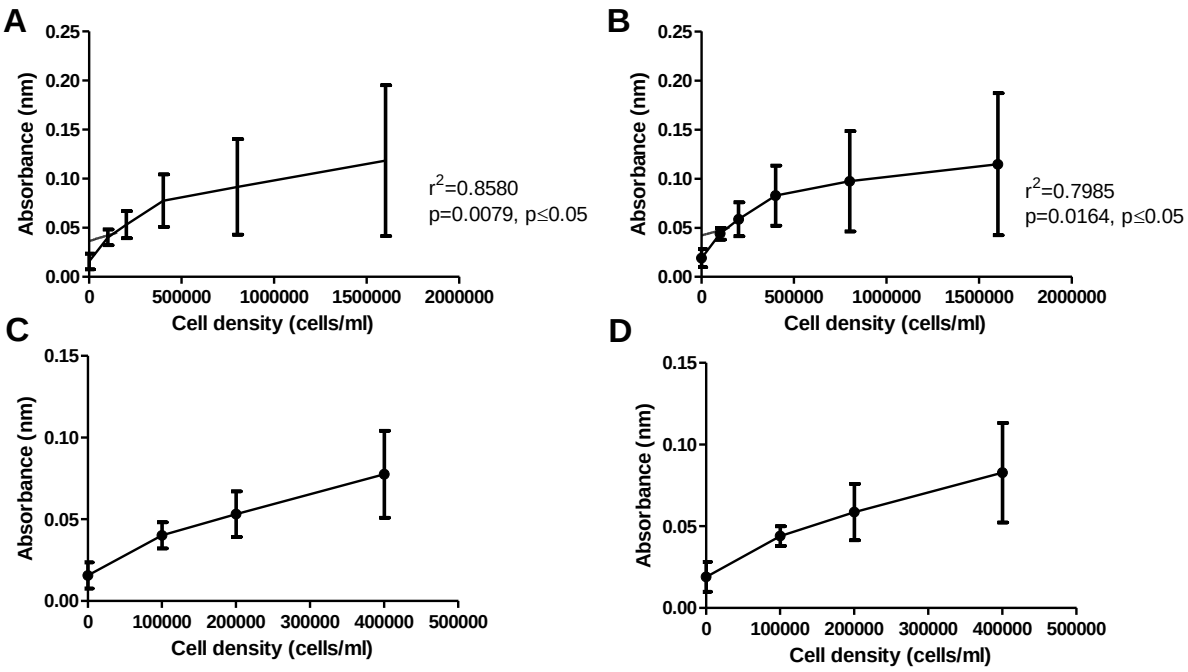


***Figure 4.5**: A. Absorbance in nm (mean ± SD) of A375 cells seeded in different cell densities (cells/ml) incubated for 24 h and determined using MTT assay, n=3, Linear regression was performed, p=0.0079, therefore, data is statistically significant; B. Absorbance in nm (mean ± SD) of HaCat cells seeded in different cell densities (cells/ml) incubated for 24 h and determined using MTT assay, n=3, Linear regression was performed, p=0.0164, therefore, data*

is scientifically significant; C. Absorbance in nm (mean ± SD) of the A375 cells seeded in different cell densities (cells/ml) to indicate linearity of cell growth; D. Absorbance in nm (mean ± SD) of the HaCat cells seeded in different cell densities (cells/ml) to indicate linearity of cell growth

This study was designed to determine the optimal cell densities by which the next studies would be performed. It was confirmed that the methods used for the growth of cell cultures, the seeding of the 96-well plates, as well as the MTT study are acceptable for further use. An optimal cell density of 100 000 cells/ml was determined for both the A375 and HaCat cell lines. This cell density will be used throughout the vesicle concentration, as well as the treatment time study.

1.3. Vesicle concentration biocompatibility study

1.3.1. Methods

A375 and HaCat cells were seeded in a 96-well plate at a density of 1.6×10^6 cells/ml, 8.0×10^5 cells/ml, $4.0.\times10^5$ cells/ml, 2.0×10^5 cells/ml and 1.0×10^5 cells/ml (as shown in Figure 4.6) and was incubated at 37ºC (5% CO_2, >90% humidity) for 24 h. This allowed the cells to adhere to the walls of the plate.

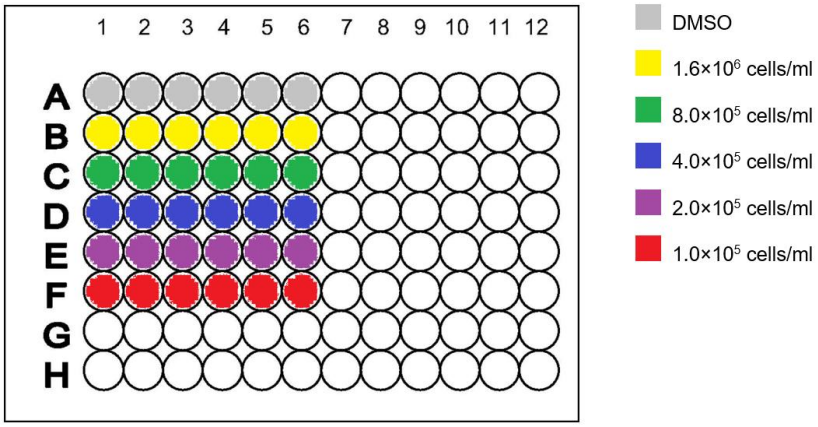


***Figure 4.6**: Vesicle concentration time study*

The cells were treated with the UDV after the incubation period. Treatment duration was 24 h. Following treatment the MTT assay was performed as described in the article. This study was performed in triplicate on separate occasions

1.4. Treatment time study

1.4.1. Methods

1.4.1.1. MTT assay

A375 and HaCat cells were seeded in a 96-well plate at a density of 1.0×10^5 cells/ml (as shown in Figure 4.7) and will be incubated at 37ºC (5% CO_2, >90% humidity) for 24 h. This allowed the cells to adhere to the walls of the plate.

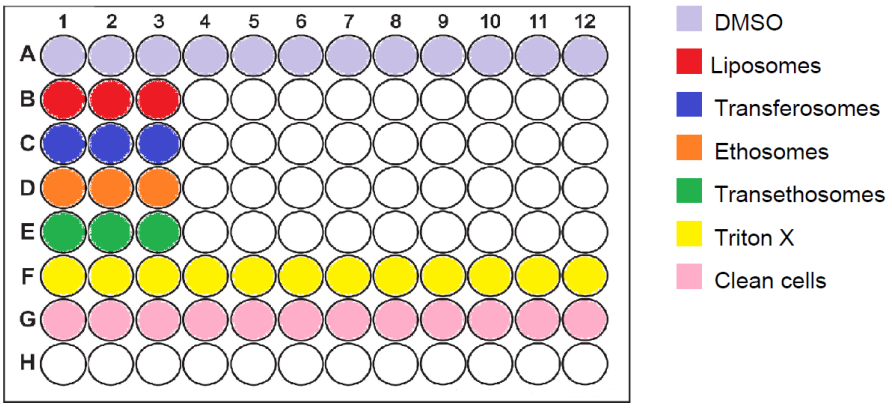


***Figure 4.7**: Seeding and treatment of the MTT 96-well plate for treatment time study*

The cells were treated with the UDV after the incubation period. Treatment duration was between 0,24 and 48 h. Following treatment the MTT assay was performed on the MTT marked plates as described in the article. This study was performed in triplicate on separate occasions.

1.4.1.2. Trypan Blue dye exclusion assay

Figure 4.8 illustrates how the 96-well plates were seeded during the treatment time study.

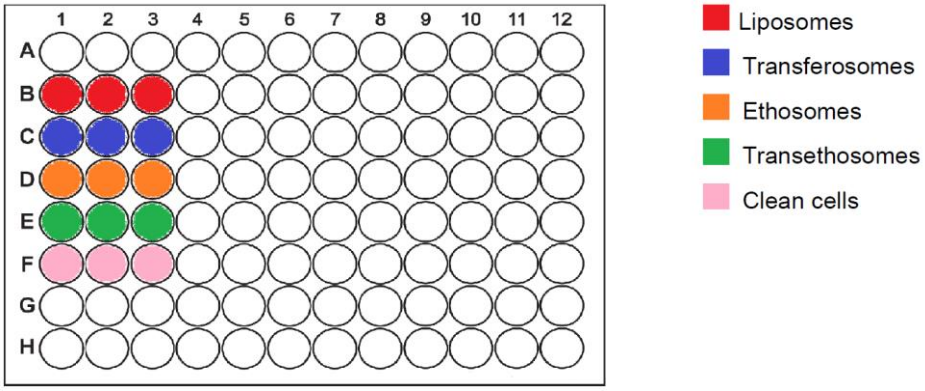


***Figure 4.8**: Seeding and treatment of the Trypan Blue 96-well plate for treatment time study*

Table 4.3 and Table 4.4 show examples of how the cells were photographed and counted before cell viability was calculated, during the Trypan Blue dye exclusion assay.

***Table 4.3**: Example of counting HaCat cells after 48 h using Trypan Blue (Liposomes)*

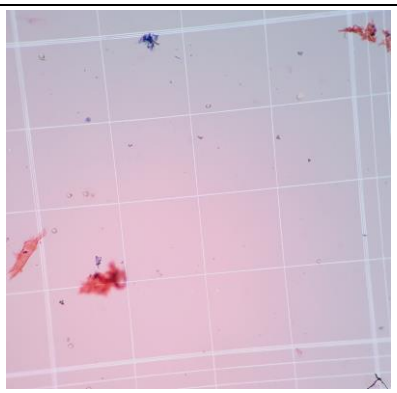
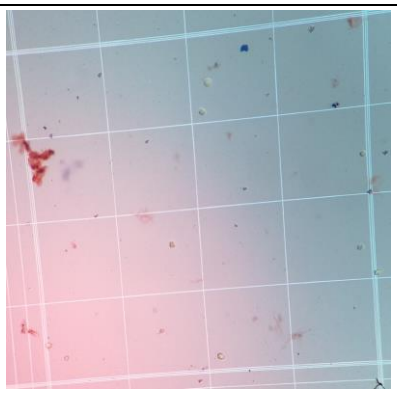
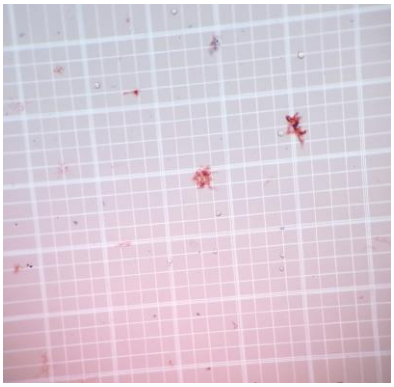
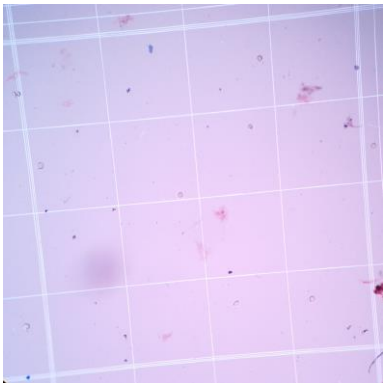
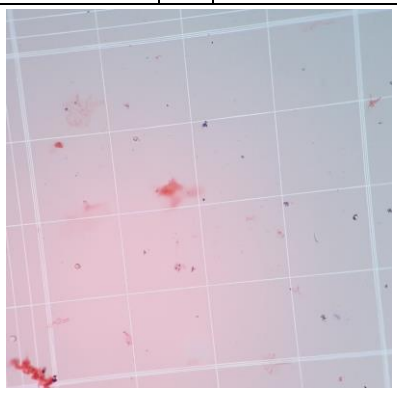
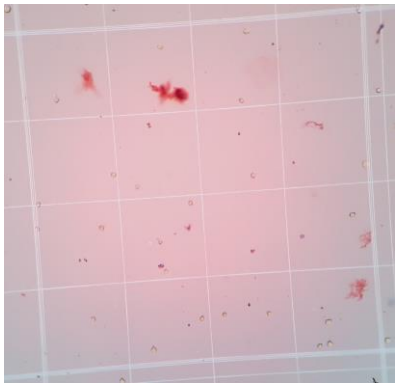
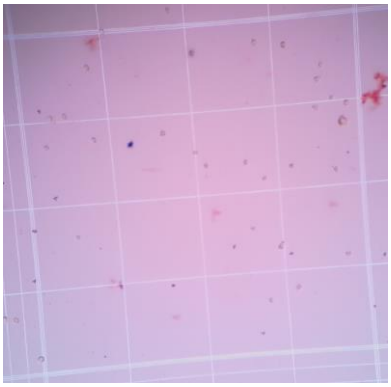
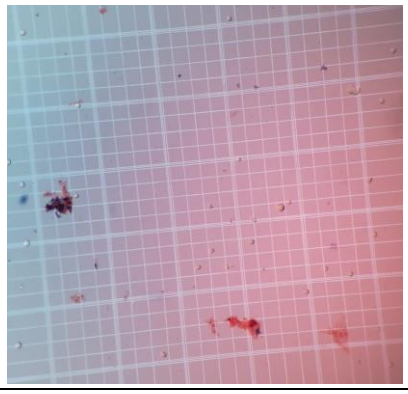
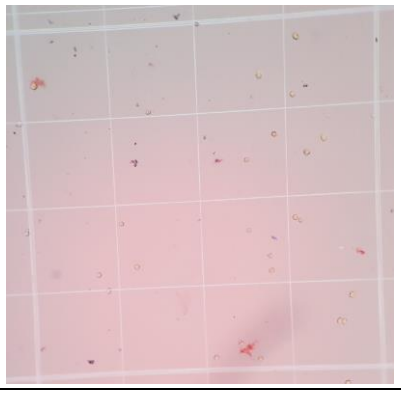
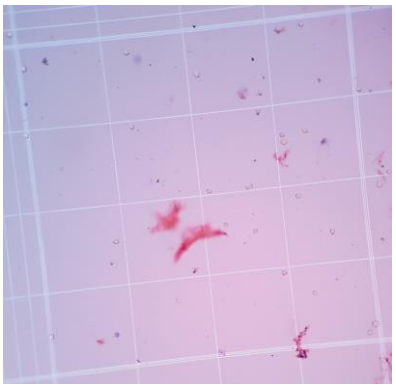
1.		2.	
3.		4.	
5.			

***Table 4.4**: Example of counting A375 cells after treatment of 48 h using Trypan Blue (Liposomes)*

1.		2.	
3.		4.	
5.			

1.5. References

1. Angius, F. and A. Floris, Liposomes and MTT cell viability assay: An incompatible affair. *Toxicology in vitro*, 2015. **29**(2): p. 314-319.

CHAPTER 5 – Conclusion

5.1. Concluding discussion

The use of transdermal and topical products have gained much attention throughout history, more so during recent years with development of novel formulations as lipid nanocarriers (LN) to enhance skin penetration [1, 2]. Accompanying these new formulations, is the concern for skin toxicity, which could be related to a wide range of parameters, such as shape and size surface area [3-6]. Toxicity would in turn have a great effect on biocompatibility, which is defined as "an expression of the benignity of the relation between a substance and its biological environment" [7]. Comprehensive characterization is the first step in determining biocompatibility of novel LN, and the next step is utilizing various cell viability assays to establish the viability of cell lines treated with the specific LN. This study focused on the ultradeformable vesicles (UDV), transferosomes, ethosomes and transethosomes, comparing them to their well-known predecessor, liposomes. The aim of this study was to determine the *in vitro* biocompatibility of the UDVs, focusing on the possible interference the lipid content of the LN could have with the standardized MTT assay. Additional to this aim, a secondary aim was to investigate whether quantitative analysis could be used as complementary means to characterize LN, in addition to the standardized DLS.

During this study the different LN were formulated and optimized, including the UDVs (transferosomes, ethosomes and transethosomes), and liposomes as a control. The LN underwent characterization by means of dynamic light scattering (DLS) utilizing the Malvern Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK), measuring diameter, PDI and zeta potential, as well as image analysis by means of the Malvern Morphologi G3 (Malvern Instruments Ltd., Malvern, Worcestershire, UK), measuring parameters concerning vesicle shape and size. These parameters include CE diameter, intensity mean, solidity, elongation, convexity, circularity and aspect ratio. The effects of storage were also determined by means of a stability study over 90 days. Due to the focus of Morphologi G3 characterization on powder particle shape up to date, little information was known about its use in lipid vesicle characterization.

The LN were successfully characterized with DLS and image analysis. The diameter of the vesicles was mostly within the micro range, but the transferosomes and transethosomes showed the smallest, and therefore the most likely to penetrate the skin for transdermal delivery. The larger size of the liposomes were similar to findings of Esposito *et al.* [8]. This smaller range was similar to Ascenso *et al.* [9] and Song *et al.* [10], who found these vesicles, especially the transethosomes were small enough to penetrate the skin, although the results obtained were much smaller vesicles than in this study, as were the findings of Bragagni *et al.* [11] who observed the size of transferosomes when loaded with the drug celecoxib. The LN proved stable over the 90-day study; the PDI of the vesicles were never below 0.6, which was an indicator that the size

distribution of the LN would be more unstable. The transferosomes and transethosomes, however, showed a stable PDI over the course of 90 days, whereas the liposomes and ethosomes were more unstable. These results were consistent with findings from Bragagni *et al.* [11], Habib *et al.* [12], Shumilov and Touitou [13] and Verma and Pathak [14].

The zeta potential of the LN were not as favorable as desired for maximum stability [9, 15, 16], due to the buffering to a neutral pH for a non-irritant application to the skin. The zeta potential of the LN remained fairly stable over the course of this study, with the transferosomes and ethosomes showing the most promise of stability over time. The use of PBS to buffer the LN systems, caused the pH to remain stable over the course of the study. Similar findings were obtained by Chen *et al.* [15], Shumilov and Touitou [13] and Vanic *et al.* [17].

Visual inspection, by means of the Malvern Morphologi G3, throughout the course of the study, concluded that the LN formed spherical vesicles, containing an aqueous phase, confirming stability over 90 days. No agglomeration of LN was observed throughout the study. After characterization by the Malvern Morphologi G3, it was concluded that elongation, convexity and circularity were the parameters that would best describe the LN. The LN showed low levels of elongation throughout the study, only varying slightly because of the elasticity of the vesicles. The vesicles showed high convexity throughout the course of 90 days, but the circularity of the vesicle only increased slightly over time. After the study was performed, it was concluded that the use of the Malvern Morphologi G3 would be best suited as a complementary means, and that the LN were relatively stable over the course of 90 days. The LN portrayed lower intensity mean values, lower elongation [18, 19] and higher circularity [18-20] when compared to previous studies analyzing powder particles, such as cement or talc [18, 21]. The convexity values observed for the LN were varied, but were lower than that observed in powder particles [18, 19]. The solidity of the LN were lower when compared to the high solidity of volcanic ash powder particles [22].

The following step investigating biocompatibility of the UDV in comparison with liposomes, was the cell viability of different cell lines when treated with the LN, utilizing human malignant melanoma cells (A375) and primary epidermal keratinocytes cells (HaCat), as well as determining whether the lipid content of these vesicles would have an effect on the absorbance of the MTT assay. Initially, a cell optimization study was performed to determine optimal seeding cell density, after which it was concluded that a cell density of 100 000 cells/ml would be seeded in each well for the following studies. One study performed was to observe the effects of different vesicle concentrations on the cell viability of the two cell lines, by means of MTT assay absorbance values. Another study observed the effect of treatment time over 48 h on the cell viability, as determined by the MTT assay, as well as the Trypan Blue dye exclusion assay, to pertain the possible interference of the UDV with the MTT assay.

It was concluded that the PC content of both the liposomes, as well as the UDVs, had a definite effect on the absorbance values of both the A375 and HaCat cells, when the MTT assay was performed during the vesicle concentration study. The absorbance values of the treated cells were considerably higher than those of their untreated counterparts (control), which confirmed the findings of Angius and Floris [23], as well as confirming that the UDV had similar interference.

During the treatment time study, a high rate of proliferation was observed for both the treated and untreated A375 cells, as determined by both the MTT and the Trypan Blue dye exclusion assay; therefore, suggesting that the liposomes, and the UDVs, did not inhibit the proliferation rate of the cells, nor were they toxic over the 48 h treatment period. The same, however, was not observed for the HaCat cell line, where cell viability started to decrease when treatment periods exceeded 24 h, suggesting that the liposomes, and the UDVs inhibited proliferation. After 48 h of treatment, signs of toxicity were observed for the cell viability, as determined by the MTT assay, although it could be speculated that a decrease in cell viability would also be observed for the Trypan Blue cells if treatment persisted longer than 48 h. Due to the excipients the LN's consisted of, low cytotoxicity should be observed, although the small size of nanocarriers portrayed a risk, due to the possibility of these vesicles interacting completely different to the cells in the body, in comparison to larger particles [24]. A previous study by Smith [25] in 1986 had already determined that smaller particles of a known non-toxic material could show cytotoxicity. Even though the excipients of which the LN consisted were generally regarded as safe (GRAS), a possible reason for the increase in cytotoxic levels over time could be due to the LN adhering to the membrane of the HaCat cells, where it would undergo degradation over time, thus releasing possible cytotoxic waste products [24].

It was, therefore, concluded that the UDVs were non-toxic to the A375 cells, as well as the HaCat cells to a certain extent, for shorter treatment periods, confirming that the UDVs were biocompatible for use as drug carriers for transdermal or topical products.

5.2. Limitations and recommendations

This study described the biocompatibility of transferosomes, ethosomes and transethosomes, by means of characterization through DLS and image analysis, as well as through cell viability studies utilizing MTT and Trypan Blue dye exclusion assays. The effect of the lipid content of these carriers on the cell viability results were also described utilizing liposomes as a control.

Firstly, this study investigated UDVs by means of comprehensive characterization, utilizing DLS and image analysis. In this study, images of the different vesicles were photographed under the 5x magnification objective, however, to ensure a clear image of the different vesicles, it is suggested that a larger magnification objective be used in future studies, as well as utilizing the Malvern Morphologi G3's feature to observe individual vesicles; this would, therefore, ensure that

images obtained of the LN, were more accurate. The use of laser diffraction could also be utilized to create a clearer characterization of the UDV. Laser diffraction is a laser beam that is passed through a dispersed sample, after which the angular variation in light intensity of the scattered light is measured [26], based on the Mie scattering theory, which could provide another perspective on the characteristics of these vesicles [27]. The characterization of the UDV could in turn be compared to other lipid carriers, such as SLN, to determine whether any similarities in structure could be present.

Lastly, the effects of the UDVs on the two skin cell lines, A375 and HaCat, were investigated. To obtain a better understanding on the effects of these drug carriers, the effect of UDVs on other skin cell lines could also be investigated. Cell lines, such as human primary epidermal melanocytes (HEMa, ATCC® PCS-200-013™), could be utilized to compare the effect of UDVs on healthy melanocytes versus the cancerous A375 line. Other cell lines include primary dermal microvascular endothelial cells (HDMVECn, ATCC® PCS-110-010™), primary dermal fibroblasts (HDFa, ATCC® PCS-201-012™) and primary epidermal keratinocytes (HEKa, ATCC® PCS-200-011™). The effects of another LN control, such as SLN, could also be used to compare their effects on the viability of the cells, to that of the UDV.

Another approach to ensure accuracy of the cell viability assays, is to select assays that utilize the same relative mechanism to determine cell viability. MTT assay measures cell viability by the uptake of MTT formazan by cells into the mitochondria, after which the dye undergoes reduction, creating a purple substance of which the wavelength is determined [28, 29]. The mechanism through which Trypan Blue dye exclusion assay works, is due to live cells that would not be taken up, however, dead cells would be stained blue, after which the live cells could be counted and viability calculated [30]. Alternatives for these assays could be the lactate dehydrogenase (LDH) cytotoxicity assay, which utilizes mitochondrial activity, and the live/dead cell assay by flow cytometry, which utilizes the exclusion technique.

UDVs were developed due to the inability of liposomes to cross the skin barrier to reach transdermal delivery [9, 31-33]. The use of transdermal delivery had been limited to a small number of drugs, due to difficulty of breaching the *stratum corneum.* Examples of these transdermal drugs are estrogen, testosterone, fentanyl, lidocaine and oxybutynin. Considering that these UDV are biocompatible with skin cells, as well as the small size of these vesicles suggesting that transdermal delivery could be possible, it is recommended that similar studies be performed, investigating the characterization, as well as the biocompatibility of drug-filled UDVs.

5.3. References

1. Kumar, L.*, et al.*, Fatty acid vesicles acting as expanding horizon for transdermal delivery*. Artificial cells, nanomedicine, and biotechnology*, 2017. **45**(2): p. 251-260.
2. Neubert, R.H., Potentials of new nanocarriers for dermal and transdermal drug delivery*. European journal of pharmaceutics and biopharmaceutics*, 2011. **77**(1): p. 1-2.
3. Pimentel, F.*, et al.*, Macroalgae-derived ingredients for cosmetic industry —an update*. Cosmetics*, 2017. **5**(1).
4. Chang, R.K.*, et al.*, Generic development of topical dermatologic products: Formulation development, process development, and testing of topical dermatologic products*. AAPS Journal*, 2013. **15**(1): p. 41-52.
5. Xia, T., N. Li, and A.E. Nel, Potential health impact of nanoparticles*. Annual review of public health*, 2009. **30**: p. 137-150.
6. Zhang, X.Q.*, et al.*, Interactions of nanomaterials and biological systems: Implications to personalized nanomedicine*. Advanced drug delivery reviews*, 2012. **64**(13): p. 1363-1384.
7. Kohane, D.S. and R. Langer, Biocompatibility and drug delivery systems*. The royal society of chemistry*, 2010. **1**: p. 441–446.
8. Esposito, E., E. Menegatti, and R. Cortesi, Ethosomes and liposomes as topical vehicles for azelaic acid: A preformulation study*. International journal of cosmetic science*, 2004. **26**(5): p. 270-271.
9. Ascenso, A.*, et al.*, Development, characterization, and skin delivery studies of related ultradeformable vesicles: Transfersomes, ethosomes, and transethosomes*. International journal of nanomedicine*, 2015. **10**: p. 5837-5851.
10. Song, C.K.*, et al.*, A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: Characterization and in vitro/in vivo evaluation*. Colloids and Surfaces B: Biointerfaces*, 2012. **92**: p. 299-304.
11. Bragagni, M.*, et al.*, Comparative study of liposomes, transfersomes and ethosomes as carriers for improving topical delivery of celecoxib*. Drug delivery*, 2012. **19**(7): p. 354-361.
12. Habib, B.A., S. Sayed, and G.M. Elsayed, Enhanced transdermal delivery of ondansetron using nanovesicular systems: Fabrication, characterization, optimization and ex-vivo permeation study-box-COX transformation practical example*. European journal of pharmaceutical science*s, 2018. **115**: p. 352-361.
13. Shumilov, M. and E. Touitou, *Buspirone transdermal administration for menopausal syndromes, in vitro and in animal model studies. International journal of pharmaceutics*, 2010. **387**(1-2): p. 26-33.
14. Verma, P. and K. Pathak, Nanosized ethanolic vesicles loaded with econazole nitrate for the treatment of deep fungal infections through topical gel formulation*. Nanomedicine*, 2012. **8**(4): p. 489-496.

15. Chen, Z.X., *et al.*, Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers. *European journal of pharmaceutical science*s, 2017. **99**: p. 240-245.
16. Abdellatif, A.A. and H.M. Tawfeek, Transfersomal nanoparticles for enhanced transdermal delivery of clindamycin. *AAPS PharmSciTech*, 2016. **17**(5): p. 1067-1074.
17. Vanic, Z., *et al.*, Characterization of various deformable liposomes with metronidazole. *Drug development and industrial pharmacy*, 2013. **39**(3): p. 481-488.
18. Ulusoy, U. and I. Kursun, Comparison of different 2D image analysis measurement techniques for the shape of talc particles produced by different media milling. *Minerals engineering*, 2011. **24**(2): p. 91-97.
19. Di Pretoro, G., *et al.*, Impact of needle-like crystals on wet and solid-lipid extrusion processes. *Powder technology*, 2015. **270**: p. 476-483.
20. Krupa, A., *et al.*, Preparation of solid self-emulsifying drug delivery systems using magnesium aluminometasilicates and fluid-bed coating process. *Powder technology*, 2014. **266**: p. 329-339.
21. Yanrong, Z., *et al.*, Characterization of the mesostructural organization of cement particles in fresh cement paste. *Construction and building materials*, 2016. **124**: p. 1038-1050.
22. Liu, E.J., K.V. Cashman, and A.C. Rust, Optimising shape analysis to quantify volcanic ash morphology. *GeoResJ*, 2015. **8**: p. 14-30.
23. Angius, F. and A. Floris, Liposomes and MTT cell viability assay: An incompatible affair. *Toxicology in vitro*, 2015. **29**(2): p. 314-319.
24. Pardeike, J., A. Hommoss, and R.H. Muller, Lipid nanoparticles (sln, nlc) in cosmetic and pharmaceutical dermal products. *International journal of pharmaceutics*, 2009. **366**(1-2): p. 170-184.
25. Smith, A., Evaluation of poly (lactic acid) as a biodegradable drug delivery system for parenteral administration. *International journal of pharmaceutics*, 1986. **30**(2-3): p. 215-220.
26. Malvern Instruments Limited, *Malvern - material relationships: Mastersizer 3000 - smarter particle sizing*, in *Malvern Instruments Ltd.*, M.I. Ltd., Editor.: Malvern, Worcestershire, UK.
27. Ltd., M.I., *Malvern mastersizer 3000 user manual*, M.I. Ltd, Editor. 2017: Malvern, Worcestershire, UK.
28. Liu, Y., *et al.*, Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of neurochemistry*, 1997. **69**: p. 581-593.
29. Diaz, G., *et al.*, Journal of neurochemistryLocalization of MTT formazan in lipid droplets. An alternative hypothesis about the nature of formazan granules and aggregates. *European journal of histochemistry*, 2007. **51**(3): p. 213-218.
30. Louis, K.S. and A.C. Siegel, *Cell viability analysis using trypan blue: Manual and automated methods. Methods in molecular biology*, 2011. **740**: p. 7-12.

31. Ascenso, A.*, et al.*, In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. *European journal of pharmaceutics and biopharmaceutics*, 2014. **88**(1): p. 48-55.
32. Cevc, G. and G. Blume, Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force*. Biochimica et Biophysica Acta*, 1992. **1104**: p. 226-232.
33. Manosroi, A.*, et al.*, Novel elastic nanovesicles for cosmeceutical and pharmaceutical applications*. Chiang Mai journal of science*, 2009. **36**(2): p. 168-178.

APPENDIX A –ETHICAL TRAINING CERTIFICATE

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support
Tel: 018 299 2092
Fax:
Email: minrie.greeff@nwu.ac.za

10 February 2017

Dear Ms Michelle Niemand (HPCSA registration number: _____)

PROOF OF ATTENDANCE

This letter certifies that you have attended the 2 day ethics training, entitled:

The Basics of Health Research Ethics

(Accreditation number: UP1163 from University of Pretoria CPD accreditation department)

presented by Prof Minrie Greeff (Head of the Health Sciences Ethics Office for Research, Training and Support) on **23 and 24 January 2017**.

This proof of attendance, as recognised by HREC and the Ethics Office, NWU, is valid for 3 years and expires on the 24th of January 2020. Where applicable, Ethics CEUs awarded: ***27 Ethics CEUs***

Yours sincerely

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Prof Awie Kotzé
Dean of Faculty of Health Sciences

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support
Tel: 018-299 2092
Fax:
Email: Minrie.Greeff@nwu.ac.za

09 February 2017

Dear Ms Michelle Niemand

PROOF OF ATTENDANCE

This letter certifies that you have attended the 1-day ethics training, entitled:

The SANS document: As regulation for research with animals

presented by Prof Christiaan B Brink (Chair of AnimCare) on the 25 February 2017.

This proof of attendance, as recognised by AnimCare and the Ethics Office, NWU, is valid for 3 years and expires on the 25th February 2020.

Yours sincerely

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Prof Awie Kotzé
Dean of Faculty of Health Sciences

APPENDIX B – EDITING CERTIFICATE

Gill Smithies

Proofreading & Language Editing Services

59, Lewis Drive, Amanzimtoti, 4126, Kwazulu Natal

Cell: 071 352 5410 E-mail: moramist@vodamail.co.za

Work Certificate

To	Michelle Volkwyn
Address	Centre of Excellence for Pharmaceutical Studies, Potchefstroom Campus, North West University
Date	13/11/2018
Subject	In vitro biocompatibility of transferosomes, ethosomes and transethosomes
Ref	GS/MV /01

I, Gill Smithies, certify that I have proofed the following dissertation,
In vitro biocompatibility of transferosomes, ethosomes and transethosomes
-Chapters 1 to 5 and Abstract,
to the standard as required by NWU, Potchefstroom Campus.

Gill Smithies

13/11/2018