STUDY OF NEW AGE LIPOSOMES: TRANSETHOSOMES

by

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Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Chemistry
Boğaziçi University

2019

STUDY OF NEW AGE LIPOSOMES: TRANSETHOSOMES

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DATE OF APPROVAL: 29.05.2019

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Naz Zeynep Atay Gök for welcoming me into her study group and making me feel like at home, for her guidance and endless patience. I feel deep gratitude and more than lucky to have studied under her guidance. Her positive attitude towards all situations has taught me to persist through all conditions with hard work.

I am thankful for the review and valuable input of my committee members Assoc. Prof. Şaron Çatak from Boğaziçi University and Assoc. Prof. Elif Çalışkan Salihi from Marmara University Faculty of Pharmacy.

I deeply appreciate the help of my fellow group member MSc Derya Karagöz. She has been the inspiration for me to go into my field of study, making me love my subject through her enthusiasm for the profession. She is a rare combination of encouragement and persistence.

I would like to thank Dr Bilge Gedik Uluocak in R&D Center at Boğaziçi University for her guidance throughout my analyses. I would also like to thank my friends Yağmur Baş and Beste Özaydın for their support, help and valuable input during my studies.

My sincere thanks belong to my family, my mother, Nesrin Aslan, my father, Erhan Aslan, my aunt Dr İnci Göl, my brother, Can Aslan and my sister-in-law Duygu Çakırsoy Aslan who have always supported me in every way in my choices and my goals. Knowing they are always there for me with their unconditional love and support guides me through hard days.

My deepest gratitude belongs to my loving husband, Cemil Güler. He never hesitated to support and guide me in everything I do. Not only he was always there for me, but he also made life so much easier and fun.

Lastly, I would like to thank Lipoid GmbH for providing technical samples that were of key importance.

This study is supported by the Boğaziçi University Research Foundation with the project number 14503.

ABSTRACT

STUDY OF NEW AGE LIPOSOMES: TRANSETHOSOMES

Liposomes are vesicles formed by the self-arrangement of amphiphilic molecules into bilayer form, enclosing an aqueous core. The fact that these vesicles resemble the skin structure makes them ideal carriers for active drug or cosmetic ingredients into the skin.

In order to effectively deliver ingredients under the skin, deformable vesicles such as ethosomes or transfersomes are designed. A new form of deformable vesicles is the transethosomes. These vesicles can deform when under stress without breaking and release their content once they are under the skin. Transethosomes are vesicles containing edge activators or permeation enhancers and ethanol.

In this study, transethosomes containing different edge activators/permeation enhancers were investigated and compared. Ethanol and oleic acid were incorporated within the lipid bilayer as permeation enhancers and polysorbate (Tween®80) was added separately as an edge activator. Effects of different preparation methods were reported in terms of size, size distribution, surface charge and morphology. Also effects of different concentrations of edge activator/permeation enhancer were investigated. Considering their wide biochemical and pharmacological benefits and the fact that transethosomes can better encapsulate lipophilic molecules, two essential oils, geraniol and linalool were encapsulated.

ÖZET

YENİ NESİL LİPOZOMLAR: TRANSETOZOMLAR

Lipozomlar amfifilik moleküllerin kendiliğinden ikili tabakalar formunda, ortalarında sulu bir kısım bırakarak oluşturdukları vesiküllerdir. Cilt yapısına benzerliklerinden dolayı bu vesiküller ilaç ve kozmetik aktif maddelerini cilt içine taşımak için ideal yapılardır.

Aktif maddeleri cilt altına taşıyabilmek için etozom ve transferzom gibi deforme olma özelliği gösteren vesiküller tasarlanmıştır. Transetozomlar deforme özelliğine sahip yeni nesil vesiküllerdir. Transetozomlar, üzerlerine bir güç uygulandığında kırılmadan deforme olarak deri altına geçer, orada bozunarak taşıdıkları aktif maddeleri deri altına ulaştırırlar. Transetozomlar kenar aktivatörü/geçirgenlik artırıcılar ve etanol kullanılarak yapılır.

Bu çalışmada, değişik kenar aktivatörü/geçirgenlik artırıcı kullanılarak yapılan transetozomlar araştırılarak karşılaştırılmıştır. Etanol ve oleik asit lipid tabakaya geçirgenlik artırıcı olarak, polisorbat (Tween®80) ise kenar aktivatörü olarak ayrıca eklenmiştir. Farklı hazırlama metotları uygulanarak elde edilen transetozomlar boyut, boyut dağılımı, yüzey yükü ve morfoloji olarak karşılaştırılmıştır. Ayrıca farklı kenar aktivatörü/geçirgenlik artırıcı konsantrasyonları denenerek farkları araştırılmıştır. Geniş biyokimyasal ve farmakolojik özellikleri nedeniyle ve transetozomların lipofilik maddeleri daha iyi enkapsüle ettikleri göz önünde bulundurularak, geraniol ve linalool esansiyel yağları enkapsüle edilmiştir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

ABSTRACT v

ÖZET vi

LIST OF FIGURES x

LIST OF TABLES xiv

LIST OF ACRONYMS / ABBREVIATIONS xv

1. INTRODUCTION 1

1.1. The Skin 1

1.1.1. Structure 1

1.1.1. Functions 5

1.1.2. Dermal Absorption 6

1.1.3. Factors Affecting Permeability 8

1.1.4. Overcoming the Barrier 9

1.1.5. Skin Aging & Use of Antioxidants 12

1.2. Lipids 15

1.2.1. Membrane Lipids 16

1.3. Vesicles and Encapsulation 19

1.3.1. Conventional Liposomes 20

1.3.2. Transfersomes 22

1.3.3. Ethosomes 23

1.3.4. Transethosomes 25

1.4. Essential Oils and their Incorporation in Vesicles 27

1.5. Transethosome Preparation Methods and their Effects 30

1.6. Vesicle Characterization 32

1.6.1. Visualization 32

1.6.2. Size and Size Distribution 33

1.6.3. Surface Charge Determination 33

2. AIM OF THE STUDY 35

3. EXPERIMENTAL 36

3.1. Materials 36

3.1.2. Lipid 36

3.1.3. Edge activator/Permeation enhancers 36

3.1.4. Encapsulated Materials 37

3.1.5. Solvents 38

3.1.6. Suspension Media 38

3.1.7. STEM Staining 38

3.2. Instruments 38

3.3. Methods 40

3.3.1. Preparation of Transethosomes....40

3.3.2. Encapsulation of Transethosomes....41

4. RESULTS & DISCUSSION....43

4.1. Comparison of Different Preparation Methods....43

4.2. Effects of the Choice and Concentrations ofaPermeation Enhancer or an Edge Activator....48

4.3. Empty vs Loaded Transethosomes with Different Materials....51

4.4. Effects of Downsizing Methods....55

4.5. Encapsulation Efficiency....56

4.6. Zeta Potential Measurements....56

5. CONCLUSIONS....58

6. FUTURE WORK....60

REFERENCES....61

APPENDIX A: DLS ANALYSIS RESULTS....77

LIST OF FIGURES

Figure 1.1. The epidermis (Kielhorn *et al.*, 2006). 2

Figure 1.2. The structure of the skin (Kielhorn *et al.*, 2006). 4

Figure 1.3. Two possible ways of absorption through the SC (Kielhorn *et al.*, 2006). 8

Figure 1.4. Comparison of different delivery methods. (Waghule *et al.*, 2019)..... 11

Figure 1.5. Radiation types in the solar spectrum (Krutmann *et al.*, 2017)..... 12

Figure 1.6. Triglycerides (Ball *et al.*, 2011). 16

Figure 1.7. Cell membrane (Ball *et al.*, 2011). 17

Figure 1.8. Self-assembly of lipids in water (Ball *et al.*, 2011). 18

Figure 1.9. Phosphatidylethanolamines and phosphatidylcholines (Ball *et al.*, 2011). 19

Figure 1.10. Interaction mechanisms of lipid vesicles with the skin (Zhai & Zhai, 2014). 20

Figure 1.11. Liposome, Transfersome and ethosome structures (Sala *et al.*, 2018).. 23

Figure 1.12. Mechanism of vesicle passage through the SC (Sala *et al.*, 2018)..... 24

Figure 1.13. Ethosomal studies in recent years (Abdulbaqi *et al.*, 2016). 25

Figure 1.14. The cold method (Abdulbaqi *et al.*, 2016). 31

Figure 1.15. Thin-film hydration method (Demetzos, 2016). 32

Figure 1.16. Zeta potential ('HORIBA', n.d.). 34

Figure 3.1. Chemical structure of Phosphatidylcholine. 36

Figure 3.2. Chemical structure of Oleic Acid. 37

Figure 3.3. Chemical structure of Tween®80. 37

Figure 3.4. Chemical structures of Geraniol (left) and Linalool (right). 37

Figure 4.1. Empty transethosomes with min oleic acid via cold method. 46

Figure 4.2. Empty transethosomes with min Tween®80 via cold method. 46

Figure 4.3. Empty transethosomes with min Oleic acid via thin-film hydration method. 47

Figure 4.4. Empty transethosomes with min Tween®80 via thin-film hydration method. 47

Figure 4.5. Empty transethosomes with max oleic acid via cold method. 50

Figure 4.6. Empty transethosomes with max Tween®80 via cold method. 50

Figure 4.7. Empty transethosomes with max Oleic acid via thin-film hydration. 51

Figure 4.8. Transethosomes with oleic acid and linalool via cold method. 53

Figure 4.9. Transethosomes with Tween®80 and linalool via cold method. 53

Figure 4.10. Transethosomes with oleic acid and geraniol via cold method. 54

Figure 4.11. Transethosomes with Tween®80 and geraniol via cold method. 54

Figure 4.12. Transethosomes with oleic acid and linalool via thin-film hydration. .. 55

Figure A.1. Transethosomes via cold method with min Tween®80. 77

Figure A.2. Transethosomes via cold method with max Tween®80. 77

Figure A.3. Transethosomes via cold method with min oleic acid. 78

Figure A.4. Transethosomes via cold method with max oleic acid. 78

Figure A.5. Transethosomes via thin-film hydration method with min Tween®80. 79

Figure A.6. Transethosomes via thin-film hydration method with max Tween®80……...79

Figure A.7. Transethosomes via thin-film hydration method with min oleic acid……80

Figure A.8. Transethosomes via thin-film hydration method with max oleic acid. 80

Figure A.9. Transethosomes via cold method with Tween®80 and linalool. 81

Figure A.10. Transethosomes via cold method with Tween®80 and geraniol. 81

Figure A.11. Transethosomes via cold method with oleic acid and linalool. 82

Figure A.12. Transethosomes via cold method with oleic acid and geraniol. 82

Figure A.13. Transethosomes via thin-film hydration method with oleic acid and linalool. 83

Figure A.14. Transethosomes via thin-film hydration method with oleic acid and geraniol. 83

Figure A.15. Transethosomes via thin-film hydration method with Tween®80 and linalool. 84

Figure A.16. Transethosomes via thin-film hydration method with Tween®80 and geraniol. 84

LIST OF TABLES

Table 4.1 Comparison of different preparation methods. 45

Table 4.2 Size (nm) and PDI of empty transethosomes for comparisonpermeation enhancer, edge activator and their concentrations. 48

Table 4.3 Size and PDI of loaded transethosomes for comparison of permeation enhancer and edge activator (nm) (PDI). 49

Table 4.4 Size (nm) and size distribution of empty and loaded transethosomes. 52

Table 4.5 Zeta Potential Values of all transethosomes. 57

LIST OF ACRONYMS / ABBREVIATIONS

DLS	Dynamic light scattering
EA	Edge activator
EtOH	Ethanol
HLB	Hydrophilic-lipophilic balance
IR	Infrared
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NaOH	Sodium hydroxide
NLC	Nanostructured lipid carrier
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PDI	Polydispersity Index
PE	Penetration enhancer
ROS	Reactive oxygen species
SC	Stratum corneum
SEM	Scanning electron microscopy
SLN	Solid lipid nanoparticles
STEM	Scanning transmission electron microscopy
SUV	Small unilamellar vesicles
TEM	Transmission electron microscopy
UV	Ultraviolet
UVR	Ultraviolet radiation
VL	Visible light

1. INTRODUCTION

1.1. The Skin

1.1.1. Structure

The outermost protective layer of our body is our skin. It protects us by acting as a barrier for foreign substances and by preventing water loss from the body. It stretches to about 1.2 – 2.2 m^2 total, weighs about 4 – 5 kg and covers the entire body; its thickness varies between 1.5 – 4.0 mm depending on the body region. The upper skin layer is the epidermis and the underlying connective fibrous tissue is the dermis. The nutrients diffuse through the various layers of these tissues and reach the epidermis.

Below the dermis, there is a mostly adipose tissue which connects the skin to the mostly muscle tissues below it. This connection helps the skin to glide, in order to cushion mechanical outer effects. Since this tissue is mostly fat, it also helps insulate the body.

The epidermis is made up of 4 distinct cell types; keratinocytes, melanocytes, Langerhans' cells and Merkel cells. The keratinocytes, which produce keratin, are in abundance in the epidermis. Keratin is the fibrous protein with a protective effect. They are produced in the deepest epidermal layer and are almost always in mitosis, so these cells migrate to the upper layers. By the time they reach the outermost layer of the skin they are dead cells, made up mostly of the keratin they have produced. The lifecycle of the keratinocytes is between 25 – 45 days. During this cycle the dead cells are shed off and are replaced by new ones.

Melanocytes are found in the deepest layer of the epidermis, they produce the pigment melanin; which is responsible for the skin color. Melanin granules also travel to the keratinocytes and form a pigment shield against UV radiation. The Langerhans' cells are made in the bone marrow and move towards the epidermis. They help activate our immune system. Merkel cells are responsible as the sensory system. They are found when crossing from the epidermis to the dermis.

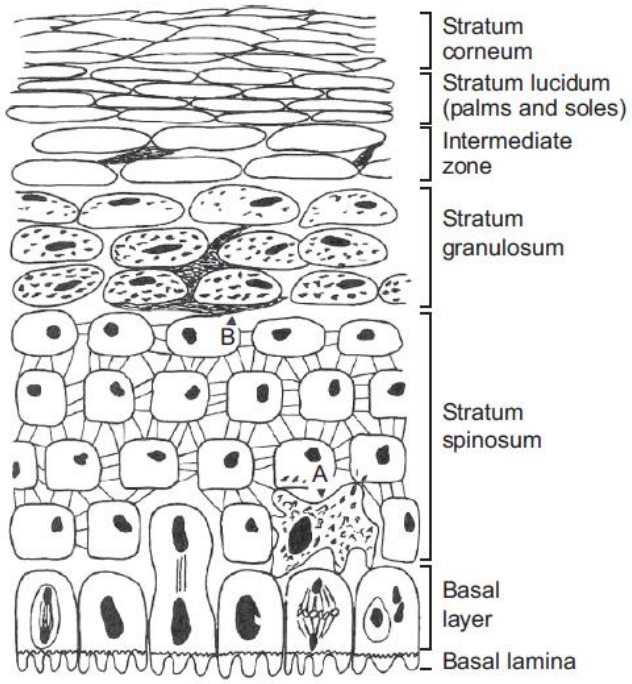

Figure 1.1. The epidermis (Kielhorn *et al.*, 2006).

The epidermis is made up of 4 or 5 layers (depending on the position in the body), which are, from deep to outer surface; stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (SC)(Figure 1.1).Skin thickness depends on the epidermal layering. Because the hands and foot soles are subjected to more and more frequent friction, the skin here is generally thicker and consists of all the 5 layers. The rest of the body lacks the stratum lucidum layer and has 4 layers. The other layers in the rest of the body are also generally thinner. (Marieb and Hoehn, 2007)

Stratum basale is the deepest epidermal layer and is attached to the dermis. This layer is ca. 70% water (Kielhorn *et al.*, 2006). Here, the keratinocytes are produced rapidly and migrate to the upper layers; stratum basale is made up of a layer of keratinocytes. Melanocytes are also found here (ca. 10 – 25 %) and they extend into the next layer (Marieb and Hoehn, 2007).

Above the stratum basale, there are several layers which make up the stratum spinosum. These layers are made up of pre-keratin filaments, Langerhans' cells and melanin granules.

As keratinocytes travel towards the surface of the skin, in the stratum granulosum, they begin to disintegrate, and from this process, the keratohyaline and lamellated granules are built up; the latter contain a glycolipid, which plays a major role in preventing water loss from the skin. Meanwhile, the lamellated granules become tougher due to cytosol proteins building up inside their plasma membranes. Also, with the lamellated granules coating their outer surfaces, these cells become more of a protective layer.

All these layers get their nutrients from the underlying dermis via capillaries. These capillaries do not reach above stratum granulosum. Therefore, above this layer the cells are dead. Above stratum granulosum there is a thin translucent layer, the stratum lucidum, which is only present in thick skin. Several layers of dead keratinocytes make up this layer. (Marieb and Hoehn, 2007)

Finally, above all these layers, the SC is found at the very top. The SC is about 20 – 30 cell layers thick; with keratin and the thickened cell plasma membranes, which makes it a very good protective layer. Also, the glycolipids between the cells prevent water loss of the skin (Marieb and Hoehn, 2007). This layer consists of ca. 5 – 20% of water and the lipid weight ratios in the intercellular space are as follows: 40 – 45% ceramides, 25% cholesterol, 15% long-chain free fatty acids and 5% other lipids. Due to its composition the SC is a dense lipid layer (Kielhorn *et al.*, 2006).

Under the epidermis there is the connective tissue, the dermis, which is rich in collagen, elastin and reticular fibers. It binds the whole body together. Right below the epidermis is the areolar connective tissue, the papillary region of the dermis with many blood vessels. It has dermal papillae on top, which indent the epidermis. This region contains capillary loops, free nerve endings (pain receptors) and Meissner's corpuscles, as touch receptors (Figure1.2). On hands and feet, these papillae are stacked and help form an epidermal ridge, which makes it easier for gripping. The epidermal ridge patterns make our unique fingerprints.

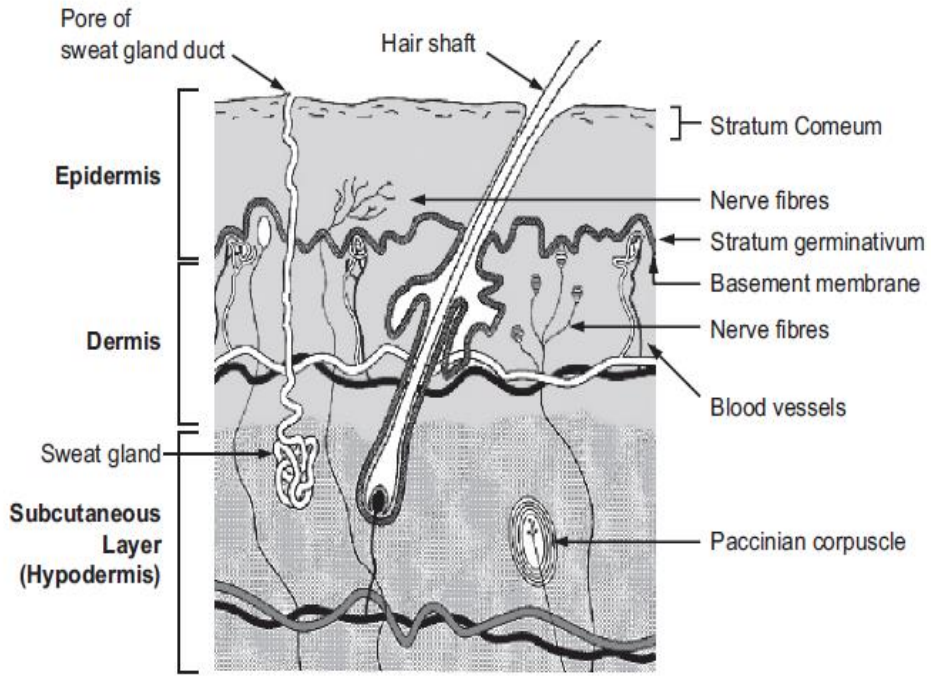

Figure 1.2. The structure of the skin (Kielhorn *et al.*, 2006).

Under the dermal papillae there is the reticular layer (ca. 80% of the dermis thickness). This layer is a dense irregular connective tissue. The extracellular matrix here contains mostly collagen fibers which are parallel to the surface. Due to the collagen fibers, the skin is strong against external forces. Also, this layer keeps the skin hydrated by binding water. The elastin fibers lend the skin stretching properties. (Marieb and Hoehn, 2007)

The skin has several types of appendages which are; apocrine and eccrine sweat glands, sebaceous glands and hair follicles. The sebaceous glands secrete sebum which keeps the skin surface acidic at ca. pH 5, which consists mainly of glycerides, free fatty acids, cholesterol, cholesterol esters, wax esters and squalene. (Kielhorn *et al*., 2006)

1.1.1. Functions

Since the skin protects what's underneath, it is subjected to outer effects the most. Our skin protects us mainly chemically, biologically and physically (mechanically). For chemical protection there is an acid mantle on the skin, formed by secretion, which hinders bacteria from multiplication. The skin also secretes sebum and human defensin, which are anti-bacterial. The melanin in the skin is a good UV shield. As for biological protection, Langerhans' cells and macrophages in the skin are parts of the immune system and helps dispose of the bacteria and viruses.

The outermost layer of the skin consists of continuous, hardened, dead skin cells, which provides physical protection. The skin is tough yet manages to be agile. The glycolipids in the epidermis prevent water-loss, as well as prevent water or water-soluble material to enter the body. These substances can enter the body through the skin: fat-soluble vitamins, steroids, oleoresins, organic solvents, heavy metal salts and penetration enhancers.

One function of our skin is temperature regulation. In normal temperatures (below 31 – 32 °C) sweat glands produce unnoticeable amounts of sweat. Upon rising body temperature, sweat glands produce significantly more sweat, which is then evaporated to keep the regular body temperature. When it is cold, the blood vessels bypass the skin by constriction, in order to keep the warmth for the body.

The sweat glands in the skin help dispose of nitrogen-containing waste such as ammonia or urea.

In order to be able to sense with our skin, it harbors cutaneous sensory receptors such as Meissner's corpuscles and Merkel discs; to feel bumps or contacts, it has the Pacinian receptors; the root hair plexuses enable us to feel the sensations on the hairs and pain is sensed by bare nerve endings in the skin.

It is a well-known fact that sunlight is necessary for vitamin D production, which in turn enables calcium absorption via the digestive tract. When the skin is subjected to sunlight, modified cholesterol molecules travelling through the dermal blood vessels, are converted into vitamin D precursors. As for other metabolic functions of the skin, the keratinocytes can disarm many carcinogens that penetrate through the skin (but can also turn harmless chemicals into carcinogens). The skin produces the protein collagenase which helps the natural turnover of collagen.

The skin serves as a blood reservoir to the body, about 5% of the body's entire blood is found travelling through the dermal vascular system. When other body parts, e.g. when hard-working muscles need more blood, the nervous system redirects the blood from the skin to the muscles. (Marieb and Hoehn, 2007)

1.1.2. Dermal Absorption

Our skin is in constant interaction with the environment; over its lifetime it gets exposed to extrinsic factors such as many products, be it of organic nature or of chemical. Although the skin acts as a good barrier, absorption does readily occur. *Dermal (percutaneous) absorption* occurs when compounds are transferred from the outside through the skin, this may be just skin-deep or materials could get transported into systemic circulation. The distinction is as follows: *penetration* is when a substance enters a particular skin layer; *permeation* is the diffusion between

distinct layers and finally *resorption* is when a substance enters through the skin and most likely ends up in systemic circulation. Dermal absorption can occur in three ways: (Figures 1.2& 1.3)

(i) *Transcellular absorption*: the material is transferred through the corneocytes, in and out of cell membranes,
(ii) *Intercellular absorption*: the material is transferred around the corneocytes,
(iii) *Appendageal absorption*: the material goes through the skin appendages.

It was shown that the distinction of the way depends on the applied concentration of the material (Bhoyar *et al.,* 2012). It is thought that even though the transcellular route is still controversial, molecules weighing less than 500 Da and show log P 1-4 can readily diffuse (Cronin *et al.,* 1999; Kang *et al.,* 2007). The intercellular spaces are not large enough to let molecules through (Sala *et al.,* 2018). There are different views about the appendageal route. Since the skin appendages cover only 0.1 – 1.0 % of the skin, it is less likely that the material is transferred via the appendages, however in body parts such as the scalp, where the hair follicles are found the most, it may present an important route. Sebaceous glands may act as drug reservoirs (Kielhorn, Melching-Kollmuß and Mangelsdorf, 2006).

The SC layer is thinner in the roots of the hair follicles and together with the invagination of the follicles, molecules' transport may be facilitated (Knorr *et al.*, 2009). This invagination can act as a drug reservoir which with the natural movement of the hairs, may direct the drug molecules to the follicles and small molecules can even get systemically absorbed (Vogt *et al.,* 2006). It has also been reported that molecules as big as 600 nm showed the highest penetration after a massage through the follicles where they are even stored for several days (Lademann *et al.,* 2015).

The lipophilic SC is the main barrier for many compounds. However, the hydrophilic epidermis and dermis can also act as barriers for very lipophilic compounds. Depending on the aimed skin strata, after diffusing through the

epidermis and the dermis, the material can join the cutaneous blood flow and resorption occurs. (Kielhorn, Melching-Kollmuß and Mangelsdorf, 2006)

Dermal absorption depends also on the physicochemical properties of the material in question. These properties are solubility, hydrogen bond formation ability and molecular weight (Potts and Guy, 1995).

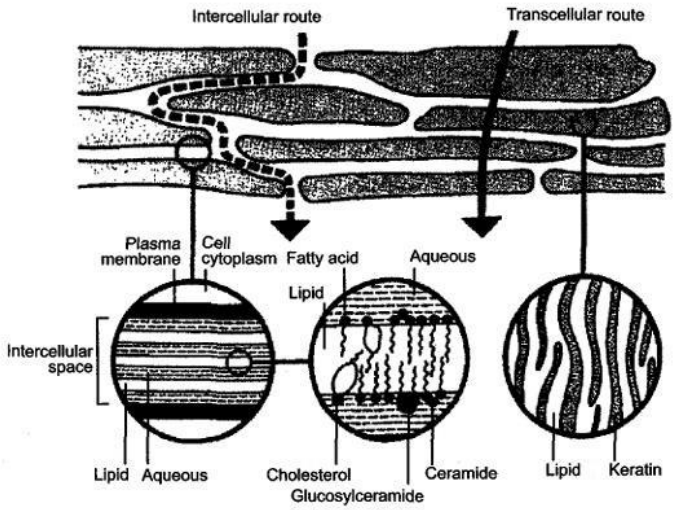

Figure 1.3. Two possible ways of absorption through the SC (Kielhorn *et al.*, 2006).

1.1.3. Factors Affecting Permeability

There are several factors that may affect skin permeability such as; species difference, anatomical site, skin condition, temperature, hydration and age. The difference in species makes itself apparent when correlating in vitro animal tests with human trials. The usual laboratory animals have more hair follicles than humans, which alters the data with appendageal absorption routes. In dermal applications, the body part in question is important. The skin thickness of different body parts and between individuals varies to a significant amount. Also the condition of a specific skin part may be altered due to pathological factors, such as

psoriasis, which makes the skin more penetrable and sensitive to outer effects. The barrier function of the skin can be altered physically or chemically on purpose. The upper layer of the skin can be partly solubilized with some polar or non-polar solvents in order to reduce its barrier properties and deliver beneficial compounds.

Increasing the skin temperature can have two effects; firstly, it directly affects the diffusion of materials and secondly, it may allow better penetration by altering the crystalline structures of the lipid bilayers of the skin. The SC consist of ca. 5 – 20% water and ca. 50% when hydrated. Although the general idea is that a hydrated skin absorbs more chemicals, there are varying results on the subject.

Some properties of the skin are altered with age: the SC is dryer, there are fewer surface lipids (decreasing activity of the sebaceous glands), the dermal-epidermal junction is flattened, and the skin capillary network is atrophied, so the viable epidermis has less nutrients coming through. Some studies indicate that the barrier function of the skin increase with aging.

1.1.4. Overcoming the Barrier

The need to overcome the SC is a topic of ongoing investigation. The lipophilic nature of the SC makes it almost impossible for (especially hydrophilic) compounds to pass through (Prausnitz, 2004). Topical delivery of active drug or cosmetic ingredients is necessary to achieve a therapeutic dosage below the SC to be effective (Waghule *et al.*, 2019). Using oral or parenteral delivery methods to treat skin problems or enhance its properties is an insufficient attempt due to low doses that reaches the skin. Increasing the dosage might help the drug or cosmetic material to reach the skin tissues, however it also might raise side effects (Sala *et al.*, 2018).

Transdermal drug delivery also has various advantages over other routes of application such as: bypassing the first-pass metabolism, sustained release

(especially beneficial among low half-life molecules), less side effects, avoiding blood concentration fluctuations and standardizing the usage among different patients(Darwhekar *et al.*, 2012; Almandil, 2016; Chathoth *et al.*, 2016)

In order to overcome this barrier, techniques are used such as lasers, radio frequency, dermabrasion, peeling and hypodermic needles. However, these are mostly invasive techniques in some cases including removal of a part of the epidermis, leading to scarring and sensitivity (Ramaut *et al.*, 2018).

Micro needles and micro needle patches are less invasive and pain-free methods, that utilize needles with sizes ranging between 25 – 2000 μm made from various materials, to open small paths in the epithelial or fibrous tissue in order to facilitate the passage of drug or cosmetic materials below, without damaging the skin (Indermun *et al.*, 2014; Rzhevskiy *et al.*, 2018). There are many studies dedicated to improving vaccines by transforming them to be applied via micro needles (or patches). Because the macrophages and Langerhans' cells in the skin lead to an increased immune response, the skin a desirable application route for vaccination (Glenn *et al.*, 2000; Bos and Luiten, 2009). Dermal vaccines are also beneficial in that they reduce waste and cost markedly and make vaccination of large masses safe, easy and pain-free. Other than cosmetology and vaccines, there are also other applications in development for micro needles in various fields such as ocular or mucosal drug delivery (Rzhevskiy *et al.*, 2018).

It has also been reported that when microneedling is performed in combination with topical ingredients, better penetration of the ingredients is achieved. Particularly the penetration of invasomes have increased, which are vesicles for enhanced skin delivery (Badran, Kuntsche and Fahr, 2009).

When the current technologies are compared with regard to ingredient delivery under the SC, it can be seen that topical creams and dermal patches are limited almost exclusively to the upper skin layer, whereas the dermal needle can deliver up to 90 – 100 % of the ingredients, with the cost of pain and application

limitations (i.e. no home application) (Figure1.4). In this sense, micro needles (and patches) are considered to be between these methods. However, micro needles have also their limitations, e.g. they can be irritating to sensitive skin, the very thin needles may break and cause problems or infections may occur if the needles aren't sterile (Waghule *et al.*, 2019). These and other possible limitations which may arise as microneedling grows more popular, require improvement on the technique of microneedling and general permeation techniques.

In the light of this information, the topic of drug and cosmetic material delivery requires further investigation of non-invasive, cost effective, pain-free and effective applications.

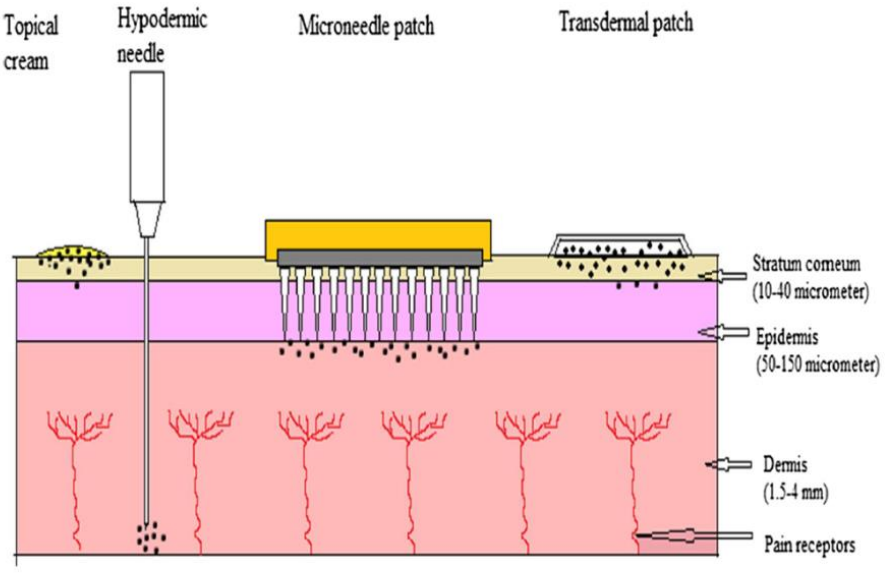

Fig. 1. Comparison of topical cream, hypodermic needle, microneedle patch and transdermal patch.

Figure 1.4. Comparison of different delivery methods. (Waghule *et al.*, 2019).

1.1.5. Skin Aging & Use of Antioxidants

The causes of skin aging can mainly be divided into two categories: intrinsic and extrinsic factors. The intrinsic aspects are genetic predisposition and genetic factors, whereas there are many extrinsic factors such as radiation, pollution, smoking and nutrition. Also to be considered are the interactions of these factors within each other. (Murina, Kerisit and Boh, 2012; Krutmann *et al.*, 2017)

The electromagnetic rays of sun's radiation are mainly classified as ultraviolet (UVA1/UVA2/UVB/UVC), infrared (IRA/IRB/IRC) and visible light (VL), which are present in different percentages.

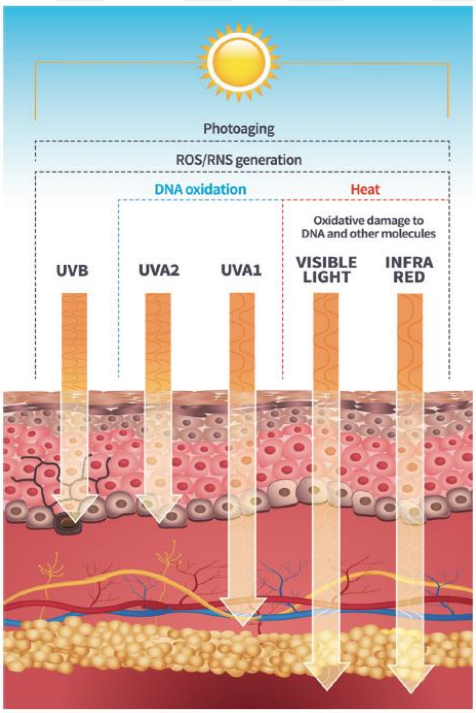

Figure 1.5. Radiation types in the solar spectrum (Krutmann *et al.*, 2017).

All radiations have different energies, get filtered in various amounts and penetrate the skin differently. (Figure 1.5). There is a wide array of studies regarding the relationship between UV radiation and skin aging; in fact the effect of UV on the skin may well be the most researched topic in this area. According to several researches, UV radiation affects all strata of the skin (dermis, epidermis and hypodermis) and all UV radiations (except UVC, which is filtered in the ozone layer) contribute to photoaging of the skin. The effects of UVR vary with differentiating genetic background due to factors such as ethnicity or age.

Skin aging is caused by both acute stress (e.g. the upregulation of extracellular matrix degrading enzymes) and chronic damage responses (e.g. mitochondrial DNA damage) (Mesa-Arango, Flórez-Muñoz and Sanclemente, 2017). UVR is responsible for over 80% of facial skin aging. It causes wrinkles, pigmentation and rough looking skin (Tobin, 2017). Since the skin gets exposed to small doses of radiation almost daily, using sunscreen is highly recommended as it is proven to delay photoaging (Mesa-Arango, Flórez-Muñoz and Sanclemente, 2017). However, it is now understood that protecting only from UVR via sunscreens is not an adequate attempt at anti-aging (Burke, 2018).

Although not as detailed as the studies regarding UV, there is increasing evidence that visible light (VL) and infrared radiation (IRR) cause dermal matrix degradation as well and alter the SC and cause pigmentation. Both UVR and IRR induce the production of reactive oxygen species (ROS) that induce the production of collagen-degrading enzymes and induce accumulation of elastin(Scharffetter-Kochanek *et al.*, 2000). The photoaging effects of ROS can be avoided and may be even reversed with the use of antioxidants such as vitamins C and E (Krutmann *et al.*, 2017; Burke, 2018). ROS generally cause oxidation of biomolecules and also lead to diverse age related alteration like Alzheimer's disease, cancer and diabetes (Edris, 2007).

Pollution occurs readily due to everyday human activities. The interaction of pollution with UVR leads to the formation of new pollutants which stay in the lower

atmosphere and affect humans directly. It is known that pollution causes skin pigmentation and wrinkling (Krutmann *et al.*, 2017). These new pollutants also impair the barrier function of the SC (Pan *et al.*, 2015). One of the most toxic environmental pollutants, ozone is formed through a series of reactions between various NO_x species, organic compounds and UVR, which in turn reacts with the unsaturated skin lipids, generating ROS. ROS further causes inflammatory molecules' generation that cause aging of the skin. They also contribute to the generation of precancers and skin cancers (Burke, 2018).

Although the exact mechanism of action is not yet clear, it is proven that nutrition plays a role in skin aging. In several studies it was found out that, diets rich in carbohydrates and sugars cause the skin to look wrinkled, while a diet high in antioxidants, vegetables, legumes and olive oil works in favor of the skin. Endogenous glycation occurs when the consumed sugars bind to proteins and lipids and form advanced glycation end products (AGEs) and exogenous glycation occurs when AGE-containing food is subjected to high temperatures. Some foods such as vitamins and flavonoids reportedly have antioxidant properties. Consuming antioxidants in the form of food or supplements is known to promote a youthful look. (Krutmann *et al.*, 2017) However it is also important to keep a good antioxidant/ROS balance, since ROS are imperative for many different metabolic activities (Mao *et al.*, 2018).

It is known and clinically proven that stress causes the skin to look aged. Although the direct relationship between stress and skin aging is yet to be investigated in detail, it is shown that chronic psychological stress leads to increased ROS production and DNA damage, which cause skin aging. It has also been reported that stress disrupts barrier properties of the skin (Dunn and Koo, 2013).

ROS damage can be prevented by the use of topical antioxidants. Antioxidants are substances that prevent oxidation and scavenge ROS (Nirmala *et al.*, 2018). UV exposure decreases the vitamins C and E from the skin's surface, so

they need to be applied onto the skin to restore the normal levels, since oral intake does not help the sufficient levels to accumulate in the skin (Burke, 2018).

Being antioxidants themselves, they are unstable molecules, so the formulation of the topical delivery system is very important. Mostly the more stable forms such as esters are preferred in formulations; however these are not metabolized by the skin to their active forms and are not absorbed percutaneously (Pinnell *et al.*, 2001). So there is a need for new topical formulations e.g. encapsulating these antioxidants for an improved delivery system to the skin (Burke, 2018).

1.2. Lipids

Unlike most groups of chemical and biochemical compounds, lipids are defined by their solubility instead of their functional groups; they are more soluble in organic solvents than in water. Lipids can mainly be distinguished as fatty acids or steroids. Fatty acids are the building blocks of all lipids, except steroids, which represent nonsaponifiable lipids, including bile salts, cholesterol and certain hormones. (Ball, Hill and Scott, 2011)

Fatty acids are carboxylic acids that are generally classified according to their carbon-carbon double bonds and the number thereof. Some naturally occurring fatty acids are lauric, palmitic, oleic and linoleic acids. Linoleic and α-linoleic acids are essential fatty acids; meaning both are necessary for normal growth and development but humans cannot synthesize them themselves; hence they must be obtained through nutrition. Oleic acid is the most abundant unsaturated fatty acid. (Ball, Hill and Scott, 2011)

Saturated fatty acids contain no carbon-carbon double bonds so these long hydrocarbon chains can pack in an ordered fashion in a crystal lattice, causing them

to have relatively high melting points. In contrast, the intermolecular attraction of unsaturated fatty acids are weaker, causing them have lower melting points, because they contain one or more carbon-carbon double bonds, which give the molecular structure bends, so that these long chains cannot pack as tightly as saturated fatty acids. (Ball, Hill and Scott, 2011)

In order to produce fats and oils, also known as triglycerides, fatty acids react with glycerol (Figure1.6). Most naturally occurring triglycerides are mixed triglycerides, meaning the three hydroxyl groups of the glycerol are esterified with different fatty acids, causing them to have different fatty acids rests. Naturally occurring fats and oils are mixtures of different triglycerides so they do not have a single chemical formula. Fats are obtained if the triglycerides are solid in 25°C whereas oils are the product if they are liquid at the same temperature. (Ball, Hill and Scott, 2011)

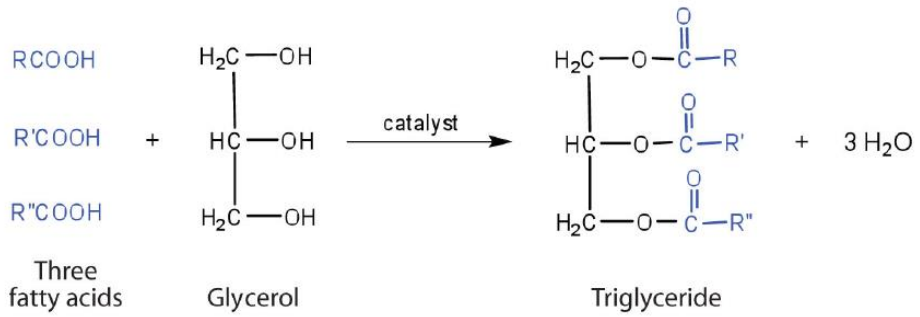

Figure 1.6. Triglycerides (Ball *et al.*, 2011).

1.2.1. Membrane Lipids

Lipids have important duties in living organisms, providing insulation, helping solubilize essential fat-soluble vitamins and storing energy. All living cells are surrounded by a cell membrane made up of lipids (Fig 1.7).

These lipids are long hydrocarbon chains with hydrophilic head groups and hydrophobic tails, which are the fatty acid portions. Therefore when in water, depending on their concentrations, lipids self-assemble to different structures such as micelles, monolayers or bilayers (Figure 1.8).

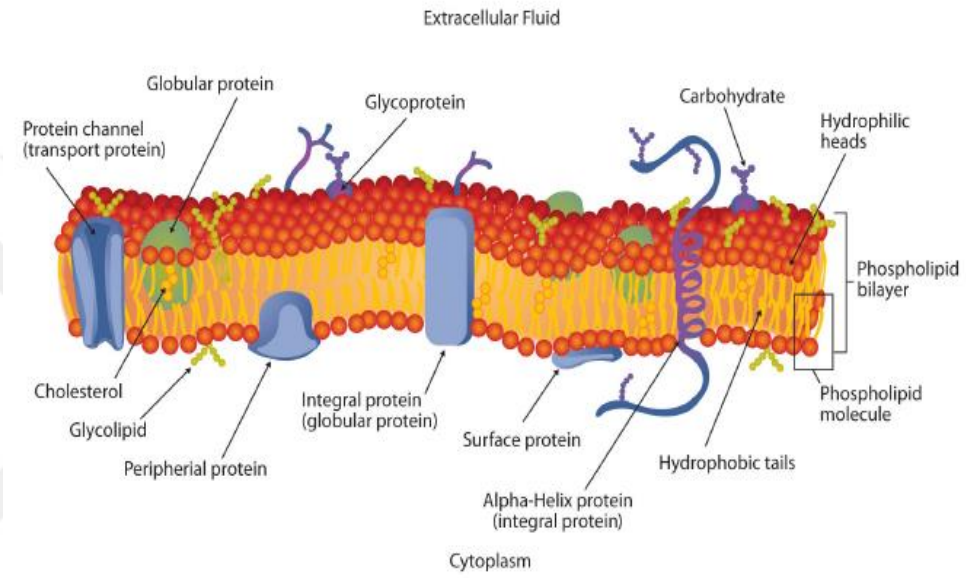

Figure 1.7. Cell membrane (Ball *et al.*, 2011).

In micelles, the hydrophilic head groups are pointed outwards the spherical organization. Monolayers are formed as a one molecule-thick layer on the surface, where the polar head groups are pointed into the water. In bilayer formation, the hydrophobic chains are facing within each other away from water molecules, while the hydrophilic head groups face the water. Dispersion forces are effective within the hydrophobic tails, which are weakened with unsaturated fats, leading them to move somewhat freely. Therefore, the membrane is said to be fluid. The membrane lipids enclosing all cells are present as bilayers (Figure 1.8). (Ball, Hill and Scott, 2011)

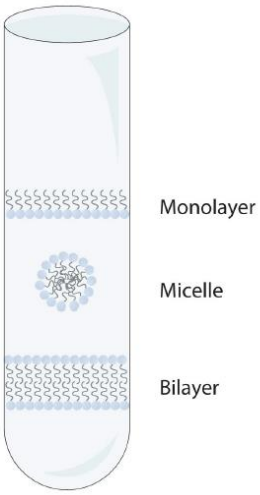

Figure 1.8. Self-assembly of lipids in water (Ball *et al.*, 2011).

Mainly, there are two types of cell membrane lipids: phospholipids and glycolipids. Glycolipids contain sugars and are found exclusively on the outer surface of the cell membranes to make cell signaling possible. Phospholipids contain phosphorus and can be divided into two categories as sphingomyelins and phosphoglycerides. The latter is the most abundant phospholipid in cell membranes and can be further distinguished as phosphatidylethanolamines and phosphatidylcholines. While phosphatidylethanolamines contain ethanolamine and are found mostly in brain tissue, phosphatidylcholines contain the choline group (Figure 1.9). (Ball, Hill and Scott, 2011)

The outer most layer of the skin, SC is made up of 30 % (mass) triglycerides and 19.3% of free fatty acids. The rest are ceramides, hydrocarbons, cholesterols and their esters (Frankiel and Woodley, no date). In order to mimic the skin lipid composition, different mixtures of lipids were examined and was concluded that the membrane proteins do not play a crucial role for the skin lipid phase behavior (Bouwstra *et al.*, 2003).

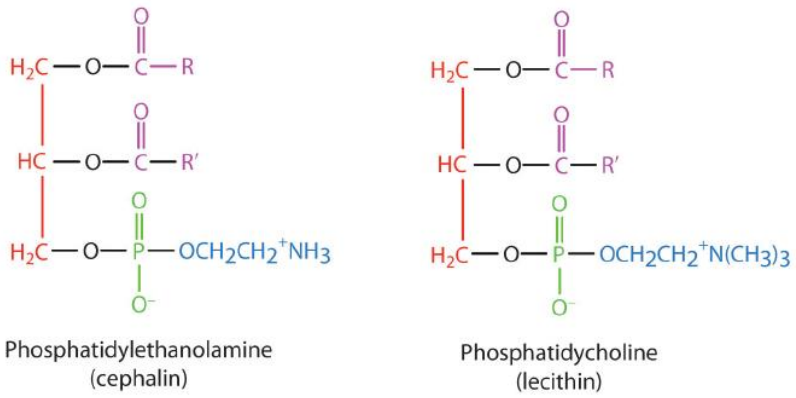

Figure 1.9. Phosphatidylethanolamines and phosphatidylcholines (Ball *et al.*, 2011).

1.3. Vesicles and Encapsulation

Vesicles are spherical structures enclosed by a lipid membrane, formed within the biological plasma membrane. They play various roles from transport to temporary storage of biological molecules. Vesicles can be useful as small reaction chambers as they are separated by the membrane from their surroundings. Artificial vesicles are called liposomes in general and are used extensively in nanotechnology.

In order to deliver active ingredients to the skin, it is essential to overcome the main barrier, SC. Since the SC is made up of lipid bilayers composed of mostly the phospholipid phosphatidylcholines, one way to encapsulate the active ingredients is to mimic the cell membrane by generating bilayer vesicles of phosphatidylcholines. Lipid vesicles are capable of self-assembling into a bilayer formation, consisting of one or several bilayers, generating aqueous compartments on the inside (Sala *et al.*, 2018).

When preparing liposomes, the water soluble compounds are dissolved in the water part of the preparation and the fat soluble compounds are dissolved in the lipid

part. In this way, the water soluble compounds are entrapped in the aqueous core whereas the fat soluble compounds locate themselves within the lipid bilayers.

Since lipid vesicles mimic the structure of SC, they show good biocompatibility with the skin. Moreover, lipid vesicles show different possible mechanisms of penetration into the skin (Figure1.10). These include loosening of the SC structure by wetting, exchanging lipids between the skin and the vesicle, fluidization of the skin or altering the polarity of the skin. (Zhai and Zhai, 2014)

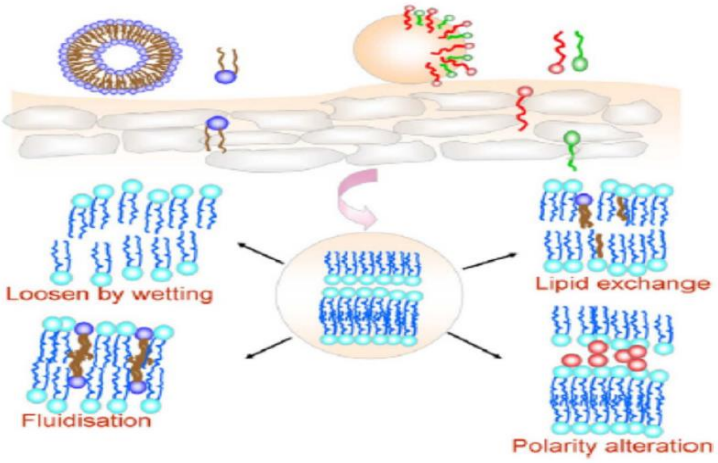

Figure 1.10.Interaction mechanisms of lipid vesicles with the skin (Zhai & Zhai, 2014).

1.3.1. Conventional Liposomes

Liposomes are formed when phospholipids self-assemble to enclose an aqueous compartment (Figure 1.11). In this sense they resemble the biological cell. Due to the aqueous compartment, liposomes are able to store aqueous compounds and due to the lipid bilayer structure, they can encapsulate lipophilic substances within the bilayer itself. Differing from the biological cell, liposomes do not contain

essential fatty acids, thus having the space for encapsulating different compounds.Liposomes, made from soya phospholipids are more fluid, lending them higher permeability, meaning the loaded active ingredients are set free more readily than from liposomes made from hydrogenated phospholipids.(Lautenschläger and Röding, 1996)

Liposomes can be produced in various sizes with different lamellarities such as multilamellar (MLV), small unilamellar (SUV) and large unilamellar (LUV) vesicles, with sizes larger than 0.5 μm, 20 – 100 nm and larger than 100 nm respectively (Sherry *et al.*, 2013). Being natural molecules themselves, phospholipids are prone to oxidation. In order to prevent oxidation, antioxidants can be incorporated (Yatvin and Lelkes, 1982).

It is proven that phospholipids promote skin passage by breaking on the surface and disrupting the SC, enabling the active ingredient to pass (Kato, Ishibashi and Miyake, 1987). However it was found out that this effect depends strongly on the elasticity of the phospholipid, hence the type of the phospholipid (Kirjavainen *et al.*, 1996).

One explanation of liposomes' SC passage is that they fuse with the SC layer and the active ingredients diffuse through the SC, increasing the mobility of lipophilic actives (Bouwstra *et al.*, 2003) (Figure 1.12). This process has also been described as liposomes forming a lipid film on the SC, leading to exchange of actives. Moreover, due to the water content of liposomes, the SC would hydrate and swell. (Sinico *et al.*, 2005)

When liposomes were first invented, it was thought that they could penetrate the SC effectively and transport active ingredients to the viable epidermis, however years of ongoing research proved this to be implausible. After the passage of liposomes under the SC, size and structure differences were observed and the passage of liposomes was attributed to the follicular route (du Plessis *et al.*, 1994; Zellmer, Pfeil and Lasch, 1995; Betz, Imboden and Imanidis, 2001).

Factors that affect skin permeation are vesicle size, zeta potential (Gillet, Lecomte, *et al.*, 2011), lipid type (Gillet, Compère, *et al.*, 2011), loading efficiency, skin disorders/barrier integrity (Raza *et al.*, 2013) and occlusion (Trauer *et al.*, 2014). It was reported that while nanoliposomes with sizes between 31 – 41 nm showed greater penetration (Hood *et al.*, 2014), lamellarity does not affect penetration capability (Szura *et al.*, no date). Although, it was found out that liposomes did not penetrate the SC effectively, it was indicated that liposomes could penetrate the skin if the barrier was impaired, due to skin conditions (Fresta and Puglisi, 1996). However, the interaction of liposomes with impaired skin is yet to be investigated further(Sala *et al.*, 2018).

1.3.2. Transfersomes

Since research showed that liposomes weren't capable of delivering actives under the SC effectively, the need for improvement has led to the discovery of deformable liposomes. One of the first types of deformable liposomes was the transfersome. First proposed in 1992, transfersomes are liposomes that are made deformable by an edge activator (Cevc and Blume, 1992), which is often a single chain surfactant such as sodium cholate, sodium deoxycholate, Tween 20 – 60 – 80 or Span 60 – 65 – 80 (Elsayed *et al.*, 2007) (Figure 1.11).

Edge activators act by disturbing the phospholipid bilayer of liposomes, not randomly, but by inserting into points of high pressure when the vesicles are under stress (Sala *et al.*, 2018). When the edge activator is inserted within the lipid bilayer, it causes discontinuities which decreases liposome stiffness (Kumar, Pathak and Bali, 2012). This effect is obtained with an edge activator ratio of smaller than 25 % (Jain *et al.*, 2003).

It is thought that the main mechanism of transfersomes' passage is that it is passively driven via osmotic strength through the SC (Choi and Maibach, 2005).

With a water content of 15 %, the SC is not hydrated when compared to the lower levels with 75 %, therefore, a water gradient forms (Kumar, Pathak and Bali, 2012). This is backed up by the observation that the passage of transfersomes was decreased through hydrated skin (Morrow *et al.*, 2007). Transfersomes can pass through the SC without breaking. FTIR spectra of skin, treated with transfersomes has shown broadening and shifting of peaks indicating structural changes (Duangjit *et al.*, 2014).

There are several effects of edge activators within transfersomes such as swelling the SC, leading to uncoiled keratin fibers (Pathan and Setty, 2009), disrupting the SC by extracting lipids (Singh *et al.*, 2015) and interaction with the vesicles (Figure 1.12).

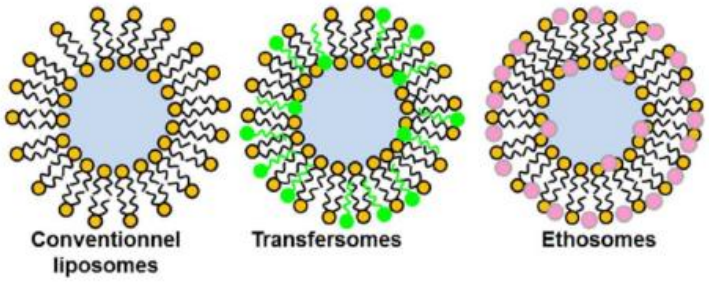

Figure 1.11. Liposome, Transfersome and ethosome structures (Sala *et al.*, 2018).

1.3.3. Ethosomes

First described by Touitou *et al.*(2000). ethosomes are vesicles that incorporate ca. 20 – 45 % ethanol within the bilayer structure. It has been observed by confocal laser scanning microscopy that ethosomes penetrate to about 80 – 200 μm deep (Godin and Touitou, 2004; Fang *et al.*, 2009). Ethosomes are shown to be suitable carries for both hydrophilic (Godin and Touitou, 2004; Dubey *et al.*,

2007)and hydrophobic drugs (Bhalaria, Naik and Misra, 2009). Ethosomes can be classified as a type of transfersomes; the difference being in ethosomes, the ethanol evaporates upon contact with skin, whereas the penetration enhancers in transfersomes do not evaporate (Ascenso *et al.*, 2015).

In ethosomes, ethanol acts as the penetration enhancer. It has many advantages such as negative surface charge leading to smaller vesicles, better stability (López-Pinto, González-Rodríguez and Rabasco, 2005) and improved skin passage (Gillet, Lecomte, *et al.*, 2011).

It has been shown that ethanol fluidizes the SC membrane by increasing the lipid's polar head groups' mobility and disrupts the SC, leading to less dense intercellular lipid domains. Ethosomes open their own passage through the SC by interacting with the disturbed skin lipids (Touitou *et al.*, 2000) (Figure 1.12)

There are also binary ethosomes, containing a second alcohol in addition to ethanol, such as propylene glycol and isopropyl alcohol (Zhou *et al.*, 2010).

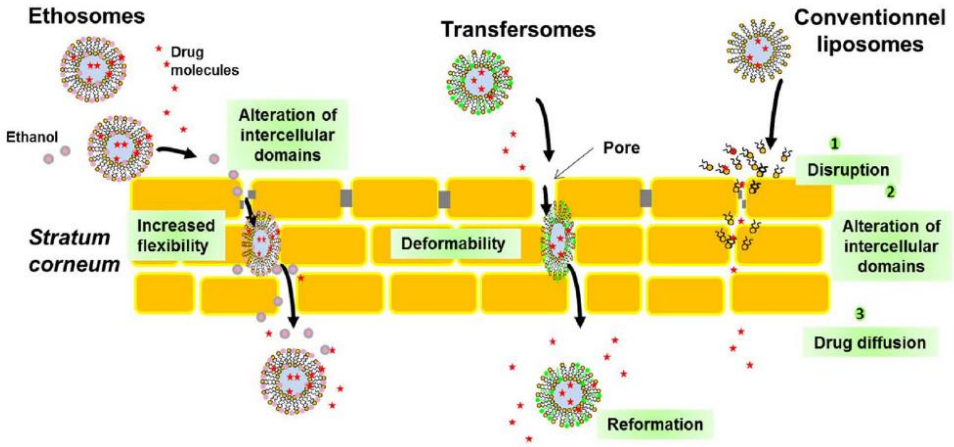

Figure 1.12. Mechanism of vesicle passage through the SC (Sala *et al.*, 2018).

1.3.4. Transethosomes

In a review, Abdulbaqi *et al.*(2016), have investigated the published articles on ethosomal (ethosomes, Transethosomes, etc.) systems. As can be seen in Figure1.13, the studies concerning ethosomal systems have increased considerably in recent years.

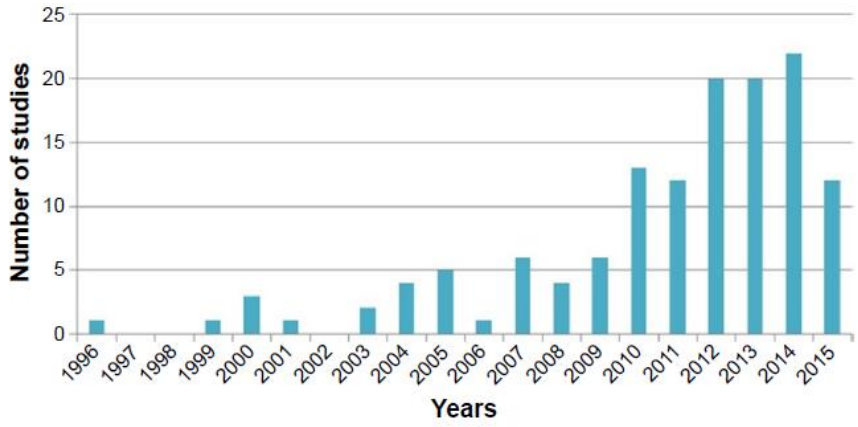

Figure 1.13. Ethosomal studies in recent years (Abdulbaqi *et al.*, 2016).

First described by Song *et al.* (2012), transethosomes are deformable liposomes containing both ethanol and edge activators or permeation enhancers. Transethosomes are said to combine the properties of both ethosomes and transfersomes. Ethanol disrupts the SC barrier and being soft and malleable, transethosomes can penetrate the SC more readily. Once under the SC, the lipid bilayers of the vesicle fuse together with the lipid bilayers of the skin and release the encapsulated active ingredient.

Song *et al.*(2012) have obtained transethosomes with Tween 80 and sodium taurocholate as edge activators and oleic acid as permeation enhancer, and encapsulated the drug fluconazole for topical delivery under the SC. When compared with the conventional liposomes and transfersomes, transethosomes were smaller

with negative zeta potentials and showed significantly higher elasticity. Oleic acid is known to intercalate between skin lipids leading to decreased phase transition temperatures (Yamane, Williams and Barry, 1995).

Superior properties such as improved elasticity and better penetration capability of transethosomes over conventional liposomes, transfersomes or ethosomes have been proven in various comparison studies (Song *et al.*, 2012; Ascenso *et al.*, 2015). It is also shown that transethosomes are more suitable for the encapsulation and delivery of lipophilic compounds (Ascenso *et al.*, 2015).

As a good permeation enhancer, the addition of ethanol leads to smaller vesicle sizes (Touitou *et al.*, 2000). The size of the vesicles has decreased as much as ca. 45 % with addition of 40 % ethanol when compared to classical liposomes (Bendas and Tadros, 2007).

However ethanol concentration must be adjusted so that it incorporates into the vesicle and not cause the vesicle to be unstable or leaky, or even solubilize it completely. Ethanol also causes the vesicles' charge to shift to negative, increasing ethanol content leads to more negative vesicles (Touitou *et al.*, 2000; Rao *et al.*, 2008). Vesicle charge affects product properties such as stability and skin interaction. The negative charge of the vesicles prevents aggregation via electrostatic repulsion. Ethanol also increases the entrapment efficiency of the vesicles (Jain *et al.*, no date) by solubilizing ingredients of different lipophilicities in a linear behavior pattern with ethanol content (Prasanthi and Lakshmi, 2012).

An important factor affecting vesicle properties such as penetration properties, zeta-potential and size of the vesicles, is the phospholipid source. Many different types of phospholipids have been used and investigated. Generally, 0.5 – 5 % of an ethosomal formulation consist of phospholipids (Limsuwan and Amnuaikit, 2012). It has been shown that the phospholipids with higher phosphatidylcholine content led to more stable vesicles (Shen *et al.*, 2015). As with ethanol content, phospholipid content is also has to be carefully adjusted during formulation as it will affect the

entrapment efficiency significantly without having much effect on the vesicle size (Liu *et al.*, 2011; Puri and Jain, 2012). After a certain phosphatidylcholine concentration, the effect is lost.

Tweens are used as edge activators in ethosomal systems, in concentrations ranging from 10 – 50 % of the total phospholipid concentration. Tween 80 was especially reported to having increased vesicle stability while improving skin penetration due to its solubilizing properties and it also prevents vesicle fusion (Bragagni *et al.*, 2012; Song *et al.*, 2012).

Oleic acid is incorporated into ethosomal systems as a penetration enhancer, affecting size, elasticity and zeta-potential. It is reported to improve skin penetration by solubilizing the SC (Song *et al.*, 2012; Ma *et al.*, 2015). Song *et al.* showed that oleic acid led to smaller vesicles with negative zeta-potentials and better penetration capabilities.

An imperative variable to consider while formulating transethosomes is the material to be encapsulated. Depending on its nature, the active ingredient may affect vesicle size and zeta-potential. Different studies, investigating various drug materials have reported both increase and decrease in vesicle size (Dayan and Touitou, 2000; Paolino *et al.*, 2012). Generally ethosomal systems have negative zeta-potentials. However it has been reported that the zeta-potential has shifted to positive values when different drugs were encapsulated (Dayan and Touitou, 2000) or shifted to be more negative in some cases (Anoop and Rakesh, 2012; Paolino *et al.*, 2012).

1.4. Essential Oils and their Incorporation in Vesicles

According to the European Pharmacopoeia, essential oils are defined as: “Odorant product, generally of a complex composition, obtained from a botanically

defined plant raw material, either by driving by steam of water, either by dry distillation or by a suitable mechanical method without heating." (*European Pharmacopoeia (Ph. Eur.) 9th Edition | EDQM*).

Essential oils are lipophilic and generally soluble in organic solvents. Only 10% of the plant species contain essential oils(Svoboda and Greenaway, 2003) and their extraction yields are as low as 1%, which makes them highly rare and expensive (Asbahani *et al.*, 2015).

In addition to their advantage of being natural, essential oilsare utilized extensively owing to their diverse biological activities; they are anti-bacterial, anti-viral, anti-fungicidal and anti-oxidant. essential oilsconsist of monoterpenes and sesquiterpenes (more than 80 %) and their oxygenated derivatives.

Being anti-oxidants themselves, essential oilsare susceptible to oxidation and degradation, their stability can be increased by encapsulation (Hong and Park, 1999). Essential oilshave several advantages to be used with liposomal systems; they reduce vesicle size, homogenize the solution, increase fluidity and protect the lipids from oxidation. Encapsulated essential oilshave increased anti-microbial properties and can be used as alternative agents to treat different diseases. (Sherry *et al.*, 2013)

The essential oils geraniol and linalool are a monoterpene alcohols, appearing as pale-yellow water-insoluble liquids and can be obtained from many flower species (Regev and Cone, 1976). They have a rose-like odor and areused widely as fragrant. Geraniol has also many biochemical and pharmacological properties some of which are; insect repellant, anthelmintic, antimicrobial, anticancer, anti-inflammatory and anti-oxidant (Chen and Viljoen, 2010).

In a study where 34 essential oils were investigated for their radical scavenging properties, geraniol has shown a remarkable anti-oxidant effect with 87.7 % (Tiwari and Kakkar, 2009). Geraniol toxicity by digestion was studied on rats both by short-term high doses and long-term low doses and showed no adverse

effects (Hagan *et al.*, 1967). The clinical sensitization test on volunteers showed also no positive results. Also no mutagenic effects were found(Ishidate *et al.*, 1984).

As geraniol is not an electrophile, it shouldn't show sensitizing properties, however it was stated in some cases that geraniol caused contact dermatitis (Cardullo, Ruszkowski and DeLeo, 1989). This is mostly attributed to either auto-oxidation of geraniol in air or CYP-mediated metabolic activation in the skin leading to the formation of allergenic compounds (Hagvall *et al.*, 2008). Since the primary route of exposure to geraniol is considered to be through the skin, dermal studies with geraniol should be conducted in the future to better understand geraniol activity and to create formulations accordingly without causing skin sensitivity.

Linalool, which is also used frequently as fragrance, also has diverse biochemical and pharmaceutical properties. It is known to be anti-inflammatory, anti-cancer, antihyperlipidemic, antimicrobial, analgesic and antidepressant but not limited to these (Pereira *et al.*, 2018).

Since essential oil extracts such as linalool show poor bioavailability(Puglia *et al.*, 2017), are volatile and therefore have a short half-life; encapsulation is a beneficial way of delivery to improve efficacy (Pereira *et al.*, 2018). There are numerous successful studies on encapsulation of linalool in β – cyclodextrins (Quintans-Júnior *et al.*, 2013), polybutylcyanoacrylates (Xiao *et al.*, 2015), solid lipid nanoparticles (SLN)and nanostructured lipid carriers (NLC) (Souto, Mehnert and Müller, 2006).Encapsulation is also advantageous for targeted drug delivery. In this way, linalool is formulated in such a way that it will only show its effects on the desired tissues, sparing healthy tissue of potential toxicity, since the toxicity of linalool is mostly doses dependent and requires further detailed investigation (Pereira *et al.*, 2018). Therefore the encapsulation of linalool in lipid delivery systems is beneficial and has room for improvement.

1.5. Transethosome Preparation Methods and their Effects

There are many methods by which ethosomal systems can be prepared. Here, two classical methods are mentioned as these were the preferred methods in this study.

A classical preparation is the cold method. This method was introduced by Touitou and is one of the simplest preparations (Touitou, 1994). Here, the organic and the aqueous phase are prepared separately at first. The phospholipids and penetration enhancer/edge activator are dissolved in ethanol at 30°C or at room temperature.

The aqueous phase can be water or a buffer solution, which is added slowly to the organic phase under stirring. If the ingredient to be encapsulated is of hydrophilic nature, it is dissolved in the aqueous phase and if it is of lipophilic nature, it is dissolved in the organic phase. (Figure 1.14)

Liposomes have long been prepared by the classical thin-film hydration method; which is slightly modified to obtain transethosomes. First, phospholipids and the penetration enhancer/edge activator are dissolved in chloroform or in a chloroform : methanol solution. Then, the solvent is evaporated with a rotary evaporator at a temperature that is above the lipid phase transition temperature, where a thin film forms at the bottom of the flask. In order to obtain transethosomes, the film is hydrated with a hydroethanolic solution or a buffer solution and transethosomes are obtained. Again, depending on the nature of the active, they are dissolved either in organic or aqueous phase. (Song *et al.*, 2012) (Figure 1.15)

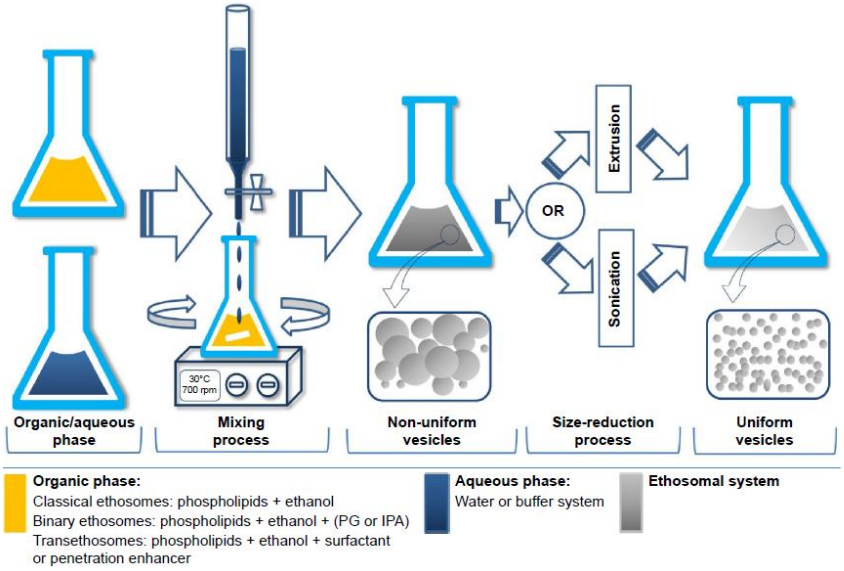

Figure 1.14. The cold method (Abdulbaqi *et al.*, 2016).

Regardless of the method of choice, the obtained vesicles are big and need to be downsized to about 200 – 400 nm (Akhtar and Pathak, 2012; Kumari and Pathak, 2013) for skin penetration to be possible in the beginning. Downsizing is usually achieved in two different ways, sonication and extrusion. Extrusion is done in classical liposome extruders for a determined number of passes through a polycarbonate membrane of the desired size (ca. 200 – 400 nm). SUVs are obtained after extrusion. Sonication (either probe or bath) has to be carefully optimized as it may lead to breakage of vesicles. It has been reported that extrusion has resulted in better entrapment capacities of vesicles than sonication (Maestrelli *et al.*, 2009).

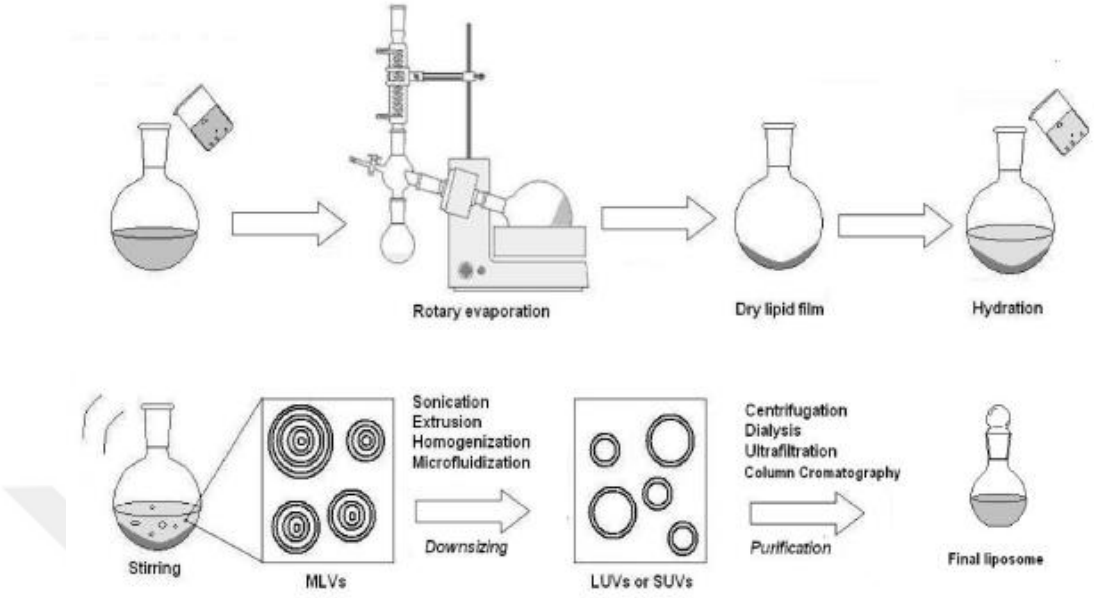

Figure 1.15. Thin-film hydration method (Demetzos, 2016).

1.6. Vesicle Characterization

1.6.1. Visualization

The morphology of liposomal vesicles are generally viewed under various electron microscopes such as Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM) or a combination of both; Scanning Transmission Electron Microscope (STEM). Like TEM, electrons pass through a thin specimen in STEM and images are formed. The difference is that in STEM, the electron beam is focused on one point first and then scanned over the sample.

In order to enhance the contrast of the viewed vesicles, negative staining is applied. 0.5 – 2 % phosphotungstic acid solution stains the “background” of the

STEM grid and illuminates the vesicles in front. The vesicle is suspended in an emulsion of high ionic strength (Oliver, 1973).

1.6.2. Size and Size Distribution

Vesicle size is one of the most important parameters for skin penetration. An easy way of determining vesicle size and size distribution is Dynamic Light Scattering (DLS). It depends on light that reflects from the suspended colloid particles that are in Brownian motion in solution. The Brownian motion of the particles cause a Doppler shift and the difference in wavelength between the initial light (usually laser) and exiting light correlates directly to the size of the particles. (Sartor, no date) DLS is a quick, cost- and time-efficient method, requiring little equipment.

Size distribution of the vesicles in solution is given by the polydispersity index (PDI) with the DLS data. It is important to have the vesicles as uniform as possible for standardized application of the final formula. Generally a PDI of < 0.2 is accepted as monodisperse and a PDI of < 0.3 is deemed acceptable for vesicle solutions.

1.6.3. Surface Charge Determination

In order to determine the surface charge of the vesicles, zeta-potential is measured. The surface charge of a particle attracts a layer of the opposite charge which is called the Stern Layer. The next layer to the Stern Layer is called the Diffused Layer, which is less tightly bound. This double layer has a Slipping Plane which is associated with the particle movement. Zeta potential is the potential difference between the medium and the surface charge of the particles (Figure 1.16).

The stability of a liposomal suspension is greatly affected by the zeta-potential, the higher the absolute value, the more stable the vesicles (Sautter, 1963). Zeta-potential is affected by several factors such as pH, particle shape, added surfactants and electrolytes (Simunkova *et al.*, 2009).

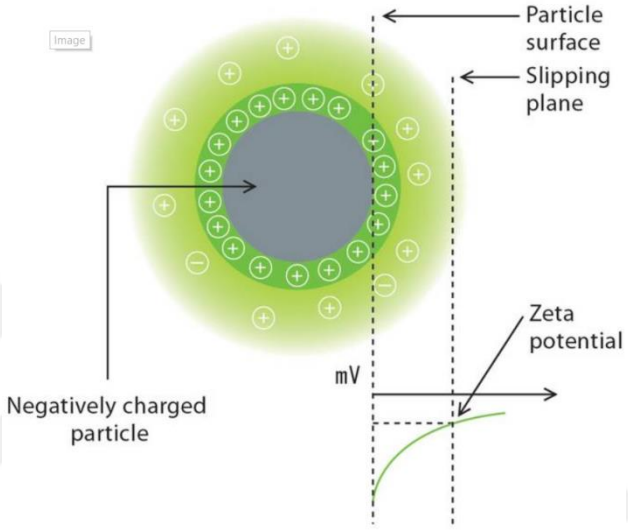

Figure 1.16. Zeta potential (‘HORIBA’, n.d.).

2. AIM OF THE STUDY

Liposomes are vesicles that form due to self-assembly of lipid bilayers. Since our skin cells comprise of lipid bilayers, liposomes are perfect vesicles that mimic the skin cells and deliver encapsulated materials into the skin. However research has shown that liposomes cannot pass the SC effectively as thought. This led to the discovery of (ultra)deformable liposomes.

Transfersomes are one of the first generation of deformable liposomes, incorporating an edge activator or a permeation enhancer. Edge activators work by disrupting the vesicle bilayer structure by relocating to points of less stress when a force is applied to the vesicle (such as going through the skin), therefore making the liposome deformable. Permeation enhancers disrupt the skin matrix and create new pathways through which the vesicles can pass. Another improvement on the liposome is the ethosomes. Ethosomes have 20 – 45 % ethanol within the bilayer structure and can be considered as transfersomes with ethanol as the permeation enhancer.

Transethosomes were made by combining the idea behind both liposomal systems; they contain both ethanol and an edge activator or a permeation enhancer. Research has shown that transethosomes' penetration capability through the SC is superior to conventional liposomes, transfersomes and ethosomes. The aim of this study is to improve this new delivery system by combining lipids with different edge activators and permeation enhancers.

Skin aging is a natural phenomenon. One of the main reasons to aging is the sun's rays. It has been proven that this process can be prevented or even reversed with the use of antioxidants. Some essential oils are natural antioxidants and it is important to keep cosmeceuticals as natural as possible. It has been shown that transethosomes are better at encapsulating lipophilic ingredients. Considering this information, two essential oils were chosen and encapsulated in this research.

3. EXPERIMENTAL

3.1. Materials

3.1.2. Lipid

Phosphatidylcholine (PC) is a natural phospholipid. Soybean phosphatidylcholine (Lipoid S 100) with 94 % PC content, in light orange colored powder form, obtained from Lipoid AG (Germany). The transition temperature is -20°C.

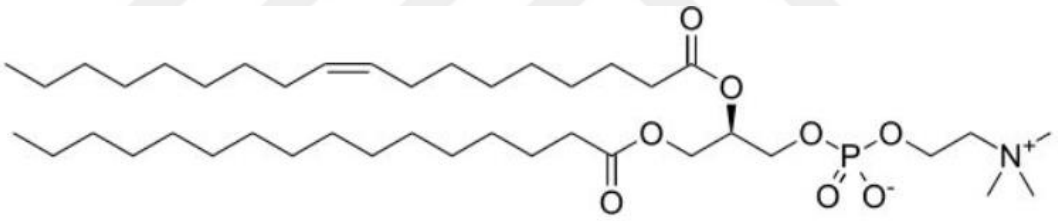

Figure 3.1. Chemical structure of Phosphatidylcholine.

3.1.3. Edge activator/Permeation enhancers

Ethanol with > 99 % purity was purchased from Merck. It is used as a permeation enhancer for the lipid bilayer system, added in the transethosome formation stage.

Oleic acid, a natural fatty acid, was purchased from Merck as colorless, odorless liquid. It is used as a permeation enhancer in the lipid bilayer.

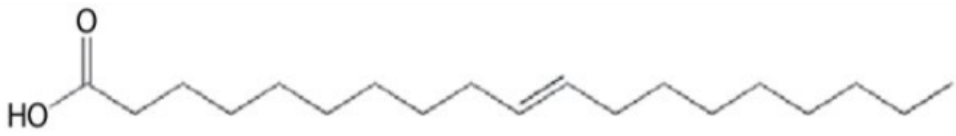

Figure 3.2. Chemical structure of Oleic Acid.

Tween®80 (Polysorbate), a nonionic surfactant and an emulsifier, was purchased from Merck in the form of yellow liquid. It is used as an edge activator in the lipid bilayer system.

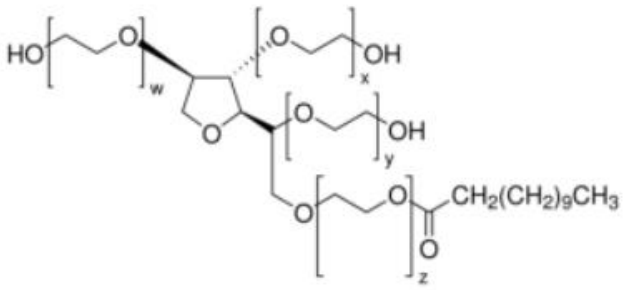

Figure 3.3. Chemical structure of Tween®80.

3.1.4. Encapsulated Materials

The essential oils geraniol and linalool were purchased from Sigma-Aldrich. They are highly odorant, yellowish, oily liquids. These two essential oils were encapsulated within the lipid bilayers of transethosomes.

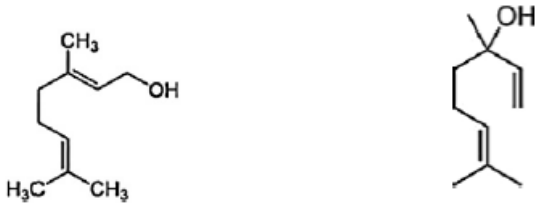

Figure 3.4. Chemical structures of Geraniol (left) and Linalool (right).

3.1.5. Solvents

Chloroform and methanol were purchased from Merck with > 99 % purities. They are used for solubilizing the lipids, essential oils and permeation enhancers prior to obtaining lipid thin film by evaporation.

3.1.6. Suspension Media

0.05 M phosphate buffer solution (PBS) was prepared in the laboratory. 1.36 g potassium dihydrogen phosphate (KH_2PO_4) from Merck was dissolved in 100 mL Type 1 water. 58.2 mL of 0.1 M sodium hydroxide solution (NaOH) from Merck was added. The final solution was obtained by adding Type 1 water to a total end solution of 200 mL. The final pH of the solution was 7.01.

3.1.7. STEM Staining

2 % ethanolic phosphotungstic acid was prepared byÇakır Kimya. It was used to as negative stain on the samples for STEM imaging.

3.2. Instruments

Rotary evaporator from Bibby was used to evaporate the organic phase to obtain the lipid thin film.

Sonicator, Bandelin Sonorex RK 52 was used to downsize transethosomes. Transethosome solutions were placed in the sonicator for ca. 40 minutes.

Water bath, Julabo SW 22 was used to keep the aqueous phase at 30°C.

MSH Basic magnetic stirrer from Yellow-line was used to heat and stir transethosomes mixtures.

Avestin Lipofast mini extruder with 100 nm polycarbonate filters was used to downsize the obtained transethosomes, by filtering 9 successive times to obtain vesicles of uniform size.

Vortex Mixer, Fusion Whirlimixer was used to break down the transethosomes to determine encapsulation efficiencies.

Centrifuge and filtration, Rotafix Hettich centrifuge was used with Amicon filtration units to remove the unencapsulated essential oils from the transethosomes.

DLSfor determination of size and size distribution,and zeta-potential measurements were performed with the 90 Plus Particle Size/Zeta Analyzer from Brookhaven Instruments. DLS measurements were performed after samples were diluted 1:10 with the ethanolic suspension medium (20% PBS/EtOH or 15% H_2O/EtOH).

SEM/STEM; XL30 ESEM-FEG/EDAX was used to visualize vesicle morphologies. A drop of the sample is put on the formvar copper grid, allowed to stay for 3-5 minutes and the excess was carefully wiped off with a filter paper. Then, a drop of the phosphotungstic acid was put on the sample on the grid and allowed for about 10 seconds and then the excess was again wiped off carefully. The grids were allowed to air dry (partially covered) for night before imaging.

UV/Vis spectrophotometer; ATİ UNICAM UV/Vis spectrophotometer was used with 1 cm quartz cuvettes to detect the encapsulation materials.

3.3. Methods

3.3.1. Preparation of Transethosomes

There are two classical methods to prepare liposomes, which were adapted to prepare transethosomes; the cold method and the thin-film evaporation method. When transethosome research is broken down according to preparation methods, thin film hydration method was performed in 10 studies whereas 6 studies investigated the cold method and 1 study has done both methods, to the best of our knowledge.In order to investigate the differences of outcome, both methods were compared in this study.

When following the cold method, 150 mg phosphatidylcholine and either 9.2 µL (min) or 18µL (max) Tween® 80 or 11 µL (min) or 22 µL (max) oleic acid were dissolved in 1.5 mL ethanol in a 25 mL round bottom flask in a water bath at 30°C±2°C while mixing on a magnetic stirrer at 700 rpm. In a separate round bottom flask, type 1 water was kept at 30°C±2°C in a water bath. After the PC and Tween® 80 or oleic acid were dissolved, 3.5 mL type 1 water was added drop-wise as 250µL / min. to the solution. After all the water was added, the solution was stirred at 30°C at 700 rpm for 20 minutes. The flask was taken out of the water bath and was allowed to cool to room temperature for 30 minutes.

The obtained vesicles are yet too large for further use. There are two main ways of downsizing vesicles, sonication and extrusion. In order to investigate effects of both downsizing methods, both were performed.

In case of extrusion, the obtained transethosomes are LUVs and were downsized to SUVs with the liposome extruder by passing the solution 9 times through 100 nm polycarbonate membranes. In case of sonication, a bath sonicator was used to downsize the vesicles for 40 minutes.

The final solution was wrapped in aluminum foil and placed in the refrigerator at 4°C in order to prevent degradation by oxidation and light.

When following the thin-film evaporation method, 360 mg phosphatidylcholine and either 40µL (min) or 80 µL (max) Tween® 80 or 50 µL (min) or 100 µL (max) oleic acid were dissolved in 12 mL of a mixture of chloroform : methanol (2:1) at 35°C±2°C in a 100 mL round bottom flask, in a water bath on a magnetic stirrer. Then, vacuum was applied and the organic solvent mixture was evaporated at 600 mbar and 35 °C±2°C in the water bath for 1 hour. The so obtained lipid thin-film was covered with a KimVipe to prevent contamination and left to dry completely overnight.

The thin film was hydrated with 5 mL PBS/EtOH for 1 hour at room temperature on the rotary evaporator. The obtained transethosomes were downsized to SUVs either with the liposome extruder by passing the solution 9 times through 100 nm polycarbonate membranes or sonicated 40 minutes with a bath sonicator. The final solution was wrapped in aluminum foil and placed in the refrigerator at 4°C in order to prevent oxidation and degradation

3.3.2. Encapsulation of Transethosomes

The essential oils geraniol and linalool were encapsulated within transethosomes. Due to the lipophilic nature of the encapsulated materials, they were -separately- added to the organic solvents. In the cold method, the essential oilswere

added to the initial ethanol solution. In the thin-film hydration method they were added to the initial chloroform : methanol solution.

4. RESULTS & DISCUSSION

4.1. Comparison of Different Preparation Methods

Within the framework of this study, empty transethosomes were prepared according to both methods with the same ratio of lipids to edge activator/permeation enhancer in order to assess the effects of the two methods, with and without any interactions from encapsulation materials. Size and Size distribution (PDI) were measured via DLS with the Particle Size Analyzer; the vesicles were visualized via stained STEM. All measurements were performed once.

Size and homogeneity of the obtained vesicles are compared in Table 4.1according to their respective methods. When comparing the empty transethosomes, the most difference can be seen when vesicle sizes from both methods are compared; the vesicles obtained from the thin-film hydration method are significantly larger than the ones obtained from the cold method.It has been reported that the vesicle size should be around 200 – 400 nm for optimal skin penetration, howeverit has also been reported that sizes around 30 – 40 nm were favorable as well, depending on application. In this sense, vesicles obtained with the cold method should show better skin penetration, however the size and PDI of the vesicles obtained with the thin-film method are also acceptable and so this method can also be used and/or further downsized via extended extrusion or sonication.

PDI gives the overall quality of the transethosome solution with respect to stability. Generally a PDI of < 0.2 indicates a monodisperse size distribution and a PDI of < 0.3 is deemed acceptable for monodisperse vesicle systems.The PDI of both methods were more or less similar and in the acceptable range.

When comparing loaded transethosomes, it can be seen that the vesicles encapsulating the essential oil linalool are smaller than the ones encapsulating

geraniol, regardless of the choice of permeation enhancer/edge activator. In the case of transethosomes prepared with Tween®80 encapsulating linalool, the vesicles are unusually large. This may be due to aggregation of vesicles. The loaded transethosomes prepared by the thin-film hydration method, show higher PDI values than those prepared with the cold method. This may be due to lipid film residues, obtained in the hydration step, which is absent for the cold method. This can be amended by a purification step.

The STEM images of both methods showed slightly deformed generally spherical vesicles, which is expected of deformable vesicles. When the images of the vesicles are observed, as it can be seen in Figures 4.1 – 4.4, the vesicles of each method look consistent within each other. When compared to each other, the vesicles from the cold method are darker with a grey hue and the vesicles of the thin film hydration method are more illuminated with less hue. The “oilier” look of the thin film hydration method vesicles may be due to traces of the lipid film, remaining after hydration.

In the light of this information, cold method proves to be the better choice of preparation in terms of size and PDI. When comparing both methods in their ease of handling, cold method requires less time and resources than the thin-film hydration method; which also indicates better scale up for mass production. Also in general, the vesicles prepared via the thin-film hydration method require more work up, since the method includes more solvents and a lipid film.

Table 4.1 Comparison of different preparation methods.

Samples	Cold Method	Thin-film Hydration Method
	Mean effective diameter (nm) (PDI)	Mean effective diameter (nm) (PDI)
Empty transethosomes with min Oleic acid	125.7 (0.197)	299.8 (0.177)
Empty transethosomes with max Oleic acid	171.8 (0.264)	247.9 (0.139)
Empty transethosomes with min Tween®80	59.9 (0.183)	186.6 (0.154)
Empty transethosomes with max Tween®80	57.4 (0.186)	261.4 (0.169)
Linalool loaded transethosomes with Oleic acid	126 (0.148)	152.3* (0.298)
Linalool loaded transethosomes with Tween®80	69.9 (0.182)	1411.3* (0.296)
Geraniol loaded transethosomes with Oleic acid	753.7* (0.215)	275.9* (0.301)
Geraniol loaded transethosomes with Tween®80	75.3 (0.188)	68.1* (0.041)

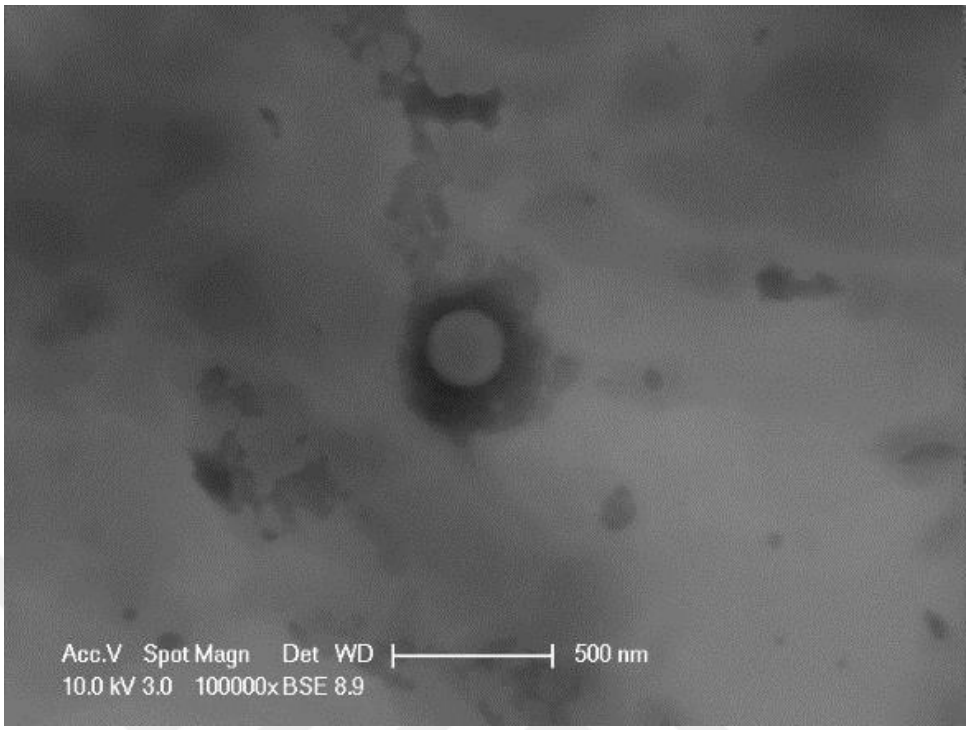

Figure 4.1. Empty transethosomes with min oleic acid via cold method.

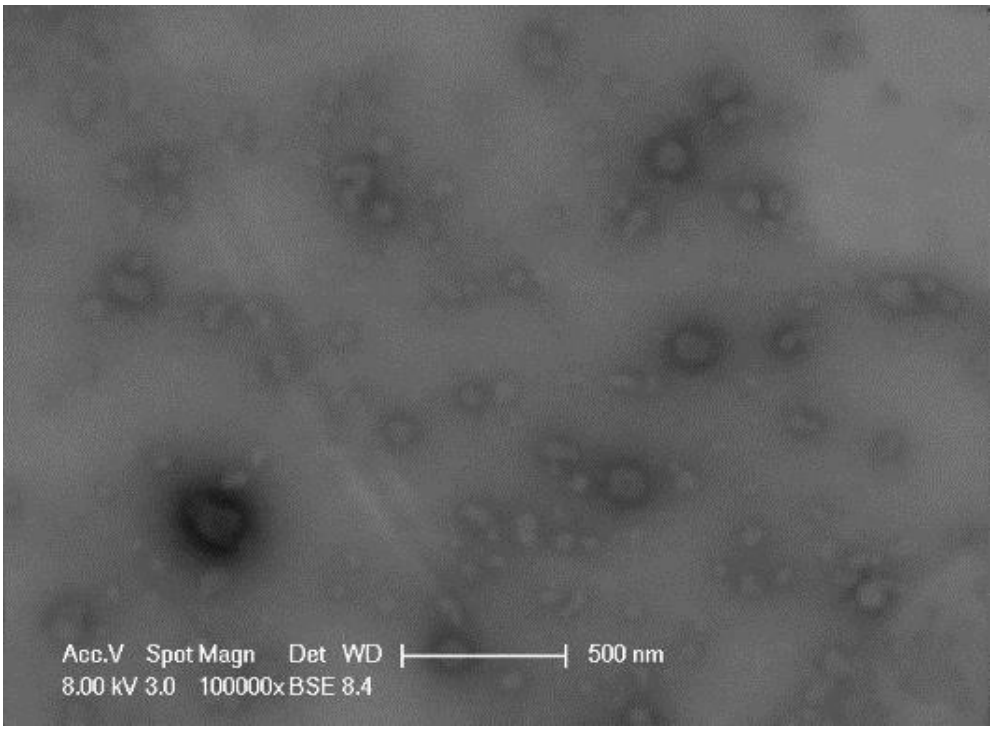

Figure 4.2. Empty transethosomes with min Tween®80 via cold method.

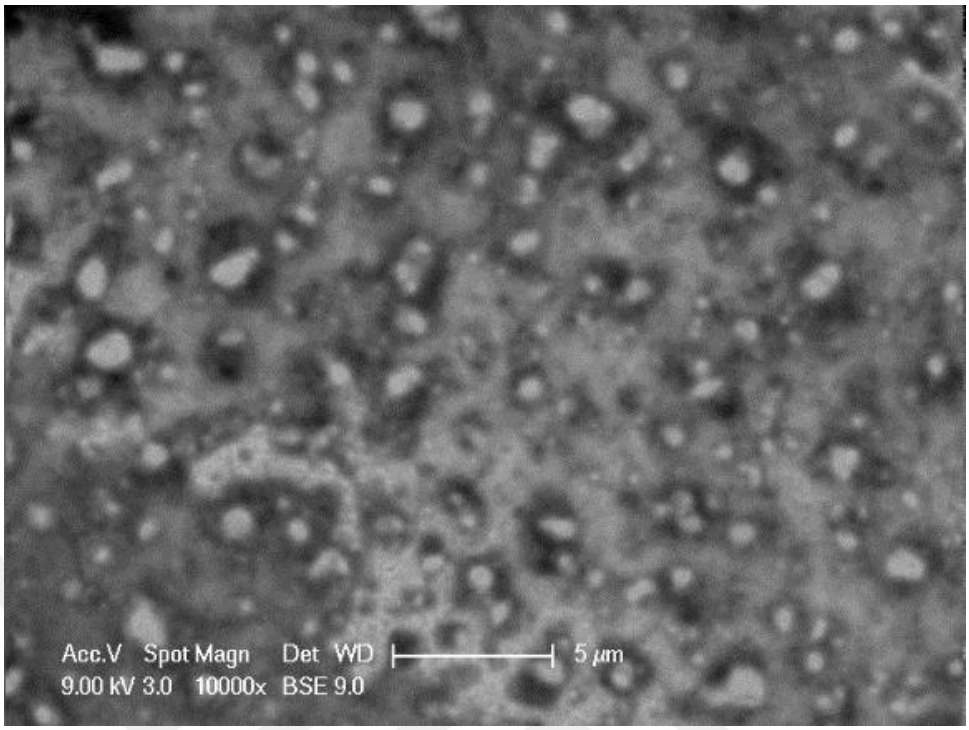

Figure 4.3. Empty transethosomes with min Oleic acid via thin-film hydration method.

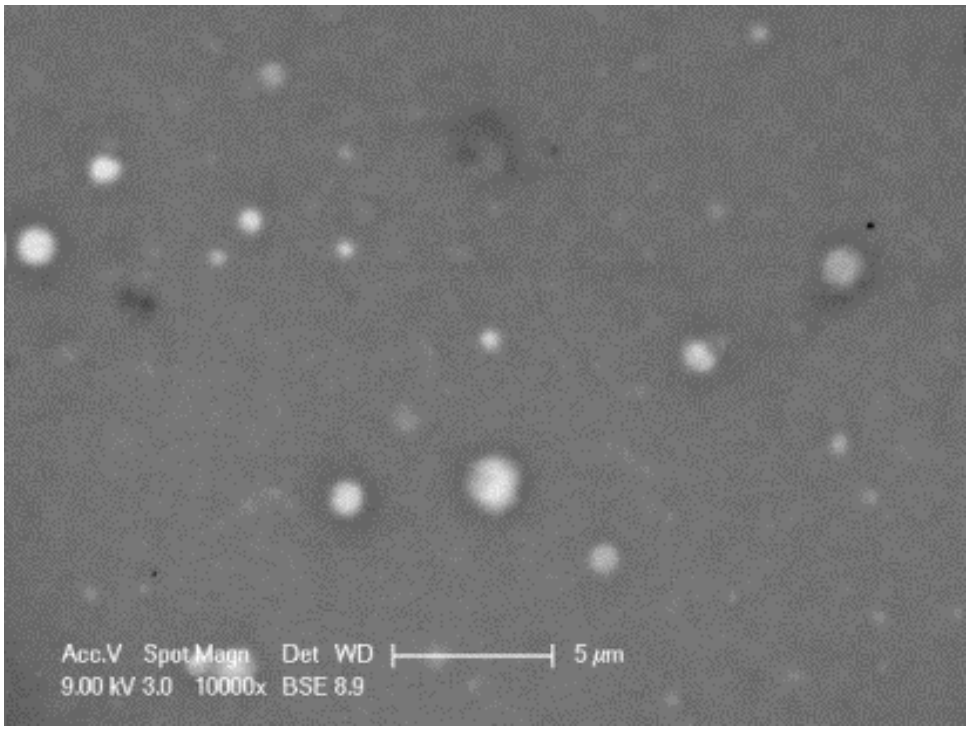

Figure 4.4. Empty transethosomes with min Tween®80 via thin-film hydration method.

4.2. Effects of the Choice and Concentrations ofaPermeation Enhancer or an Edge Activator

Oleic acid was the choice of permeation enhancer in this study. For both methods,0.2 % (w/w) oleic acid at minimum and 0.4 % (w/w) at maximum of phosphatidylcholinewere used to make empty vesicleswith 3 % (w/w) phosphatidylcholine.

Tween®80 was chosen as the edge activator and was used 0.2 % (w/w)at minimum and 0.4 % (w/w) at maximum of the phosphatidylcholinewith 3 % (w/w) phosphatidylcholine.

For the cold method, as can be seen in Table 4.2, the vesicle sizes with oleic acid are significantly bigger than those with Tween®80. However, the PDIs are compliant and similar. Since generally, smaller vesicles show better permeation through the skin, here the Tween® 80 vesicles are smaller, therefore better. For the thin-film hydration method, the size and PDI values didn't show significant fluctuations.

Table 4.2 Size (nm) and PDI of empty transethosomes for comparison of permeation enhancer, edge activator and their concentrations.

Method	Oleic acid		Tween®80	
	Min(nm) (PDI)	Max(nm) (PDI)	Min(nm) (PDI)	Max(nm) (PDI)
Cold Method	125.7 (0.197)	171.8 (0.264)	59.9 (0.183)	57.4 (0.186)
Thin-film Hydration Method	299.8 (0.177)	247.9 (0.139)	186.6 (0.154)	261.4 (0.169)

As for concentrations of the permeation enhancer and the edge activator,it can be seen in Table 4.2, for the cold method, that increasing the oleic acid concentration lead to larger vesicles whereas increasing the Tween®80 concentration did not show significant changes. For the thin-film hydration method, increasing the oleic acid concentration lead to smaller vesicles, whereas an increased Tween®80 concentration resulted in larger vesicles.

The STEM images of vesicles with higher concentrations (max) show an "oily" look between the vesicles(Figure 4.4 – 4.7). It can be concluded that doubling the permeation enhancer and the edge activator concentrations apparently lead to free permeation enhancer and the edge activator between the vesicles. Therefore 0.2 % (w/w) of phosphatidylcholine is the optimalpermeation enhancer and the edge activator concentration. The loading of transethosomes were continued with the minimum concentration.

In Table 4.3, it can be seen that at the same concentrations, oleic acid lead to larger vesicles than Tween®80, with one exception. This may have been cause due to aggregation of vesicles.

Table 4.3 Size and PDI of loaded transethosomes for comparison of permeation enhancer and edge activator (nm) (PDI).

Method	Oleic acid (min)		Tween®80 (min)	
	Linalool(nm) (PDI)	Geraniol(nm) (PDI)	Linalool(nm) (PDI)	Geraniol(nm) (PDI)
Cold Method	126 (0.148)	753.7 (0.215)	69.9 (0.182)	75.3 (0.188)
Thin-film Hydration Method	152.3 (0.298)	275.9 (0.301)	1411.3 (0.296)	68.1 (0.041)

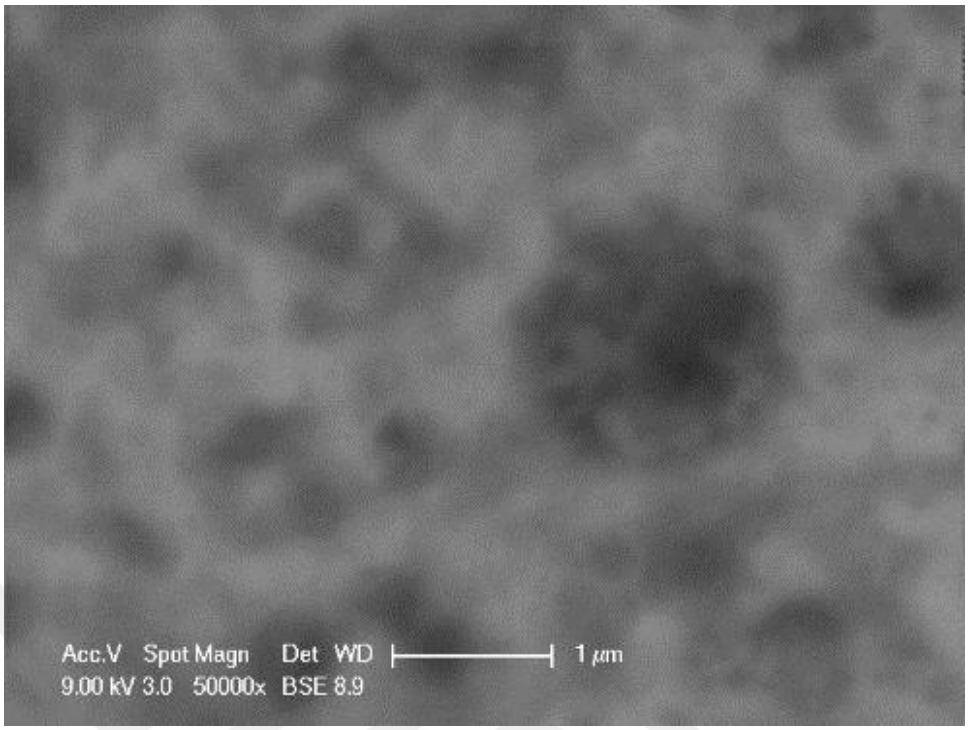

Figure 4.5. Empty transethosomes with max oleic acid via cold method.

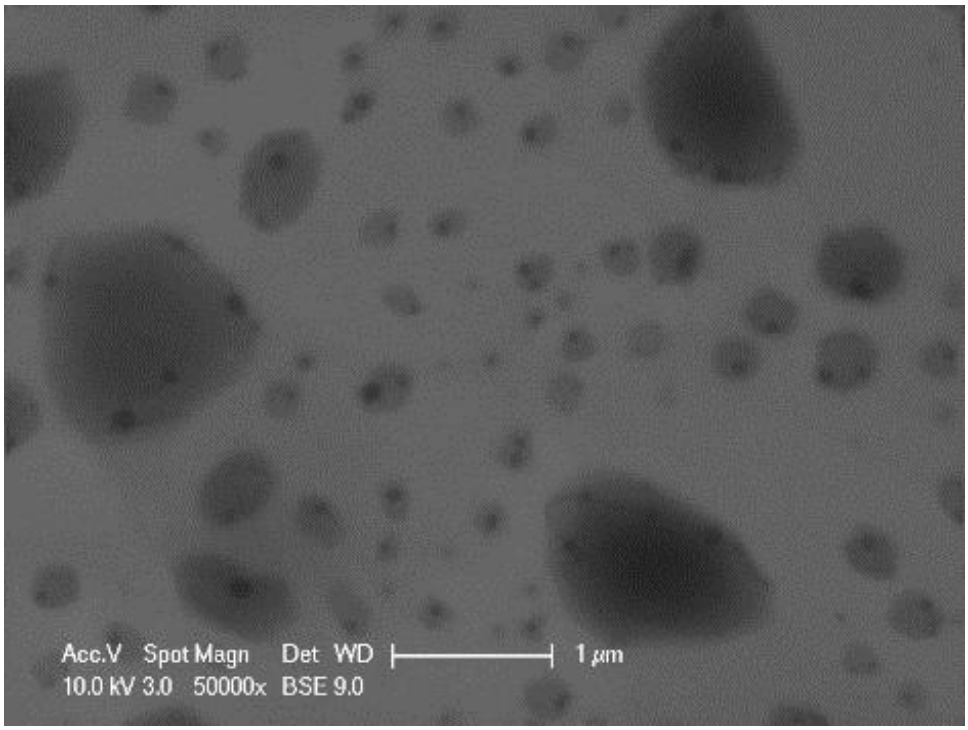

Figure 4.6. Empty transethosomes with max Tween®80 via cold method.

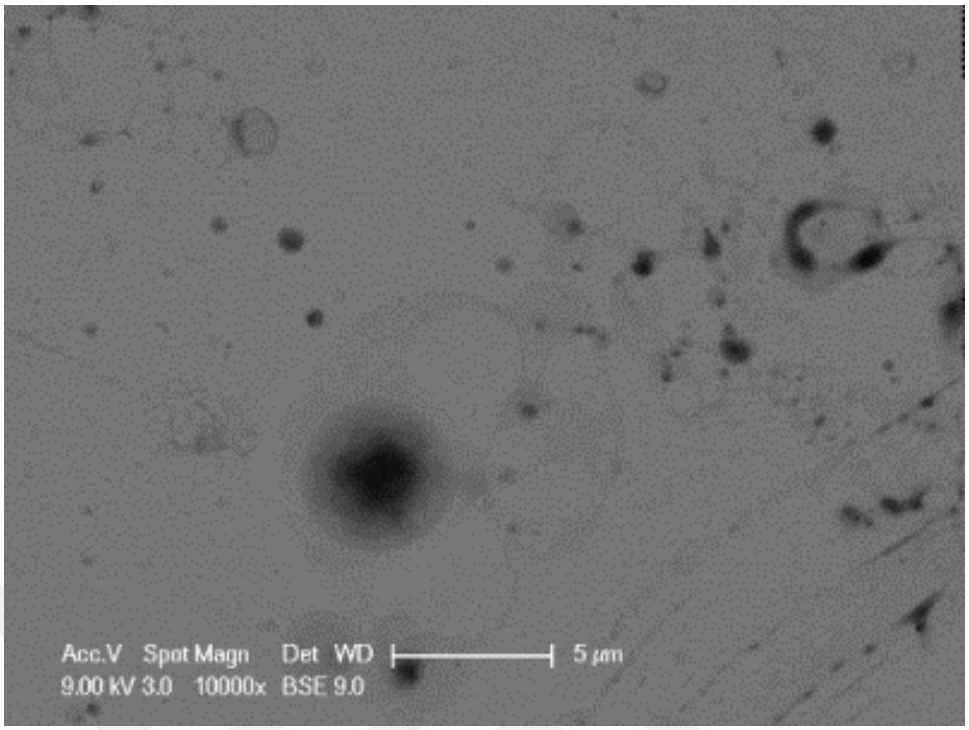

Figure 4.7. Empty transethosomes with max Oleic acid via thin-film hydration.

4.3. Empty vs Loaded Transethosomeswith Different Materials

Since transethosomes are proven to encapsulate lipophilic compounds better than they do hydrophilic compounds and for their aforementioned benefits, the two essential oils, geraniol and linalool were the choices of encapsulation materials.

As it can be seen in Table 4.4,when all empty and loaded vesicles prepared via the cold method were compared, the sizes and PDI values of empty vesicles and linalool loaded vesicles were almost identical, with one exception. Geraniol loading with oleic acid via the cold method was not favorable in terms of size.

For the vesicles prepared by the thin-film hydration method with oleic acid and linalool, empty vesicles were found to be almost twice as large as the loaded

vesicles, whereas the difference is much smaller for geraniol loaded vesicles. The largest difference can be seen when the vesicles with Tween®80 and linalool are compared; this may be due to aggregation of vesicles. The vesicles with Tween®80 and geraniol resulted in very small vesicles. It can be seen that geraniol loading is much more favorable with Tween®80.

Table 4.4 Size (nm) and size distribution of empty and loaded transethosomes.

Method	Empty(nm) (PDI)		Linalool loaded(nm) (PDI)		Geraniol loaded(nm) (PDI)	
	Oleic acid	Tween ®80	Oleic acid	Tween® 80	Oleic acid	Tween® 80
Cold Method	125.7 (0.197)	59.9 (0.183)	126 (0.148)	69.9 (0.182)	753.7 (0.215)	75.3 (0.188)
Thin-film Hydration Method	299.8 (0.177)	186.6 (0.154)	152.3 (0.298)	1411.3 (0.296)	275.9 (0.301)	68.1 (0.041)

PDI values remained stable both for empty and loaded vesicles within the cold method, however the difference was larger for the empty and loaded vesicles prepared via the thin-film hydration method.

STEM images of both loaded and empty vesicles were compliant with near spherical shape which is expected of deformable vesicles (Figures 4.9 – 12). Generally the transethosomes prepared via the thin-film hydration method were harder to visualize with STEM; they formed a thick layer on the STEM grid. This may be attributed to the fact that the lipid film wasn’t completely disintegrated during the hydration step.

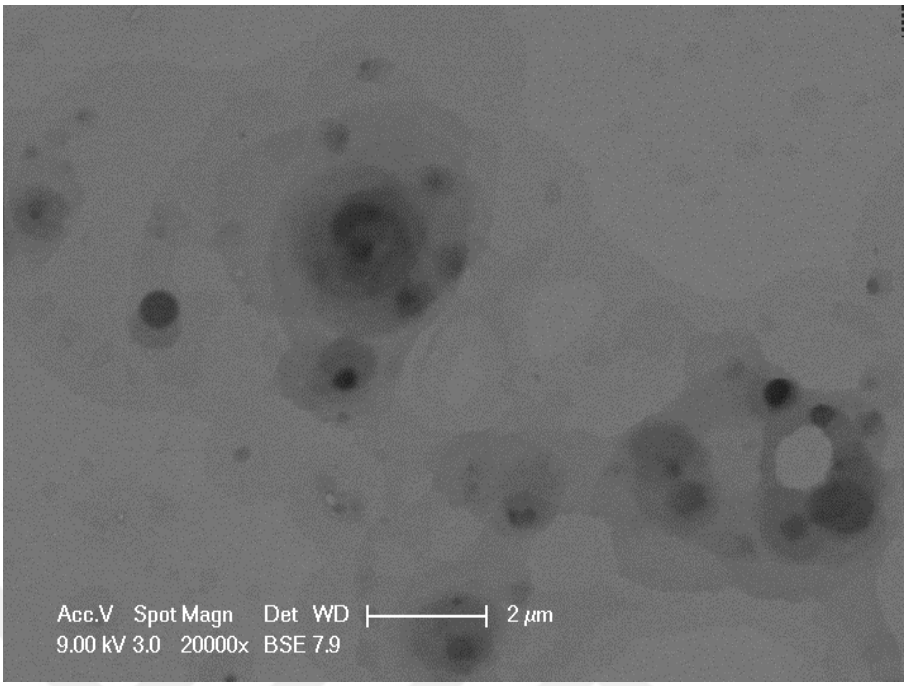

Figure 4.8. Transethosomes with oleic acid and linalool via cold method.

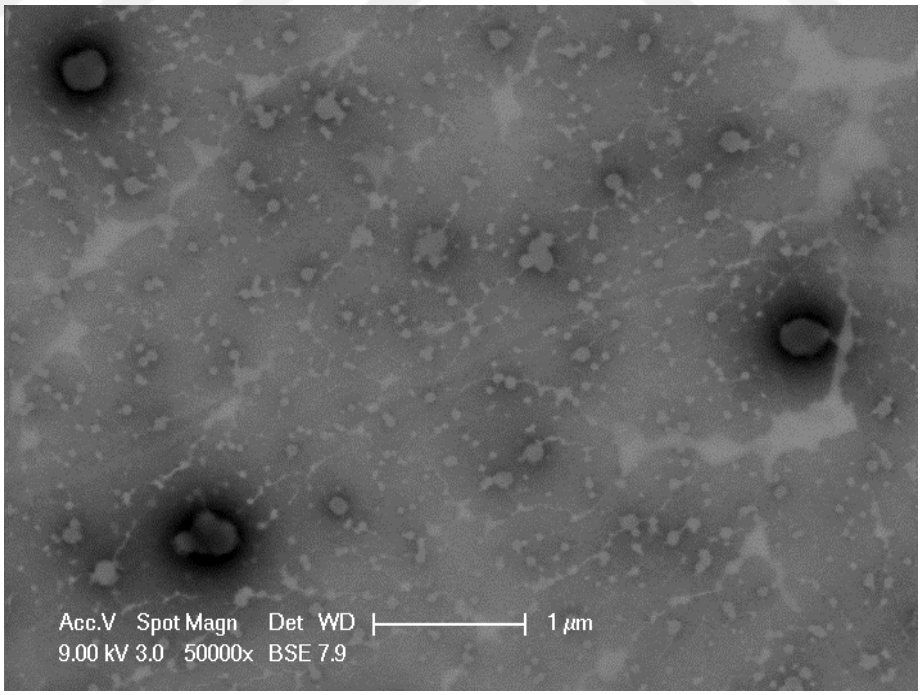

Figure 4.9. Transethosomes with Tween®80 and linalool via cold method.

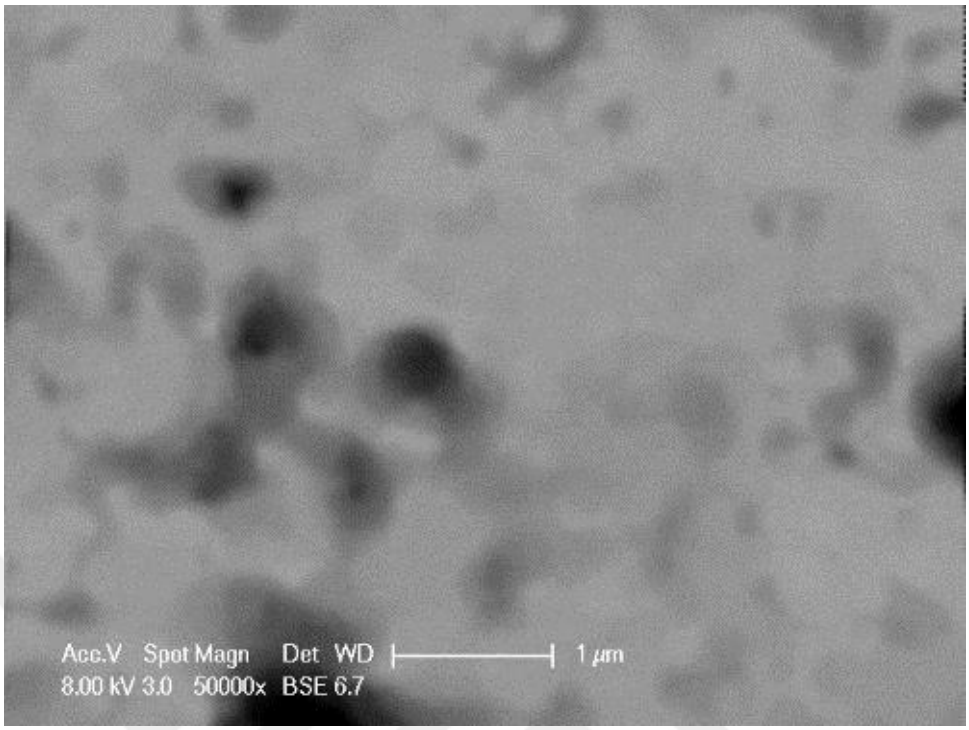

Figure 4.10. Transethosomes with oleic acid and geraniol via cold method.

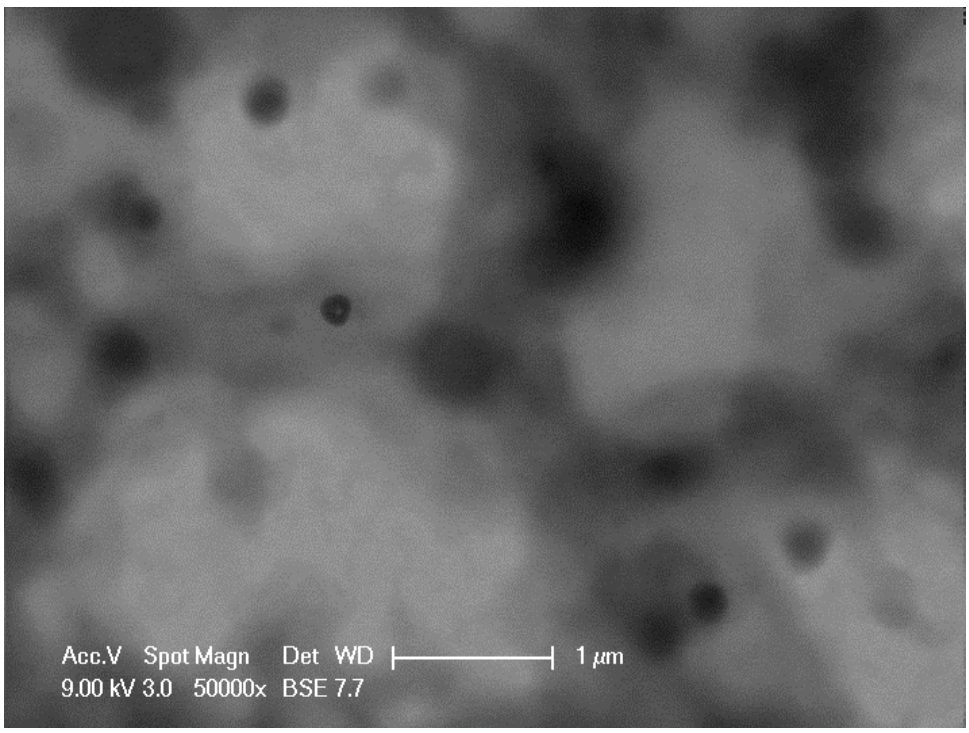

Figure 4.11. Transethosomes with Tween®80 and geraniol via cold method.

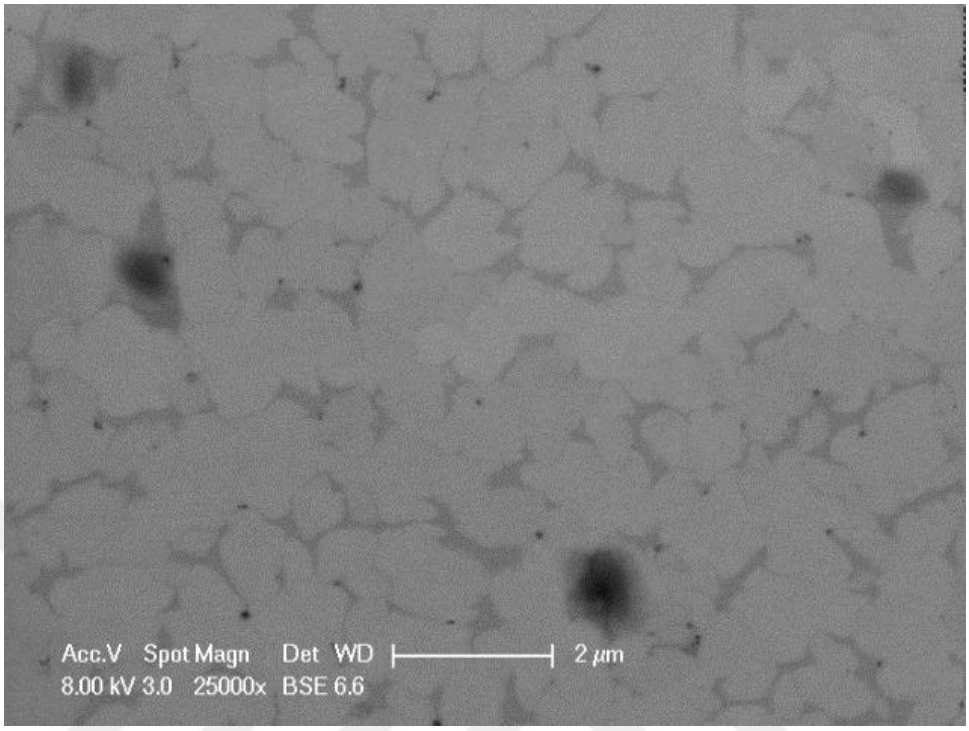

Figure 4.12. Transethosomes with oleic acid and linalool via thin-film hydration.

4.4. Effects of Downsizing Methods

Five out of eight of the loaded transethosomes were sonicated (marked with an *) and the rest were extruded. As it can be seen in Table 4.1, with one exception, extrusion resulted in smaller vesicles. Depending on the desired vesicle size, both downsizing methods can be used without much difference in PDI values.

Also an interesting occurrence was observed during sonication of the vesicles. When the transethosome solutions were sonicated in a 100 mL or 250 mL round bottom flask, the expected transition from a milky suspension to a translucent, bluish lipid bilayer vesicle look did not happen. Only when the solution was transferred to a 50 mL round bottom flask the transformation was achieved in a short

time. This observation indicates that the size of the vessel is important not only in the lipid formation step but in the downsizing step as well.

4.5. Encapsulation Efficiency

Using a UV/Vis spectrophotometer, maximum absorbance for linalool was found to be 2.899 at 209 nm and two peaks were determined for geraniol as 3.076 at 212 nm and 3.085 at 220 nm, where 20 mg of the essential oils were dissolved in 10 mL solvent mixture. The free essential oils in the transethosome samples were separated using centrifugation at 60000 rpm and were controlled systematically with the UV/Vis spectrophotometer until the free oil peaks were vanished. Then the transethosomes were broken down with 1:1 methanol with subsequent vortexing and the released encapsulated materials were detected with UV/Vis spectrophotometer. The best results were obtained for the vesicles prepared via the thin-film hydration method with oleic acid and linalool with and absorbance of 0.218 resulting in 18.75% encapsulation efficiency and for the vesicles prepared via the thin-film hydration method with oleic acid and geraniol with an absorbance of 0.313 corresponding to an encapsulation efficiency of 35%.

4.6. Zeta Potential Measurements

Although, it depends on the encapsulation material, due to their ethanol content, transethosomes are generally expected to have negative zeta potentials. As it can be seen in table 4.5, all obtained results are below zero as expected, with some exceptions. However these values are near zero with 0.4, 0.5 and 0.7. Zeta potential is a measure to predict the stability of the vesicle suspension. When the vesicles all have the same charge, they repel each other and the aggregation of vesicles is delayed, thus stability is achieved.

Table 4.5 Zeta Potential Values of all Transethosomes.

Samples	Zeta Potentials	
	Cold Method	Thin-film Hydration Method
Empty transethosomes with min Oleic acid	-21.50	-5.21
Empty transethosomes with max Oleic acid	-11.89	-14
Empty transethosomes with min Tween®80	-10.31	0.454
Empty transethosomes with max Tween®80	-1.94	0.560
Linalool loaded transethosomes with Oleic acid	-14.57	-7.11×10^{-3}
Linalool loaded transethosomes with Tween®80	-3.92	0.747
Geraniol loaded transethosomes with Oleic acid	-3.17	-0.248
Geraniol loaded transethosomes with Tween®80	-2.3	-0.235

5. CONCLUSIONS

Within the frameworks of this study, different variables of a new type of deformable liposomes, the transethosomes, were investigated. Effects of different preparation and downsizing methods, permeation enhancer/edge activator choice and different encapsulation materials were reported.

The size and homogeneity of transethosomes were examined via DLS and visualized by STEM, which are key parameters in skin permeation and product formulation. In order to assess the encapsulation properties of the vesicles, two essential oils were encapsulated. Linalool and geraniol are widely used ingredients for their diverse properties.

When the two preparation methods are compared, the cold method was found to be superior to the thin-film hydration method for several reasons such as; ease of handling, less work up and cheaper due to fewer ingredients, better PDI and smaller vesicles.

Comparing Tween®80 and oleic acid, PDI values were not far apart but Tween®80 resulted in smaller vesicles, hence it is regarded as the better choice for better skin permeation. Considering the oily residues in the final product, a Tween®80 concentration of 0.2% of the lipid concentration proved to be optimal.

Looking at empty and loaded transethosomes, lipophilic loading between the lipid bilayers did not alter the size of the vesicles too much within the cold method preparations. However the loaded vesicles prepared via the thin-film hydration method showed larger PDI values, i.e. broader size distribution, than the empty vesicles. This, in turn,again favors the cold method for loading lipophilic ingredients.

As for the choice of downsizing method, sonication resulted generally in larger transethosomes with higher PDI values. When smaller vesicles are the goal, extrusion seems to be the better choice.

Lastly, transethosomes showed good encapsulation efficiency values for lipophilic materials, as expected.

Although here, the transethosomes were examined with essential oils for their many benefits including theiranti-oxidant applications, this new type of vesicles can be and are being investigated widely to be used to encapsulate various active ingredients from drugs to immunizations. This study was an attempt at improving transethosomes for better skin permeation to different layers of the skin, leaving the choice of permeation depth to be optimized for desired applications in the future by trying to show an example in the way of customizing this delivery system.

In conclusion, their ease of preparation using few and safe materials makes transethosomes a good choice of delivery system. Here, the observed properties of transethosomes indicate that this new type of (ultra-)deformable liposomes can be used for many applications, including transdermal delivery.

6. FUTURE WORK

Better methods should be developed and optimized for testing the encapsulation efficiencies of transethosomes.

The effective permeation of transethosomes through the skin can be tested via systems such as Franz diffusion cell.

Like liposomes or other ethanolic systems, transethosomes can be incorporated into systems like gel, cream or patches.

Transethosomes can be studied to encapsulate various hydrophilic ingredients. A dual encapsulation of both hydrophilic ingredients in the aqueous core and lipophilic ingredients between the bilayers can be of notable interest.

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APPENDIX A: DLS ANALYSIS RESULTS

The following pages show the DLS analyses results of the prepared samples.

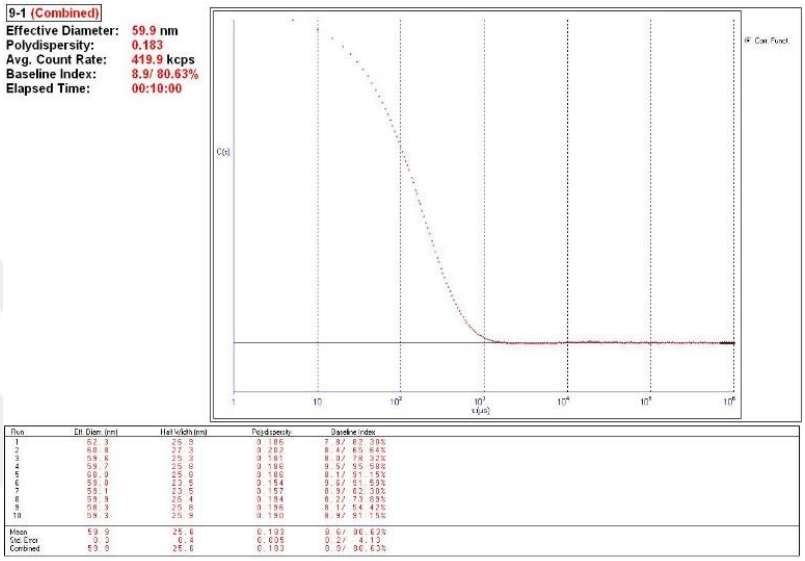


Figure A.1. Transethosomes via cold method with min Tween®80.

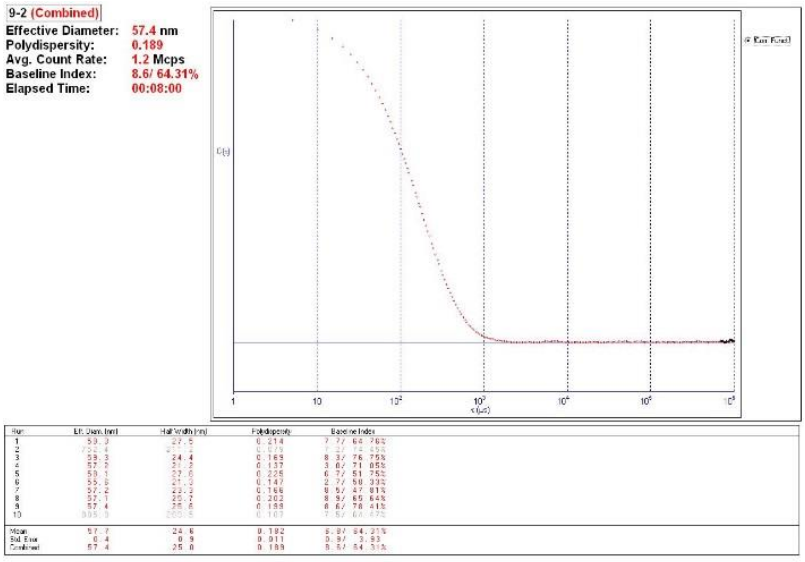
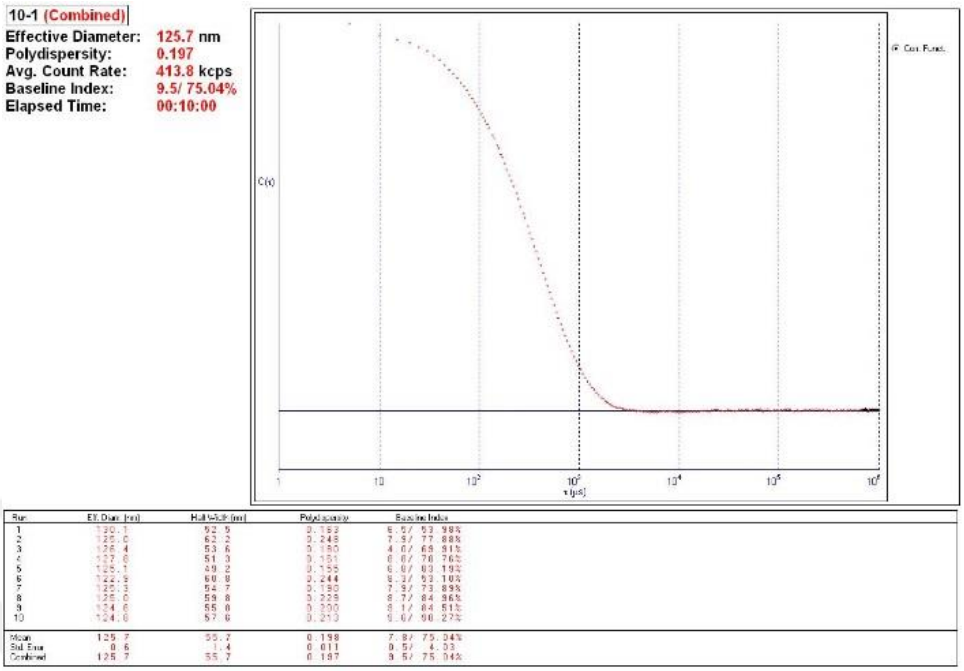
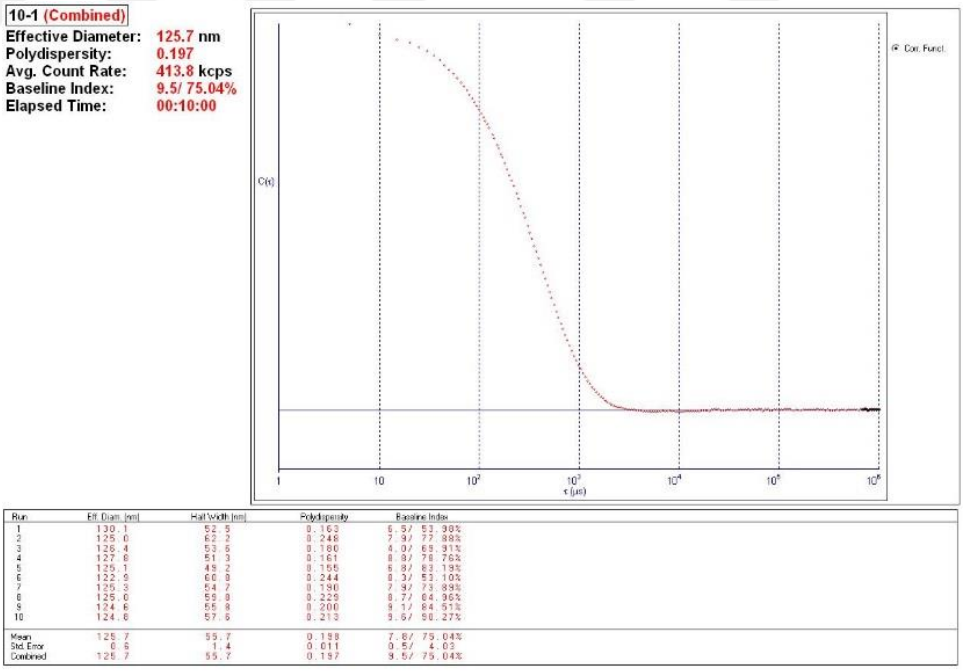


Figure A.2. Transethosomes via cold method with max Tween®80.



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	130.1	52.5	0.163	6.5/ 53.98%
2	125.0	62.2	0.246	7.9/ 77.88%
3	126.4	53.6	0.180	4.0/ 68.91%
4	127.8	51.3	0.161	8.8/ 78.76%
5	125.1	49.2	0.156	6.8/ 83.19%
6	122.9	60.9	0.244	8.3/ 53.10%
7	125.3	54.7	0.190	7.9/ 73.89%
8	125.6	59.8	0.229	8.7/ 84.96%
9	124.6	55.8	0.200	9.1/ 84.51%
10	124.6	57.6	0.213	9.5/ 90.27%
Mean	125.7	55.7	0.198	7.8/ 75.04%
Std. Error	0.6	1.4	0.011	0.5/ 4.03
Combined	125.7	55.7	0.197	9.5/ 75.04%

Figure A.3. Transethosomes via cold method with min oleic acid.



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	130.1	52.5	0.163	6.5/ 53.98%
2	125.0	62.2	0.246	7.9/ 77.88%
3	126.4	53.6	0.180	4.0/ 68.91%
4	127.8	51.3	0.161	8.8/ 78.76%
5	125.1	49.2	0.156	6.8/ 83.19%
6	122.9	60.9	0.244	8.3/ 53.10%
7	125.3	54.7	0.190	7.9/ 73.89%
8	125.6	59.8	0.229	8.7/ 84.96%
9	124.6	55.8	0.200	9.1/ 84.51%
10	124.6	57.6	0.213	9.5/ 90.27%
Mean	125.7	55.7	0.198	7.8/ 75.04%
Std. Error	0.6	1.4	0.011	0.5/ 4.03
Combined	125.7	55.7	0.197	9.5/ 75.04%

Figure A.4. Transethosomes via cold method with max oleic acid.

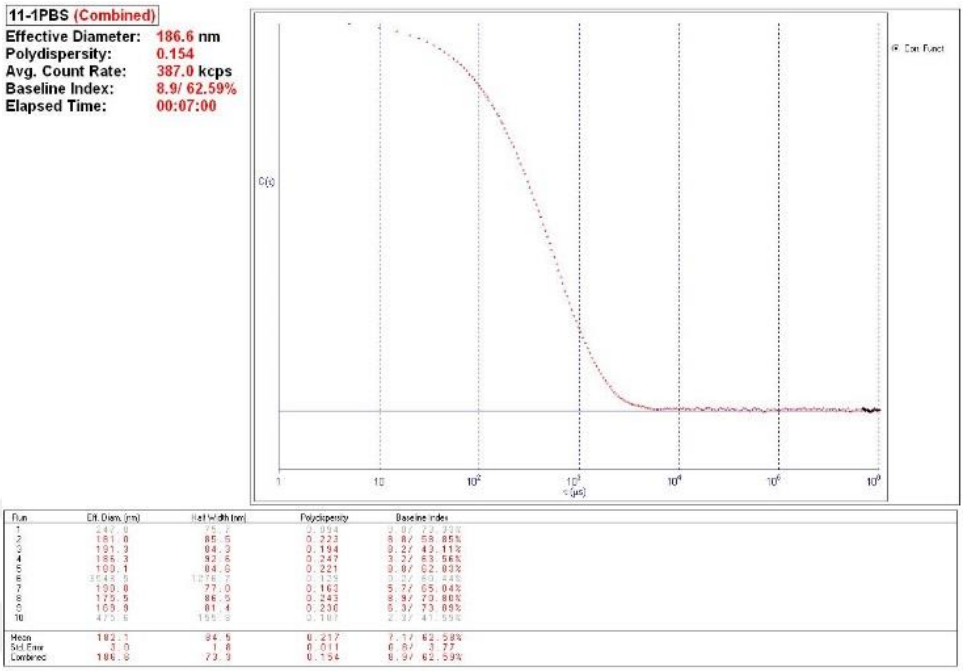


Figure A.5. Transethosomes via thin-film hydration method with min Tween®80.

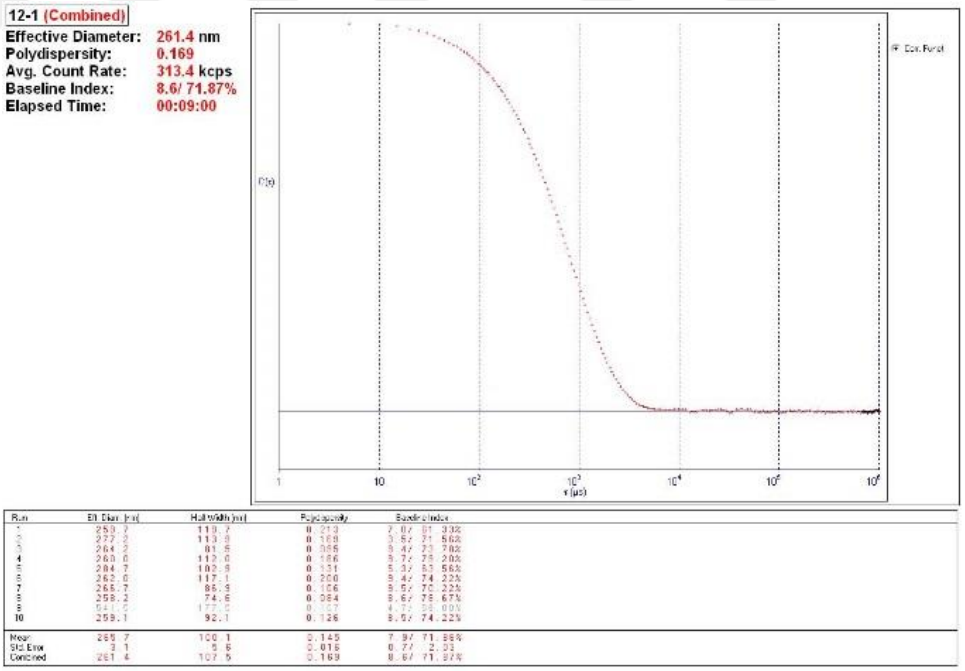


Figure A.6. Transethosomes via thin-film hydration method with max Tween®80.

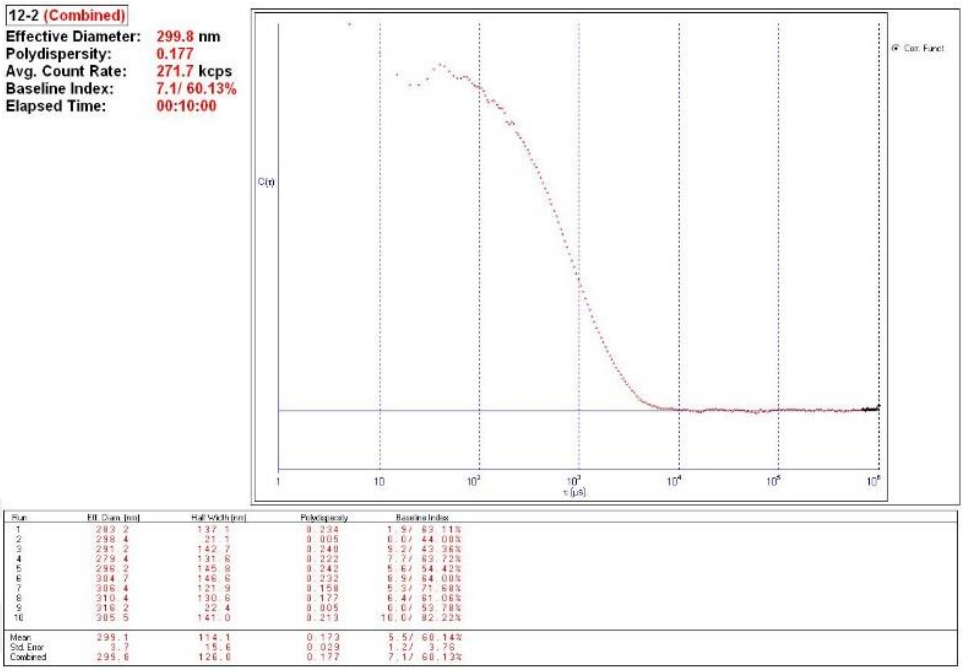


Figure A.7. Transethosomes via thin-film hydration method with min oleic acid.

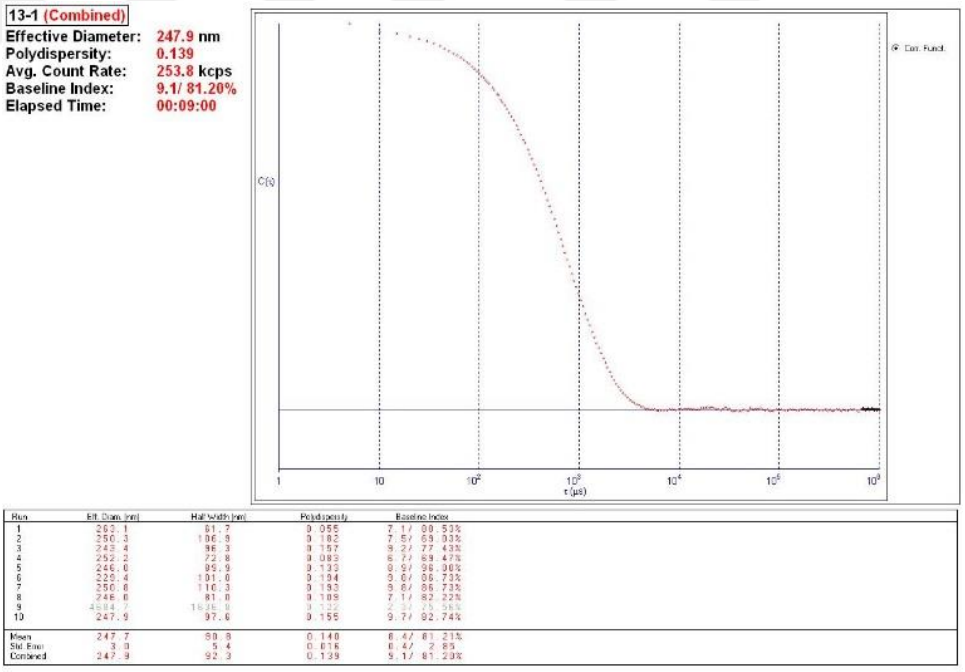


Figure A.8. Transethosomes via thin-film hydration method with max oleic acid.

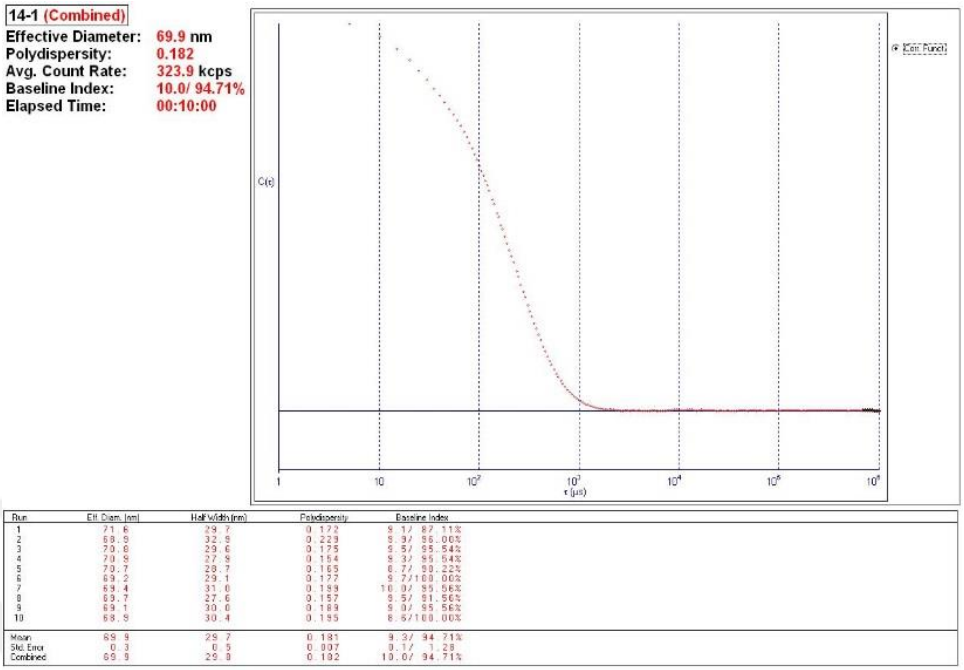


Figure A.9. Transethosomes via cold method with Tween®80 and linalool.

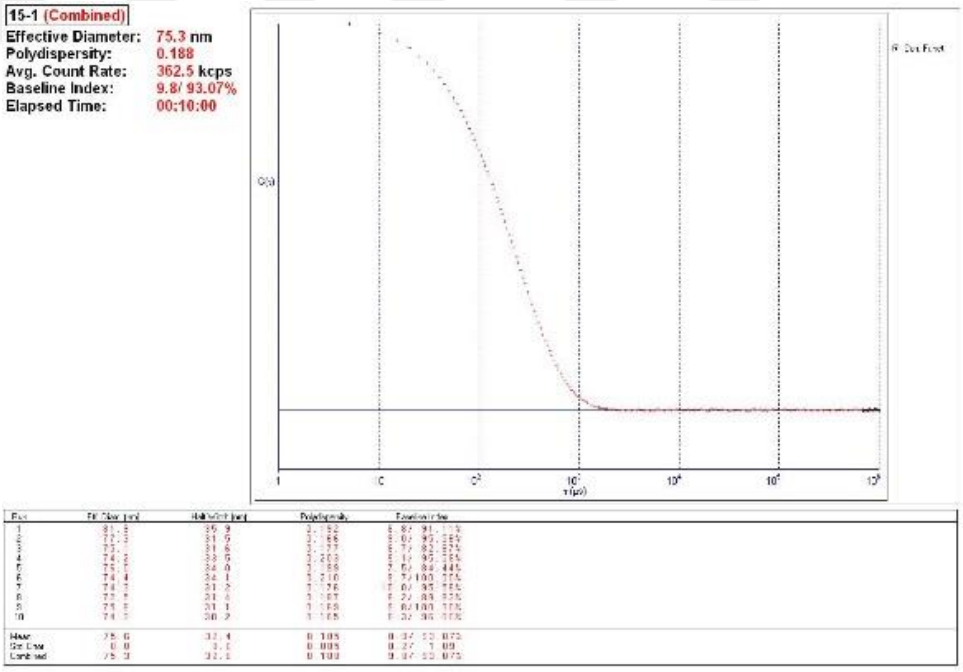


Figure A.10. Transethosomes via cold method with Tween®80 and geraniol.

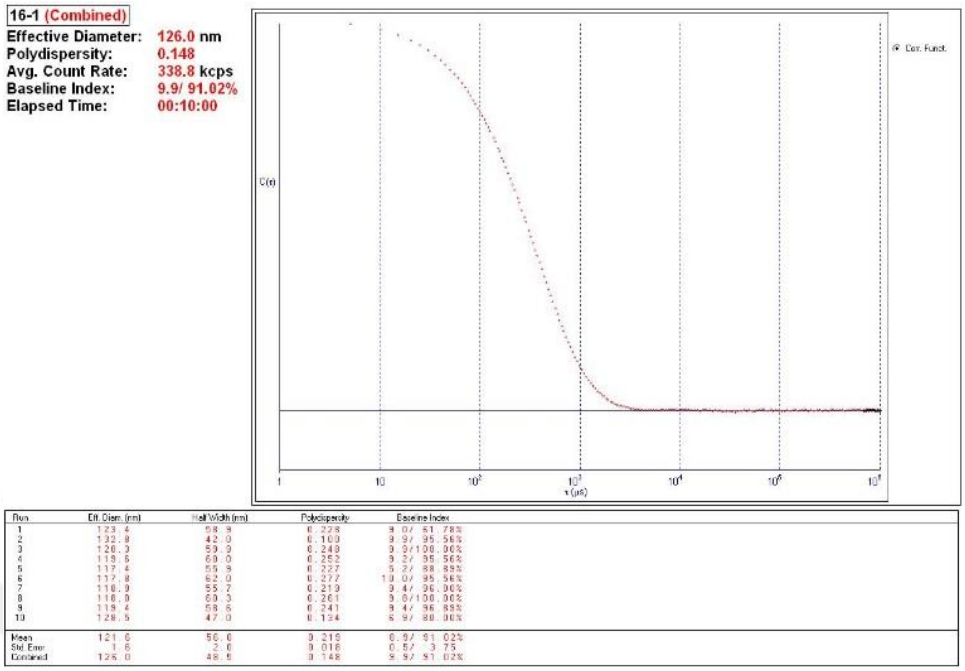


Figure A.11. Transethosomes via cold method with oleic acid and linalool.

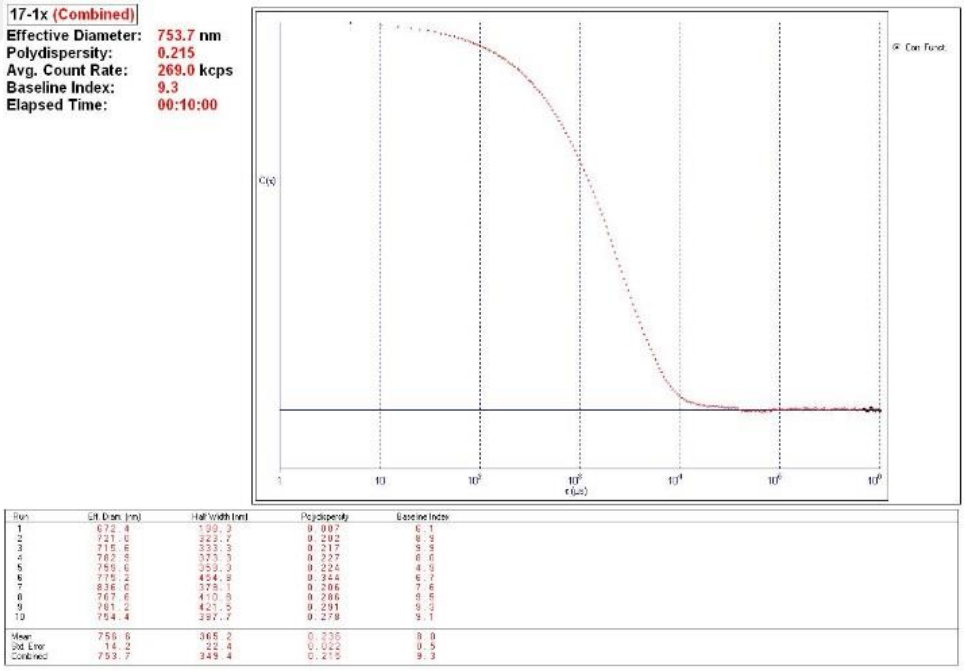


Figure A.12. Transethosomes via cold method with oleic acid and geraniol.

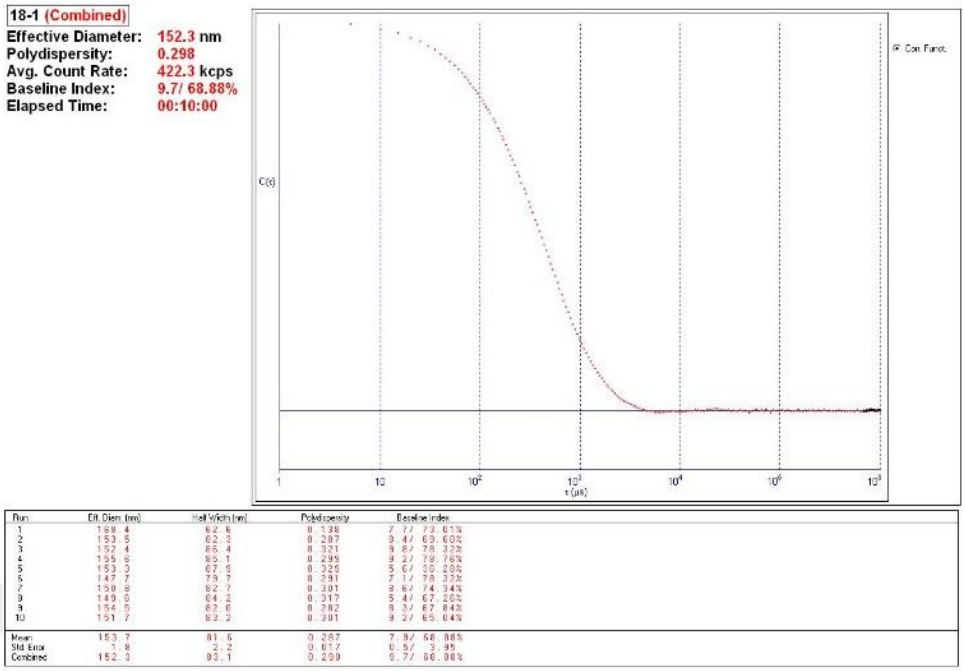


Figure A.13. Transethosomes via thin-film hydration method with oleic acid and linalool.

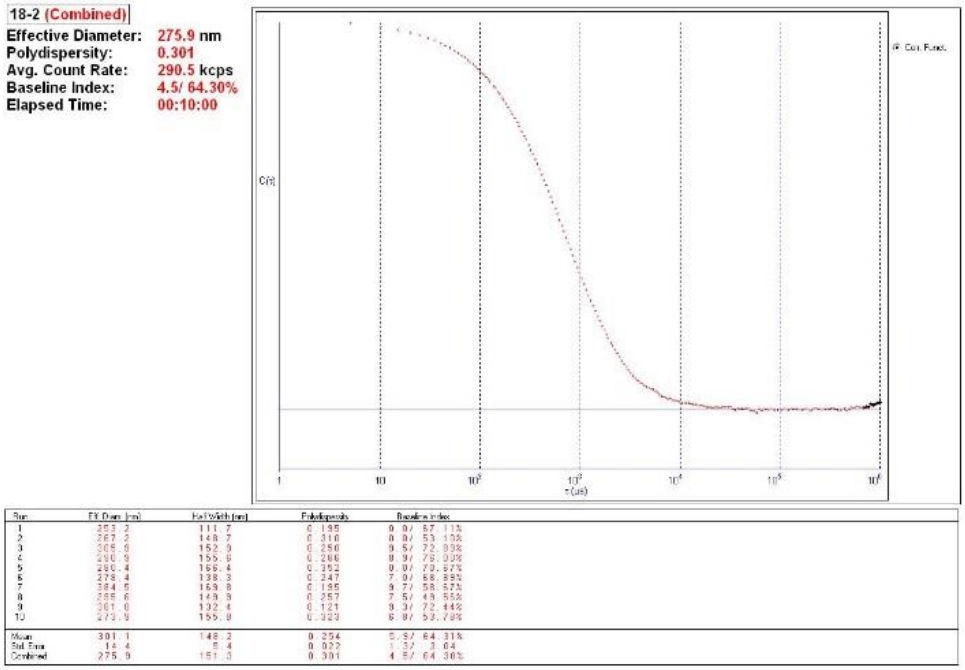


Figure A.14. Transethosomes via thin-film hydration method with oleic acid and geraniol.

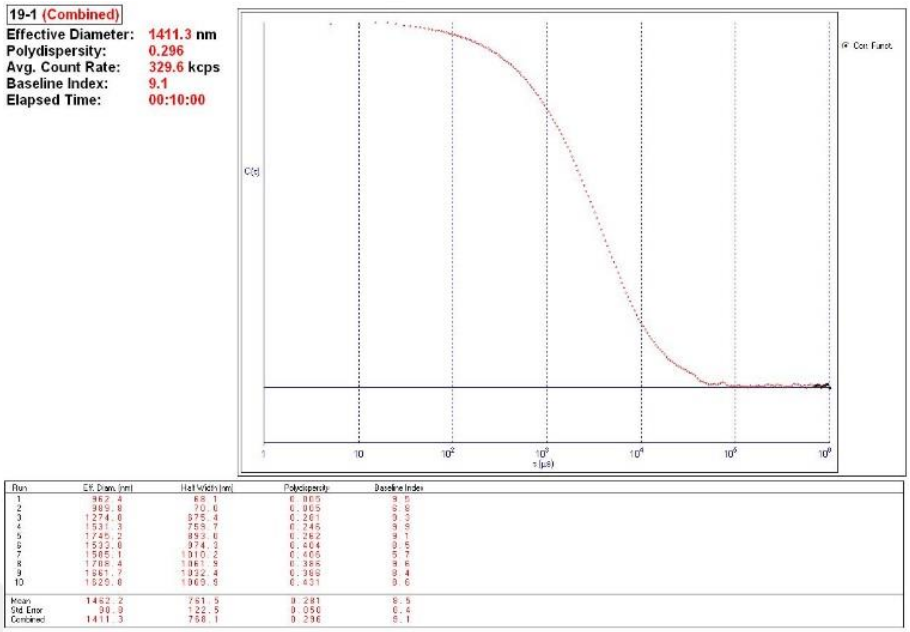


Figure A.15. Transethosomes via thin-film hydration method with Tween®80 and linalool.

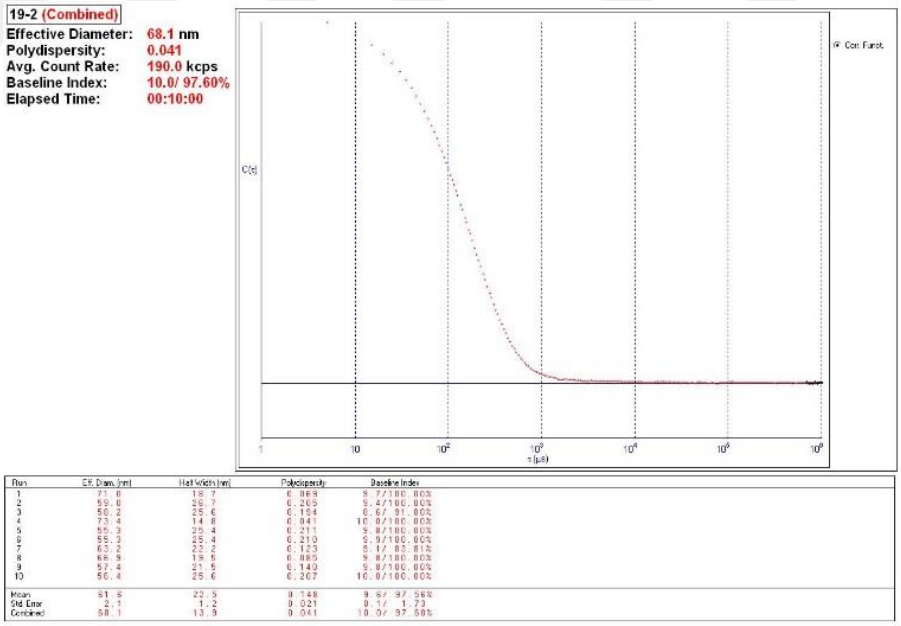


Figure A.16. Transethosomes via thin-film hydration method with Tween®80 and geraniol.