T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY

PREPARATION AND EVALUATION OF TRANSFEROSOMES LOADED WITH MULTIPLE ANTIWRINKLE AGENTS

MASTER THESIS

MELTEM MACİT, BSc. Chem. Eng.

ISTANBUL-2015

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Assist. Prof. Dr. M. Abdur ROUF

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Assct. Prof.Dr. Erdal CEVHER
Istanbul University
Department of Pharmaceutical Technology

Ast.Prof.Dr. Gülengül DUMAN
Yeditepe University
Department of Pharmaceutical Technology

Ast.Prof.Dr.M. Abdur ROUF (Advisor and Chair of the Jury)
Yeditepe University
Department of Pharmaceutical Technology

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated..24.08.2015... and numbered ..2015/22-4.

Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgement has been made in the text.

15.07.2015

Meltem MACİT

DEDICATION

To my precious family…

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LIST OF SYMBOLS AND ABBREVIATIONS

σ	Standard deviation
%	Percent
&	And
˚C	degree Celsius
µ	Average concentration
λmax	Wave length of maximum absorption
µg	Microgram
C_a	Actual concentration
Ca	Calcium
C_f	Found concentration
cm^{-1}	per centimeter
cP	Centipoise
CV	Coefficient of variation
d-nm	Diameter in nanometer
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GL21	Carbopol Ultrez 21 gel incorporating liposomes
GL394	Carbopol 934 gel incorporating liposomes
GT21	Carbopol Ultrez 21 gel incorporating transferosomes
GT934	Carbopol 934 gel incorporating transferosomes
h	Hour
L	Liter
LCE	Liposomes formulation code
mbar	Millibar
mg	Milligram
min	Minutes
mL	Milliliter
mM	Millimole
MMP	Matrix metalloproteinases
mV	Millivolt
N	Number of replicates
Na-EDTA	Sodium ethylenediaminetetraacetic acid
nm	Nanometer
PBS	Phosphate buffered saline
PDI	Polydispersity index
Phospholipon 90 H	Hydrogenated phospholipid
R^2	Correlation coefficient
rpm	Revolutions per minute
SD	Standard deviation
TEA	Triethanolamine
TSE	Transferosomes formulation code
UV	Ultraviolet

UV-VIS	Ultraviolet-Visible
w/w	weight by weight
x_i	Individual concentration
Zn	Zinc

ABSTRACT

Macit, M. (2015). Preparation and Evaluation of Transferosomes Loaded with Multiple Antiwrinkle Agents. Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Technology, MSc Thesis, Istanbul. The degeneration of extracellular matrix and higher pH of wrinkled skin logically require matrix metalloproteinase inhibitor, eg, ethylenediaminetetraacetic acid (EDTA) and formulation pH of around 5.5. Alpha-hydroxy acid, eg, glycolic acid improves photodamaged skin. Vitamin E (α-tocopherol) is an antioxidant protectant of skin membrane. For carrying these multiple antiwrinkle agents deeper into the skin, deformable lipid vesicle, transferosome, seems to be a good choice as, at the same time, they have the capability to load both water- and lipid-soluble agents and to penetrate through tiny skin pore. Hydrogenated phosphatidylcholine and Tween 80 were used to prepare transferosome by thin-film method. Liposome was prepared in the same way except Tween 80 was replaced by cholesterol. Vitamin E (α-tocopherol), glycolic acid and EDTA were loaded into both transferosome and liposome during their preparation. Phosphate buffered saline (PBS, pH 5.5) was used during hydration of lipid films. The average sizes of transferosome and liposomes were 50 and 177 nm, respectively. Their polydispersity indexes, 0.244 and 0.374, respectively, showed that they were homogenous in sizes in each class. As was expected their zeta-potentials were negligible, namely, 0.9 to 1.0 mV. As measured by a validated UV-VIS spectrophotometric method, the vitamin E loading efficiency of transferosome, 96.66 %, was better than that of liposome, 86.71 % with a vitamin E to lipid ratio of 3.41 and 3.05 mM per 100 mM lipid, respectively. Finally, the lipid vesicles were incorporated separately into Carbopol Ultrez 21 and Carbopol 934 gels with a final concentration of vitamin E, glycolic acid and EDTA in gels being 0.005, 1 and 0.1 % (w/w), respectively, and a pH of 5.0 – 6.2. Rheograms of all the four gel formulations showed a plastic behavior with a yield value. The plastic viscosities of these gels were 1.9925 & 1.9692 cP for transferosome and liposome in Carbopol Ultrez 21 and 0.5368 & 0.5851 cP in Carbopol 934. There was no effect of vesicle types on gel viscosity. To conclude that transferosome loading several antiwrinkle agents is a suitable candidate for evaluation for *in vivo* efficacy.

Key words: antiwrinkle, transferosome, vitamin E, EDTA, gel

ÖZET

Macit, M. (2015). Birden Çok Kırışıklık Önleyici Madde Taşıyan Transferozomların Hazırlanışı ve Değerlendirilmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Teknoloji ABD, Yüksek Lisans Tezi, İstanbul.

Kırışık derinin hücre-dışı matriks dejenerasyonu ve yüksek pH'ı mantıksal olarak matriks metalloproteinaz inhibitörü, örneğin, etilendiamintetraasetik asit (EDTA) ve yaklaşık 5.5 civarında bir formülasyon pH'ı gerektirir. Glikolik asit gibi alfa-hidroksi asitler, güneş ışınlarının hasar verdiği cildi iyileştirir. E vitamini (α-tokoferol) deri membranının bir antioksidan koruyucusudur. Derinin altına bu kırışık önleyen maddeleri taşımak için deforme olabilen lipit veziküller, transferozomlar, iyi bir seçim gibi görünmektedir. Çünkü, hem suda hem lipidte çözünür maddeleri taşıma ve derinin gözeneklerinden geçebilme özelliğine sahiptir. İnce film tabakası yöntemi ile transferozomların hazırlanışında hidrojene fosfatidilkolin ve Tween 80 kullanılmıştır. Lipozomlar hazırlanırken Tween 80 yerine kolesterol kullanılmıştır. Bunların hazırlanması sırasında E vitamin, glikolik asit ve EDTA, hem transferozom hem de lipozom içine yüklenmiştir. Lipit filmlerin hidrasyonu esnasında fosfat tamponlu tuzlu su (PBS, pH 5.5) kullanılmıştır. Transferozom ve lipozomların ortalama boyutları sırasıyla 50 ve 177 nm olarak ölçülmüştür. Bunların polidispersite endeksleri, sırası ile 0.244 ve 0.374 olarak hesaplanmıştır. Bu değerler, veziküllerin homojen olduğunu göstermektedir. Beklendiği gibi bunların zeta-potansiyelleri, 0.9 ile 1.0 mV arasında olup ihmal edilebilir düzeydedir. Validasyonu yapılmış UV-VIS spektrofotometri ölçüm sonuçlarına göre, transferozomlara E vitamini yükleme veriminin (% 96.66) lipozomlara göre (% 86.71) daha iyi olduğu görülmüştür; lipid – vitamin oranına bakıldığında 100 mM lipid başına sırasıyla 3.41 ve 3.05 mM E vitamini yüklenmiştir. Son olarak, lipid vezikülleri Carbopol Ultrez 21 ve Carbopol 934 polimerleri kullanılarak hazırlanan jellere eklenerek E vitamini, glikolik asit ve EDTA'nin son konsantrasyonları sırası ile % 0.005, % 1.0 ve % 0.1 (a/a) olarak ve jellerin pH'ları ise 5.0 ile 6.2 arasında belirlenmiştir. Hazırlanmış dört jel formülasyonlarının her birine ait reogramlar verim değerine sahip bir plastik davranış göstermiştir. Polimeri Carbopol Ultrez 21 olan ve sırasıyla transferozom ve lipozom eklenen jellerin plastik viskozitesi 1.9925 ve 1.9692 cP, polimeri Carbopol 934 olan jellerin viskozitesi ise 0.5368 ve 0.5851 cP olarak hesaplanmıştır. Jellerin viskozitesine vezikül tiplerinin hiçbir etkisi bulunmamıştır. Sonuç olarak, kırışıklık önleyen birden çok madde taşıyan bu transferozomlar *in vivo* etkinliğinin değerlendirilmesi için uygun adaylardır.

Anahtar Kelimeler: kırışıklık önleme, transferozom, vitamin E, EDTA, jel

1. INTRODUCTION AND OBJECTIVE

Wrinkled skin shows higher stratum corneum pH (1) which inhibit enzymatic lipid processing. Another important feature is degeneration of extracellular matrix primarily due to the activity of Zn & Ca dependent matrix metalloproteinases, MMP, at neutral pH (1). EDTA is a strong metal ion chelating broad spectrum MMP inhibitor (2). Improvement can be obtained in multiple signs of photodamaged skin by the sustained topical application of alpha-hydroxy acids, eg, glycolic acid (3). Vitamin E is essential for the stabilization of biological membranes, and the major free-radical chain-breaking antioxidant in the membrane (4).

These antiwrinkle agents are not very effective when applied as conventional cosmetic formulation. This is mainly because of their inability to penetrate through the skin barrier of stratum corneum. There are several approaches to increase penetration through skin like chemical penetration enhancer, microneedles, ultrasonic or electrical penetration enhancement. With disadvantages of each of these methods, they are not suitable for regular usage. However, transferosome is an interesting delivery systems with almost problem-free characteristics.

Transferosomes are deformable lipid vesicles. Their lipid bilayer and water core allow loading of both lipid soluble materials like vitamin E and water soluble materials like glycolic acid and EDTA, respectively (5). Because of their structural flexibility, they can squeeze through much smaller pores than their own size. It was reported that they can penetrate through 10 times smaller pore size (6,7) whereas skin pore size is reported to be in the range of 20–40 nm (8). As such, a suitable limit of transferosome size should be less than 300 nm.

The aqueous vesicular suspension do not have enough viscosity. So they are not suitable for topical application. Among the cosmetic forms, polymeric hydrogel is the most suitable form for incorporation of lipid vesicles. Because their formulations are simple and does not contain any ingredient which may destabilize lipid vesicle like transferosome and liposome.

Therefore, the aim of this study was to prepare and evaluate transferosomes loaded with above multiple antiwrinkle agents and incorporate them into gel formulation for easy topical application. The desired pH of the formulations was around 5.5 which is the value for healthy skin. For comparison with transferosome, a liposome formulation were also intended as transferosomes are basically a type of liposome where cholesterol component is replaced by surfactant to provide elasticity.

2. GENERAL INFORMATION

2.1. Skin Layers, Functions and Penetration through the Skin

The skin is the biggest organ and it covers all of the human body. It contains three layers, namely, sorted from outside to inside, epidermis, dermis and hypodermis (9). Its most important function is to form a physical barrier to the environment, to balance the water, electrolytes content in the body and to protect against micro-organisms, ultraviolet radiation, toxicants and impacts. Compounds with appropriate properties can penetrate through the skin and reach systemic circulation.

2.1.1. Skin Layers

Epidermis

It is the outer layer of the skin. It can be divided into 5 layers (Figure 2.1). They are, sorted from outside to inside, stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spinosum and stratum basale (10). They include some special cells which detect the impacts coming from the environment such as hits, cold-hot, soft-hard.

In the outer most layer of the epidermis, the keratinocytes maturation process is finished. The cells of the layer is called corneocytes. They are enveloped by protein and it provides strength the SC. The structure provides the natural physical and water-retaining barrier to the skin. Stratum lucidum is a clear and trans lure layer. Stratum granulosum and spinosum is called malphigian layer. Some special cells are located in these layers such as langerhans, dermosomes lamellar granules. The stratum basale layer lie above the dermis and contains melanocytes and tactile cells which is connected to the sensory nerve ending (9).

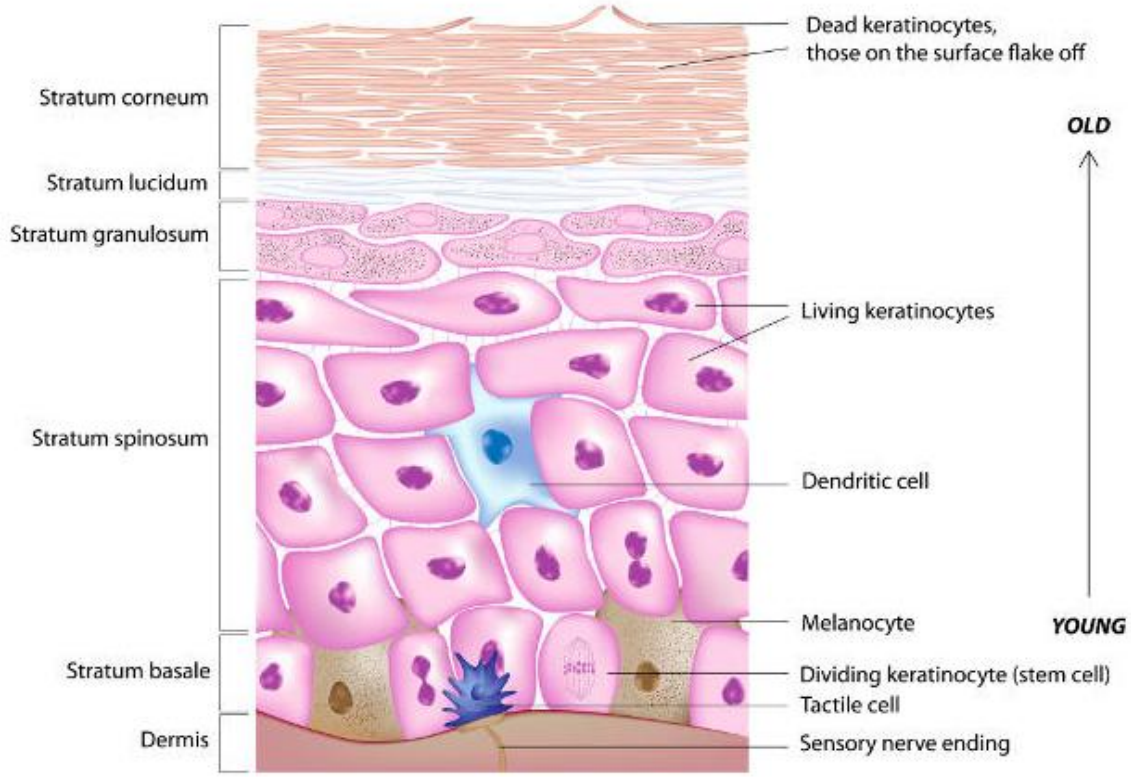

Figure 2.1. Structure of the epidermis (11)

Dermis

Dermis is the second major layer of the skin, just below basement membrane (Figure 2.2). It provides thermoregulation and vascular connection to move nutrients to epidermis. It includes hair root, sebaceous gland and fibroblasts and provides elasticity to the skin with the help of the elastin (12).

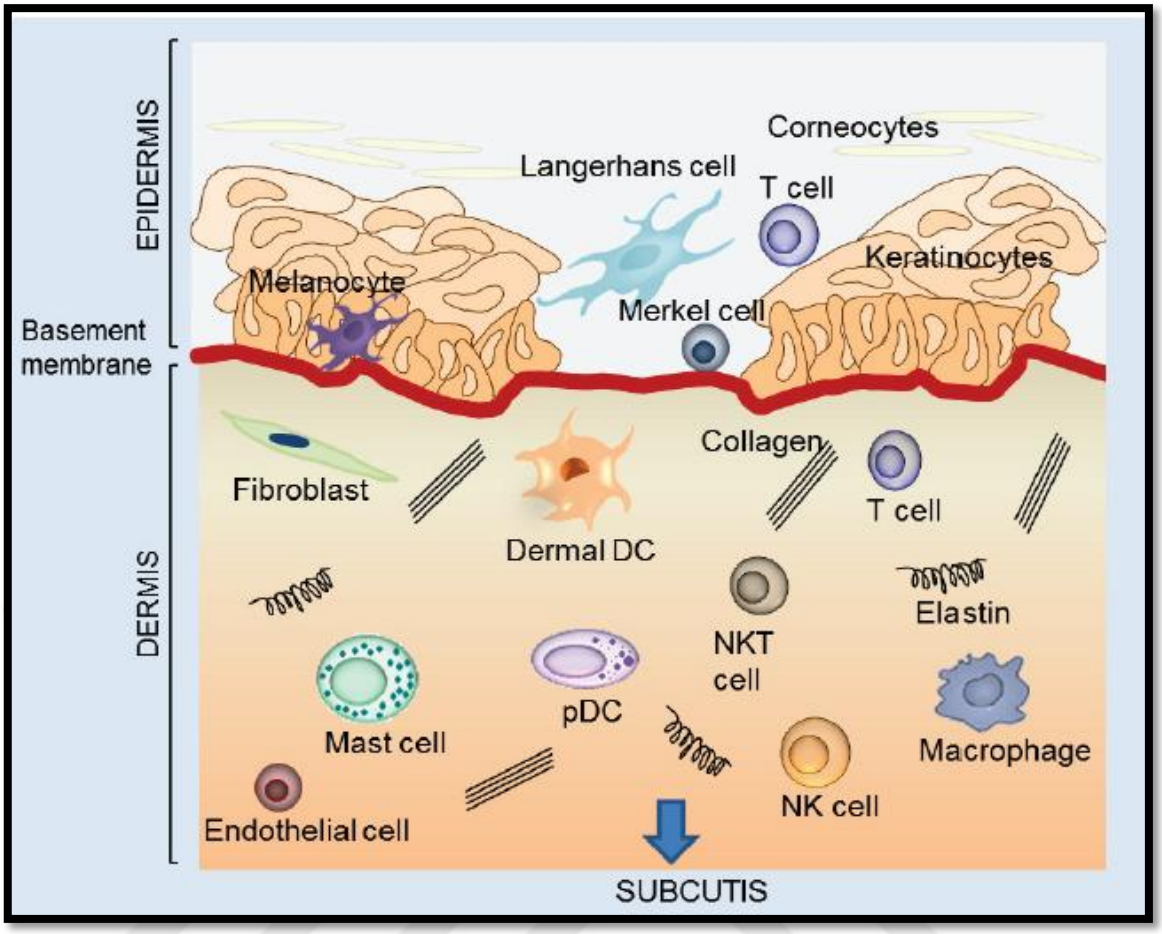

Figure 2.2. Epidermis, basement membrane and dermis (10)

It divides into two zones. The first zone of the dermis is the papillary layer which includes the vascular connective tissue and elastin fibers. The second reticular layer is composed of irregular tissue (13).

Hypodermis

This is the third and the last major layer of the skin. It includes adipose tissues which insulate skin preventing heat loss and. It has large blood vessels which are suppliers of the blood to dermis (10).

2.1.2. Skin Functions

Skin has four main functions: protection, thermal regulation, sensation and endocrine function.

Protection

The dead cells of SC protects from mechanical impacts to a certain level, fluids-loss, radiation and toxicants and microorganism (14). The skin behaves as a barrier covering the body with oily coat. Radiation is very harmful for the internal tissues. However, UV radiation is a cause of skin aging.

Thermal Regulation

The second important function of the skin is regulation of the body temperature. Fatty tissues prevent the heat loss in the cold condition. Vasoconstriction, vasodilation and sweat glands secretions are other mechanism to regulate the body temperature (15).

Sensation

The skin fells superficial touch, pain, temperature, deep pressure and vibration. The touch and pain feelings are transmitted to the brain by nerves and the brain percepts these feelings and shows reaction to the senses. Temperature, pressure and the vibration are percepted by same processes (16).

Endocrine Function

The skin possesses the capacity to generate several hormones and substances with hormone-like activity. These substances appear to act through paracrine, autocrine, intracrine and endocrine mechanisms. Human skin produces hormones which are released in the circulation and are important for functions of the entire human organism. Major examples are sex steroids, whereas a large proportion of androgens and estrogens in men and women are synthesized locally in peripheral target tissues from the inactive adrenal precursors DHEA and androstenedione. The skin is the unique site of cholecalciferol synthesis (15).

2.1.3. Penetration through the Skin

The term dermal absorption means the journey of some compounds into through the skin into the systemic circulation. Some reference (17) describes this process as being consists of three steps: penetration, permeation and resorption. The first step describes the access of a compounds into a skin layer. The second step is continuous

penetration among the layers from up to deep. The last step is identified as the attendance of the compound in the systemic circulation.

The structure of the skin is an important factor for controlling the penetration rate. SC provide protection against the penetration of the most compounds. A highly hydrophilic substance is not able to enter the hydrophobic SC layer. Therefore, it cannot penetrate the skin easily. Hydrophobic compounds can penetrate easily but because aqueous environment of blood, they remain inside the skin and are stored there (18) Therefore, only those compounds which have appropriate hydrophilic-lipophilic balance can penetrate through the skin easily. There are three dermal absorption routes: intercellular, intracellular and follicular pathways. The intercellular pathway is through the intercellular lipids. Intracellular pathway is through the cells. The follicular pathway is not used very much since the fraction of skin area they occupy is very small (18).

2.2. Aging and the Skin

2.2.1 Aging Theories

Aging is a complex biological and physiological process affected by a combination of intrinsic and extrinsic factors. Several molecular mechanisms play a role in aging and several theories were proposed to explain the mechanism of aging (19).

Programming Aging Theory

According to this theory, genes determine of the duration life. So, the genes determine when aging starts and how it continue (19).

Depreciation Theory

Cells, tissues and the vital parts of body are deformed with time. They work and depreciate as the time goes on. This theory was proposed by German biologist Dr. August Weismann in 1882 but it is considered still acceptable todays (19).

Velocity of Life Theory

Proposed in 1908 by Dr. Rubner, this theory supported that the if organism's basal metabolism is fast, the duration of life is short. It implies that consumption of excessive oxygen and production of lot of reactive oxygen species are the causes of aging (19).

Free Radical Theor

According to this theory, superoxide and other free radicals destroy the structure of cell's macromolecules (Figure). It was accumulated in the cells and increased the amount of damage and to arrive the level of the organ. Therefore, firstly cells after organs lost their functions. These macromolecules, nucleic acids, lipids, carbohydrates and proteins, were exposed to free radical attack (20).

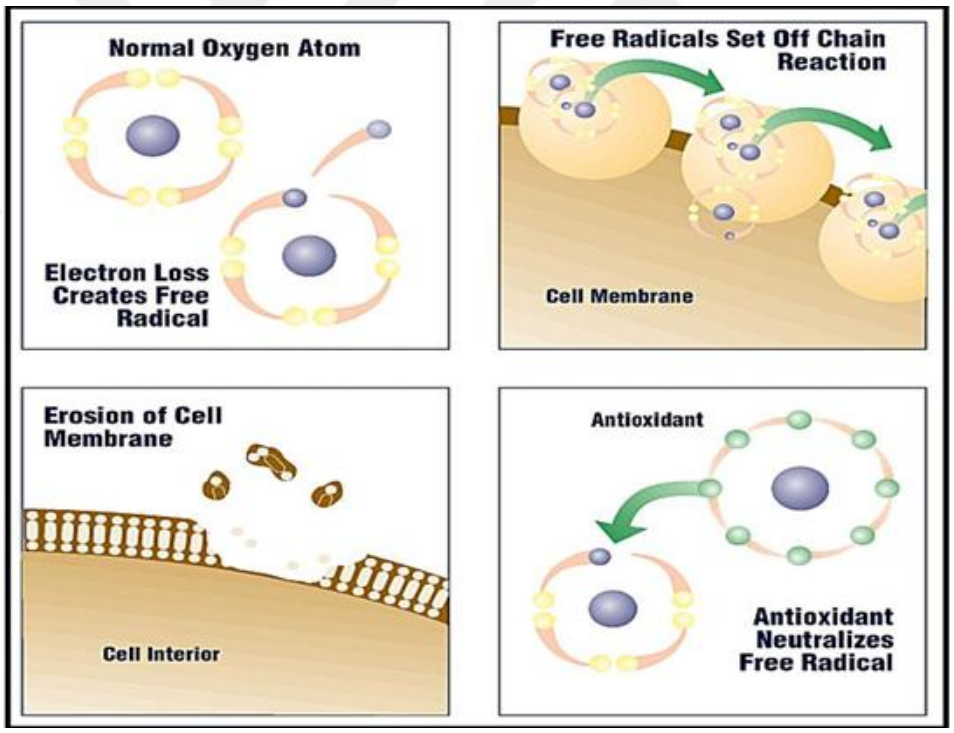

Figure 2.3. Free radical destruction of cell membranes (21)

Lysosomal Membrane Theory

Richard Hochschild proposed this theory in 1971. According to this theory, the free radicals and lipid peroxidation destabilize the lysosome membrane, and as result lysosomal activity increases (19).

Membrane Theory

Professor Imre Zs-Nagy proposed this theory. According to this theory, changes of permeability of cell membrane and increase in their rigidity are cause of aging (22).

Mitochondrial Membrane Depreciation Hypothesis

Professor Jaime Miquel proposed this theory. According to this theory, lipid peroxidation and free radicals depreciate mitochondrial membrane, the mitochondria loose their functions and could not synthesize enough ATP to fulfill the needs of the cell. As a result, decrease in physiological performance, protein synthesis and resistance to the environment stress factors are the causes of the aging (23).

Telomere Theory

As the cells divide, telomeres, the end part of each DNA, shorten. It is suggested that, human cells have the capacity to divide 50 times. After that cells loose their capacity to divide. As a result, body cannot repair normal wear and tear (24-25).

2.2.2. Skin Aging

In the skin aging processes, some changes of skin functions occurs. They include decrease in the recovery capacity, barrier function, cleaning capacity, resistance to mechanical impact, performance of immune system, thermoregulation ability, production of sweat and sebum, synthesis of vitamin D. As a result, wrinkles, loss of flexibility, spots and some skin disease (26-27) and a variety of other symptoms (Figure 2.4) appears (28). Aged skin become inelastic, and can recover itself more slowly after injury.

Figure 2.4. Changes in aging skin (28)

As we age, skin collagen production slows, and elastin, the substance that enables skin to snap back into place, becomes less spring. Dead skin cells do not shed as quickly and turnover of new skin cells decrease. These changes are reported schematically in Figure 2.5 (29).

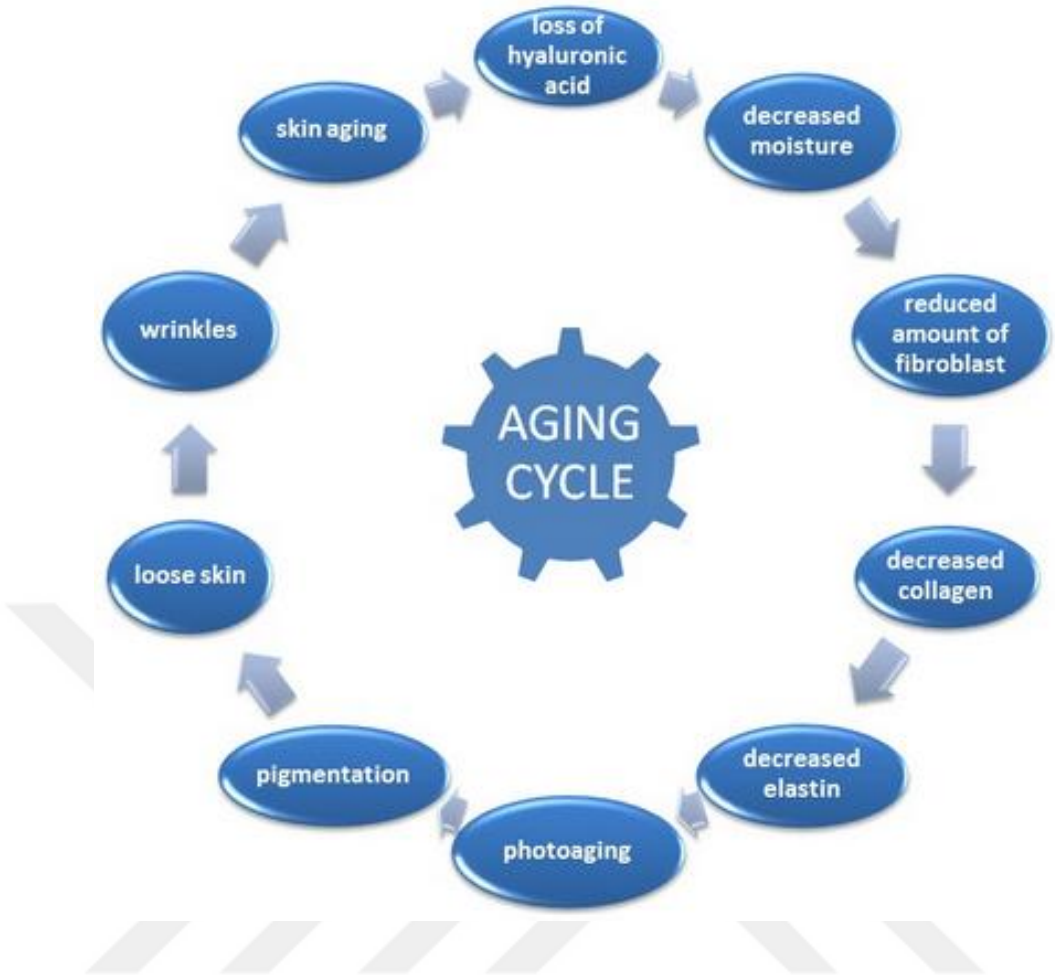

Figure 2.5. Skin aging cycle (29)

The sign of the youthful skin is the amount of collagen and its synthesis. Reduced synthesis of collagen is a prominent feature of skin aging (30). Collagen, produced by fibroblasts, plays an essential role on skin structure and accounts for as much as 70% of the weight of the skin. In aged skin, collagen is not only decreased, collagen fibers begin the cross-link, resulting in losing skin elasticity, due to the external factors like exposure the UVA radiation. Inflammatory reactions are also seen during the production of free oxygen radicals. In addition, wrinkled skin shows a higher stratum corneum pH impeding enzymatic lipid processing, degeneration of extracellular matrix primarily due to the activity of Zn & Ca dependent matrix metalloproteinases, MMP, at neutral pH (1).

Intrinsic and Extrinsic Skin Aging

Cutaneous aging included two distinct occurrence: intrinsic aging and extrinsic aging. Intrinsic aging is a universal inevitable change attributable to the passage of time alone, and it appears to be related to the genetic factors.

Extrinsic aging is the superposition on intrinsic aging of changes attributable to chronic environmental insults, sun exposure. It is neither universal nor inevitable. Extrinsic skin aging is also termed photoaging, reflecting the large and well-studied role of chronic sun exposure (1). Skin is not only the largest organ of the body, it is the only organ that is chronically exposed to the environment and on display. The rate of the extrinsic aging depends on the level of exposure to environmental factors (Figure 2.5). The damages in the skin caused by solar UV radiation. As one ages, sun-exposed areas of skin, generally face, neck, upper chest, forearms and hands are wrinkled. Because, these areas are the least protected from the sun. The effect is more pronounced in lightly pigmented persons.

The extrinsic skin aging's commonly defined manifestations are coarse wrinkles, solar elastosis and pigment irregularities (26).

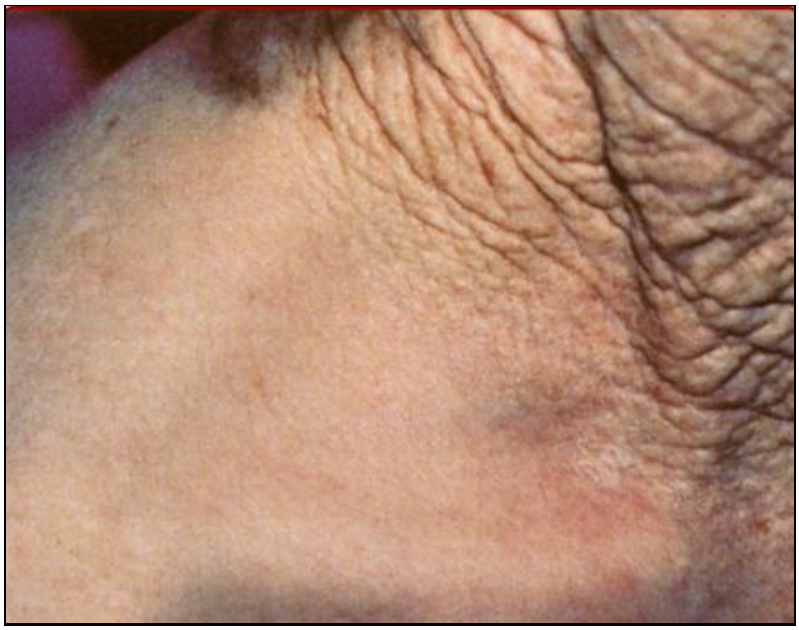

Figure 2.5. Chronologically aged the same aged but ravaging photoaging above collar line (1**)**

Strategies for Reducing Photodamage to Skin

Some strategies to reduce the photodamage to the skin are as follows (31).

- Protection from the UV penetration with physical and chemical sun protector.
- Overcoming reactive oxygen radicals by use of the antioxidants.
- Regular moisturization
- Application of product of skin repair like occlusive oils, skin lipids, plants and animal extracts, biological factors, alpha hydroxy acids, retinoids and some vitamins.
- Decreasing the inflammation with anti-inflammatory composites like cyclooxygenase inhibitors.

2.3 Antiwrinkle Agents

All scientific evidence points to UV radiation as the primary cause of skin wrinkles. Adequate sun avoidance and sunscreen use are partially prophylactic in the prevention of wrinkle formation (32). Antiwrinkle agents can be categorized in the following groups.

2.3.1 Moisturizers

Definite effects on skin appearance, and potentially on structure and function, can be achieved with moisturizers. Improvement in stratum-corneum structure and hydration and decrease in transepidermal water loss can be quickly achieved and may result in improvement in the appearance of wrinkles (32).

2.3.2 Alpha-Hydroxy Acids

There is substantial evidence that meaningful improvement can be obtained in multiple signs and symptoms of photodamaged skin by the sustained topical application of alpha-hydroxy acids, eg, glycolic acid. Specifically, wrinkle effacement has been

shown in multiple, well-designed and executed clinical trails using clinical and instrumental endpoints (3).

2.3.3 Retinoids

Evidence of wrinkle effacement by topical application of retinoids has been extensively shown in numerous, large, published clinical trials (33-35). Tretinoin (all transretinoic acid) has been the most studied but results with topical isotretinoin (13 cisretinoic acid) appear comparable. Retinol, the parent compound, may require metabolism to the purported active transretinoic acid for pharmacological effect and is increasingly incorporated in cosmetic products claiming benefit in wrinkle appearance. Similarly, retinaldehyde has been shown to be active in wrinkle effacement.

2.3.4 Vitamins

Many vitamins, including vitamins A, C, D, and E, are vital in normal metabolic processes, and clinical skin changes resulting from their deficiencies were identified. Retinoids (vitamin A class), as mentioned above, at pharmaceutical concentrations are the most thoroughly substantiated class in their general effect in photoaging and specific effect on wrinkles.

Vitamin E is an widely studied antioxidant. A four-week study of 5% RRR alpha-tocopherol naturally occurring oil-in-water (o/w) cream applied to the crows feet area showed, by optical profilometry, decreased skin roughness, length of facial lines, and depth of wrinkles compared with placebo (36). Vitamin E is also used in sun care products in order to improve the functions of sun filters in the sun. Among the different types of tocopherol, the alpha-tocopherol has the highest biological potency (37).

An increasing number of vitamin C-containing topical products are being marketed with claims of improvement in skin wrinkling. Vitamin D analogues have been highly successful in treatment of psoriasis and because of their modulating effect on keratinization, they have potential in preventing photoaging (32).

2.3.5 Hormones

Estrogens and their decrease at menopause have profound effects, especially on epithelium of the skin and vagina. Wrinkle effacement has been convincingly shown in at least one controlled clinical trail of topical application of 0.01% estradiol or 0.3% estriol-containing preparations (38). Other studies have shown beneficial changes in skin thickness and texture with topical estrogen application (39).

2.3.6 Etyhlenediaminetetraacetic Acid, EDTA

Ultraviolet radiation induces several members of the matrix metalloproteinase family that degrade collagen fibrils and other components of the dermis. The matrix metalloproteinases are a multigene family of tightly regulated calcium and zinc-dependent enzymes. Elevated matrix metalloproteinases activity in photodamaged skin resulted in breakdown of the collagen (40). EDTA is a strong metal ion chelating broad spectrum MMP inhibitor which is reported to modulate MMP activity (2).

2.3.7 Miscellaneous Agents

Hyaluronic acid is a normal component of epidermis and especially dermis. Stimulation of hyaluronic-acid production in skin by a device that produces a specific pulsed electromagnetic field (electrorydesis) produced improvement in appearance of wrinkles in a small study (41). Natural cartilage polysaccharides as oral formulations derived from cartilage of marine fish have purported to improve dermal thickness and elasticity (42). Resveratrol (3,4',5-trihydroxystilbene) is polyphenolic compounds found in grapes, wine, grape juice and peanuts. Resveratrol induces antioxidant enzymes, with evidence of antioxidant effects (43).

2.4. Novel Cosmetic Delivery Systems

Delivery systems which are used for cosmetic active substance are classified into four major groups (44). They are shown in Table 2.1. One objective in the design of novel drug delivery systems is controlled delivery of the active to its site of action at an appropriate rate. Encapsulation techniques are used in pharmaceuticals, cosmetics, veterinary application, food, copying systems, laundry products, agricultural uses,

pigments, and other lesser well-known uses to control the delivery of encapsulated agents as well as to protect those agents from degradation.

Table 2.1. Novel Cosmetic Carrier System (44)

Types of Delivery Systems	**Examples**
I. Vesicular Delivery systems	Liposomes
	Niosomes
	Transferosomes
	Silicone vesicles and Matrices
	Microemulsions
	Nanoemulsions
	Multiple emulsions
II. Particulate Systems	Microparticulates
	Porous polymeric systems
	Nanoparticulates
III. Other Delivery systems	Cyclodextrin complexes
	Carbosomes
	Dendrimers and hyperbranched polymers
	Nano Crystals
	Liquid crystals
IV. Delivery Devices	Iontophoresis
	Cosmetic patches

2.4.1 Vesicular Carrier System

Majority of the delivery systems can be categorized as vesicular systems. Some active substances carried by these delivery systems, their applications and available marketed preparations are given in Table 2.2. (45). Various vesicular systems are described in the following subsections. Transferosome, being the chosen delivery system for this thesis work, is described separately in section 2.5.

Table 2.2. Several active ingredients carried by vesicular delivery systems (45)

S.N.	Novel Trends Cosmetic	Active ingredients	Available Market preparation	Application
1.	Liposome	Ascorbyl palmitate, Tocopherol, retinol. Coenzyme Q10, Niacinamide	Rovisome ACE Plus Ageless Facelift cream	Anti-ageing, wrinkle reduction Anti-ageing, anti-oxidative, wrinkle reductio
2.	Niosome	methotrexate	methotrexate gel	Treatment of Psoriasis NSAIDS, ↑ anti-inflammatory action
3.	Micro-emulsion	VitaminC (5%)	Ultimate Anti-Ageing Cream	Anti-ageing, wrinkle reduction
4.	Nano-emulsion	CoenzymeQ10, Vitamin E acetate. Vitamins A,E,C	Nano-Lipobelle H-EQ10 cream Nano-Lipobelle H-AECL	Anti-ageing, anti – inflammatory. Anti-wrinkle, antiageing.

Liposome

Liposomes are phospholipid vesicles. Phospholipids in contact with water forms bilayer where their hydrophobic parts are shielded from water, and hydrophilic head region remain in contact with water. For further thermodynamic stability, the bilayers take a spherical shape, avoiding edge contact with water, encircling an aqueous core (Figure 2.6) (46). The bilayer structure is very similar to the cell membranes. The lipid bilayer is not stable, so, usually cholesterol, as is present in cell membranes, is added to impart rigidity and stability to the liposome structure.

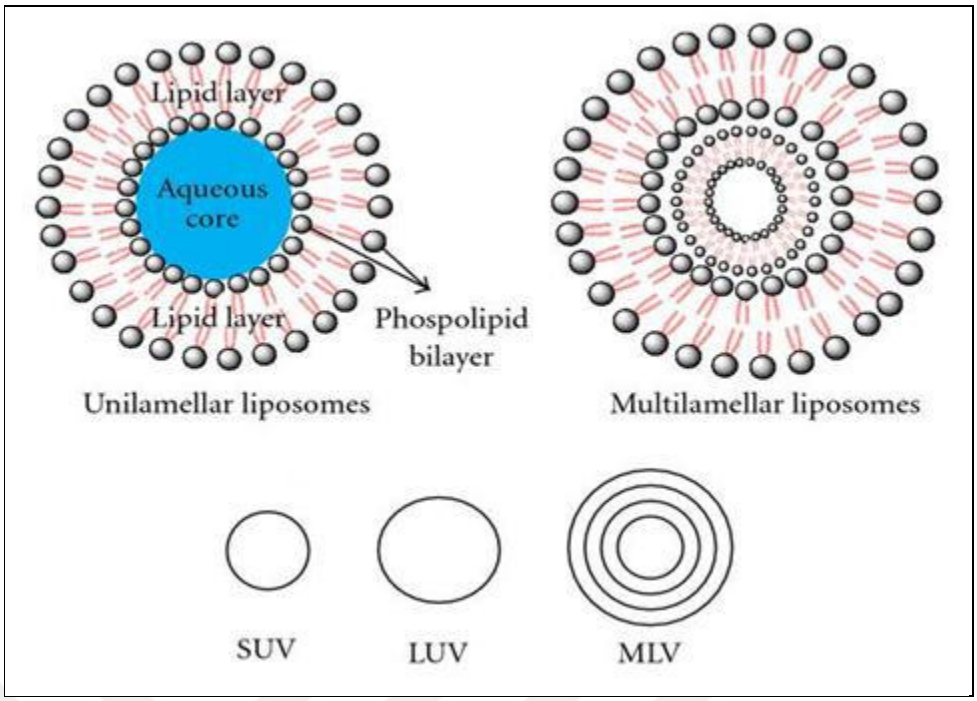

Figure 2.6. Liposome structure and their types (46).

Depending on the condition of preparation, liposomes can be unilamellar, oligolamellar or multilamellar vesicles (Figure 2.6). Unilamellar liposome vary in sizes from 20 nm to 1000 nm. Oligolamellar type consists 2-10 lipids bilayers surrounding a large internal volume. Multilamellar vesicles have more than 10 lipid bilayers and are the largest in size.

Preparation Methods for small unilamellar vesicles (SUV) include sonication method (47), ethanol injection method (48), detergent dialysis method (49), and reverse micelle method (50). Preparation methods for multilamellar vesicles (MLV) and large unilamellar vesicles include ether ınfusion method (51), calcium induced fusion method (52), reverse phase evaporation method (53).

Liposomes are is the most studied delivery system for cosmetic actives because of their many advantages. They can be made in a wide range of sizes from 15 nm up to several μm. The main constituents of liposomes, phospholipid and cholesterol, are biocompatible. Their preparation methods are simple and easy. They can be used to upload lipid soluble ingredients within the lipid bilayer structure and water soluble ingredients in the inner aqueous core.

Niosomes

Niosomes are non-ionic surfactant vesicles which have a structure similar to liposomes but non-ionic surfactant, instead of phospholipid, forms the bilayer structure. They are more stable but have more skin irritation risk than liposomes (54).

Silicone Vesicles and Matrices

Silicone elastomer blends comprise lightly cross-linked siloxane gels swollen in diluents such as cyclopentasiloxane or low viscosity dimethicone. Recently, silicone manufacturers have studied the potential for using this blends for delivery of actives such as fragrances, vitamins and sunscreens. Being matrix type, they are not true vesicle type delivery system. However, in the mid 1990s, fundamental research highlighted the ability of a new class of proprietary silicone polyethers (dimethicone copolyol) to form vesicles. These silicone-based vesicles are structurally comparable to liposomes (55-57).

Multiple Emulsion

While the normal emulsion systems are either water in oil (W/O) or oil in water (O/W), most commonly used multiple emulsion systems are either water in oil in water (W/O/W) or oil in water in oil (O/W/O). In multiple emulsion system, solute has to transfer from inner to outer miscible phase through the middle immiscible phase. So they can act as delivery system (58-59).

Microemulsion

The microemulsion is a dispersion system. Oil, water, surfactant and co-surfactant form a dynamic two phase system. They are thermodynamically stable and have advantage of homogeneity, as distinct from conventional emulsions and nanoemulsions. They are used wide variety of cosmetics products (60). They can exist as bicontinuous systems and have important applications, such as the delivery of food, consumer products and pharmaceuticals, as well as in petrochemical recovery (61).

Nanoemulsions

They include very fine oil-in-water dispersion and their droplet sizes are smaller than 100 nm. Nanoemulsion is different from the microemulsion, metastable state and very fragile system. They are formulated with compatible vehicles and additives. They provide enhanced solubility of active substance. They are used in cosmetics, drugs and pharmaceuticals areas (44).

2.4.2. Particulate Systems

Microparticulate System

Microparticles sizes lie between 0.5-1000 μm. They are of two types: microcapsules which has core and shell structure, and microspheres which is spherical matrix type bead (Figure 2.7).

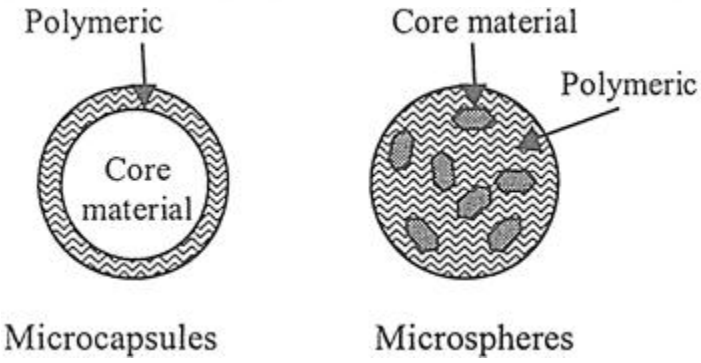

Figure 2.7. Schematic representation of microparticles (62)

Commercial microparticles typically have a diameter between 1 and 1000 mm and contain 10 to 90 wt% core. Most capsule shell materials are organic polymers, but fat and waxes are also used. Various types of physical structures of the product of microencapsulation such as mononuclear spheres, multinuclear spheres, multinuclear irregular particles, and so on can be obtained depending on the manufacturing process (62).

Microsponge system

Microsponge system are spherical beads composed of thousands of small particles wrapped together to form a microscopic sphere capable of binding, suspending, or entrapping a range of substances. The outer surface is porous, allowing the controlled flow. Microsponges can be incorporated into gels, creams, liquids, powders, or other formulations, and can release ingredients depending on their temperature, moisture, friction, volatility of the entrapped ingredient, or time (62).

Nanoparticles

Nanoparticles can be defined as submicron (<l μm) systems, but are not necessarily made of polymers. According to the process used for the preparation of nanoparticles, nanocapsules or nanospheres can be obtained. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymeric membrane; nanospheres are matrix systems in which the drug is dispersed throughout the particles (62). Solid lipid nanoparticle are colloidal carriers consists of triglyceride in solid state, used for carrying poorly water soluble drug and cosmetic actives (63).

2.4.3. Other Delivery System

Liquid Crystal

Liquid crystal is a state of material between the liquid and the crystalline structure. Various intermediate phases may exist between solid and liquid states. At high surfactant concentration, several liquid crystalline phases can be seen to have formed. In surfactant–water systems, with increase in surfactant concentration, the hexagonal, cubic liquid crystalline, and lamellae phase are produced. The hexagonal phase consists of long rod micelles of surfactants hexagonally arranged. The lamellae phase comprises surfactant bilayers separated by water layers. The water layers vary in thickness from 10 A˚ to several 100A˚ (64).

Cyclodextrin Complexes

Cyclodextrins are cyclic oligosaccharides including a minimum of six d-(+)-glucopyranoses attached by a (1, 4) glucoside bonds. There are three types of cyclodextrins: alpha, beta and gamma which contain 6, 7 and 8 glucose units, respectively. As a result, they have different ring sizes and solubilities. All have internal, relatively hydrophobic cavity and hydrophilic outer surface. So they can form complexes with molecules of appropriate size which can fit to the cavity sizes. This complexation increases aqueous solubility of insoluble actives. In addition, the complexes stabilize active ingredient against oxidative, photolytic and thermal degradations. They also prolongs the effect of fragrances (65).

Dendrimers and Hyperbranched Polymers

Dendrimers are unimolecular, monodisperse, star shaped nanostructures. They are about 20 nm in size and have well-defined symmetrical branches and dense functional end groups at the surface. They are used in hair care, skin care, nail care, artificial skin tanning, creams and gels formulations (44).

Nanocrystals

Nanocrystals of active substances have quite different properties from their molecular forms. They are prepared in the size range of 10-400 nm. This is a very recent development in field of delivery systems (44).

2.4.4. Delivery Devices

Iontophoresis

Iontophoresis is painless procedure in which water soluble materials are delivered into the skin using mild electric currents. It has gained a great deal attention in recent years. The device include electrical power source, electrodes and active materials solution in water. Initially only charged molecules were put under an electrode with the

same charge. Electrical repulsion pushes the molecule into the skin. However, later it was found that nonelectrolyte molecules can also be pushed into skin by the phenomenon of electroosmosis (44).

Cosmetic Patches

Transdermal patches have several layers in their construction. The first layer, from outside to inside, is protector. In some patches, the active substance is mixed with adhesive substances or polymeric formulation to release the active by diffusion. In other patches, the active is kept as a reservoir separated from skin by a release controlling membrane. The last layer is the protectant release liner which is removed prior to use. Pressure-sensitive adhesives materials used in the patch ensure intimate contact between patch and skin (66).

2.5. Transferosome

2.5.1 Definition of Transferosome

The term transferosome was introduced in 1991 by Gregor Cevc (67). It was registered as a trade mark by IDEA AG, a German company, as Transfersomes® and they began to use in delivery system. Literally transferosome means ‘carrying’ body and is derived from the Latin word ‘tranferre’ meaning ‘to carry across’ and the Greek word ‘soma for ‘body’ (68). Gregor Cevc (67) showed that, by modifying the chemical composition of bilayers of liposomes so as to decrease its Young’s modulus nearly tenfold below that of conventional liposomes, the resulting deformable liposomes (transferosome) could successfully penetrate the 10–20 μm thick barrier comprised of stacked corneocytes embedded in a non-phospholipid lipid matrix. For changing the rigidity of conventional liposomes, bilayer forming phospholipids plus a given amount of edge activators, eg, surfactants/hydrophilic detergents of high mobility such as sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, Tween 80, and dipotassium glycyrrhizinate were used with the elimination of cholesterol. Thus, they retain the liposomes’ capability to carry both lipophilic and hydrophilic substances (Figure 2.8), with additional property of being deformable,

elastic or flexible. This deformability enables them to transfer/carry actives across SC, and hence the name transferosome.

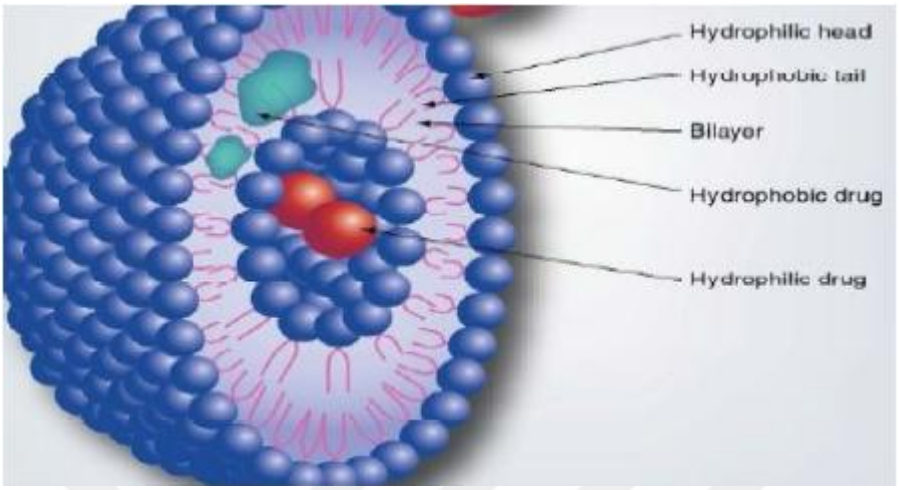

Figure 2.8. Structure of transferosome (69)

2.5.2 Important Features of Transferosome

Important characteristics of transferosomes can be summarized as follows (69).

- Transferosomes are elastic and deformable lipid vesicles, the deformability being provided by some edge activator like surfactants.
- They have an infrastructure capable of loading both hydrophobic and hydrophilic substances together and thus provide formulator with the opportunity to include substances with wide range of solubility in the formulation**.**
- They can deform and get into the narrow skin pores 10 times less than their own diameter without measurable loss. Skin pore size is reported to be in the range of 20–40 nm (8). Considering an average size of 30 nm, a maximum 300 nm size vesicle should be appropriate for transdermal application.
- They are biocompatible and biodegradable as they are made from natural phospholipids similar to liposomes.
- They have a high entrapment efficiency.
- They protect the actives from chemical or metabolic degradation.
- They act as depot and release their contents slowly and gradually.

2.5.3. Penetration Mechanisms of Transferosome

It is understandable that relatively rigid vesicle, like conventional liposome, will find it difficult to penetrate through a pore which is smaller than its own size, but for a deformable vesicle like transferosome it is an easy process (Figure 2.9). Because it can take elongated shape while squeezing through smaller pores and reform after passage. However, the intriguing question was what is the driving force that pushes or pulls the transferosome across tiny skin pore?

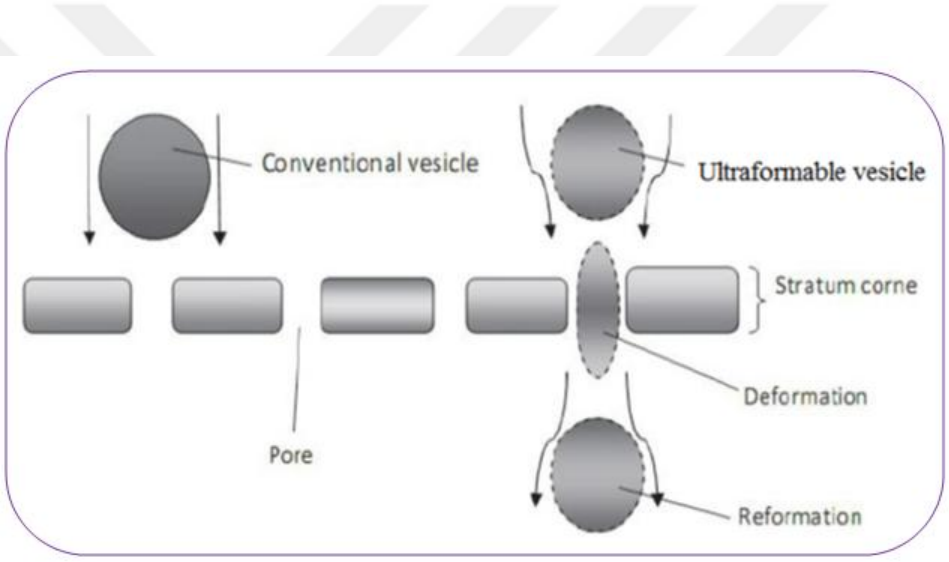

Figure 2.9. The penetration mechanism of the transferosome (5)

Cevc and Blume (70) suggested that elastic vesicles (transferosomes) are most efficient under non-occlusive conditions, that is when skin can evaporate moisture and become dry. Non-occlusive conditions are necessary in order to create a transepidermal osmotic gradient, which is believed to be the driving force for elastic transport into the skin. The osmotic gradient is caused by the difference in water concentrations between the skin surface and skin interior. The lipids feel the osmotic gradient and try to escape complete drying by moving along the gradient into the skin. At this moment, deformable property facilitates their rapid penetration through the intercellular lipid pathway of the SC.

2.5.4 Characterization of Transferosome

Transferosomes, like liposomes, are characterized by the following methods (71-73).

Vesicle Size and Charge

The vesicle size is determined by using the dynamic light scattering method or photon correlation spectroscopy. The dynamic light scattering method require slightly turbid vesicle suspension. Zeta-sizer (Malvern, UK) directly measures vesicle size and charge on the basis of movement of vesicle when exposed to laser light or put between electrodes. The related software automatically calculates the polydispersity index which gives indication of size homogeneity.

Vesicle Shape and Type

They are screened by transmission electron microscopy or the phase contrast microscopy. Electronic microscopy of freeze-fractured vesicle are used to see the number of bilayer in vesicles.

Number of Vesicle per Cubic mm

The number of vesicle per cubic mm of vesicle suspension can be counted with Haemocytometer and optical microscope after appropriate dilution.

Degree of Deformability

Transferosomes are extruded at 2.5 bars pressure through polycarbonate membrane filter having a pore diameter which is half the size of vesicles (before passes through membrane); and deformability is calculated by the following formula.

$$D = J X \left(\frac{r_v}{r_p}\right)^2$$

where,

D = deformability of vesicle membrane,

J = amount of suspension, which was extruded during 5 min

r_v = size of vesicles (after passage through membrane) and

r_p = pore size of the membrane

Entrapment Efficiency

Entrapment efficiency is the percentage of active substance that is loaded into vesicle. Generally a purification method like ultracentrifugation is used to separate vesicles from unentrapped actives. After analysis, entrapment efficiency is calculated by the following formula.

Entrapment efficiency (%) = (amount entrapped/ total amount added) × 100

Confocal Scanning Laser Microscopy to Study Penetration Ability

Different fluorescence markers are loaded into transferosome for studies with confocal scanning laser microscopy such as fluorescein- DHPE (1, 2- dihexadecanoyl- sn- glycero- 3- phosphoethanolamine- N- (5 - fluoresdenthiocarbamoyl), triethyl- ammonium salt), Rhodamine- DHPE (1, 2- dihexadecanoyl- sn- glycero- 3ogisogietgabikanube- NLissamineTmrhodamine- B- sulfonyl), triethanol- amine salt), NBD- PE (1, 2- dihexadecanoyl- sn-glycero- 3- phosphoethanolamine- N- (7-nitro- Benz-2- oxa- 1,3- diazol- 4- yl) triethanolamine salt), and Nile red. After treating the skin with transferosome, a mechanical cross-section of the transferosome treated skin is performed with a special cross-section device. The cross-sectioned skin is mounted in a sample holder in such way that the freshly cut surface remain attached to the cover glass. Then, microscopic observation of fluorescence reveals how deep the transferosome has penetrated.

2.5.5 Application of Transferosome

Transferosomes have been used to deliver proteins and peptides (74) across skin as they are destroyed by the gastrointesinal hostile environment. It is also reported to deliver non-steroidal anti-inflammatory drugs to decrease the gastrointestinal side effects (75), to provide transdermal immunization with tetanus toxoid. Transferosome is also used to target peripheral tissue due to the minimization of the carrier-associated drug clearance through cutaneous blood vessels plexus (73). However, its most promising application is going to be the delivery of cosmetic active ingredients into the targeted region of skin.

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3. MATERIALS AND METHODS

3.1. Materials

The materials used in this study are given in Table 3.1.

Table 3.1. List of Materials

Materials	Company
Alpha tocopherol (Vitamin E)	BASF, Germany
Cholesterol	Merck, Germany
Carbopol 934	Lubrizol, USA
Carbopol Ultrez 21	Lubrizol, USA
Ethylenediaminetetraacetic acid	Cliarant,Germany
Glycerin	Evyap, Turkey
Glycolic acid	Du pont, USA
Methyl Paraben	Cliarant, Germany
Phospholipon 90 H	Lipoid, Switzerland
Potassium chloride	Sigma-Aldrich, Germany
Potassium diphosphate	Riedel-de haen, Germany
Propyl Paraben	Cliarant, Germany
Sodium chloride	Sigma-Aldrich, Germany
Sodium monophosphate	Riedel-de haen, Germany
TEA	Arkem, Holland
Tween 80	Sigma-Aldrich, Germany

3.2. Equipments

The equipments used in this study are listed in Table 3.2.

Table 3.2. List of Equipments

Equipment Names and Models	Company
Extruder	Liposofast, Canada
Lyophilizatior L 2-4	Christ, Germany
Magnetic stirrer	Isolab, Germany
Polycarbonate filter, Whatman® Nuclepore Track-Etched	Sigma-Aldrich, Germany
Probe sonicator	Bandelin, Germany
Rotary evaporator	Heidolph, Germany
Spectrophotometer	Thermo Scientific, Evolution 300 UV-VIS, USA
Ultracentrifuge	Beckman coulter, USA
Viscometer	BROOKFIELD DV-II+ Pro, USA
Vortex	Scilogex, USA
Zeta-sizer	Malvern, UK

3.3 Methods

3.3.1. Preparation of Phosphate Buffered Saline (PBS)

Phosphate Buffered Saline (PBS) was prepared with the components listed in Table 3.3 according to the Cold Harbour Spring Phosphate Buffered Saline Protocol (76) with slight modification for pH adjustment to 5.5.

Table 3.3. Composition of PBS

Components	Amounts, mM
Sodium monophosphate	0.56
Potassium diphosphate	11.46
Sodium chloride	137.00
Potassium chloride	2.70

Sodium monophosphate, potassium diphosphate, sodium chloride and potassium chloride were weighed out into a beaker. They were dissolved in distilled water and the volume was completed to 1 L. Finally, the pH was measured with a calibrated pH meter and adjusted to 5.5 with acid or base.

3.3.2. Establishment and Validation of Calibration Curve for Vitamin E

The previously reported UV-VIS spectrophotometric analytical method (77) was validated by determining the following validation characteristics: specifity, linearity, sensitivity, and accuracy & precision (78). At first, a stock solution of vitamin E in chloroform (1 mg/ mL) was prepared and necessary dilution was performed to carry out this validation study. The absorption spectrum was taken between 200 and 800 nm using a quartz cell.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present in the course of analysis. Vitamin E alone, vesicular (transferosome and liposome) vitamin E samples were dissolved/diluted in chloroform and were analyzed. Spectra were compared to see whether λ_{max} of vitamin E is affected or not by other substances.

Linearity

Linearity was demonstrated using a 6 points (0.05, 0.075, 0.1, 0.125, 0.150, 0.175 mg of vitamin E per mL of chloroform) calibration curve. The absorbance values were measured 3 times for each point. After that, average absorbance for each concentration point was plotted as a function of alpha tocopherol concentration. Linearity was observed by calculation of a regression line by the method of least squares and by visual inspection of the plot.

Sensitivity

Sensitivity of the method was determined by finding the detection and quantitation limit. Determination of the signal-to-noise ratio was performed by comparing measured absorption from samples of known low concentrations of vitamin E with those of chloroform blank. Minimum concentration at which sample absorption to blank absorption were 3:1 and 10:1 were accepted as detection limit and quantitation limit, respectively**.**

Accuracy & Precision

The accuracy of an analytical procedure means the closeness of the value found to the reference value. On the other hand, precision means degree of scatter among a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels: repeatability (intra-assay precision), intermediate (inter-assay precision) and reproducibility (between laboratories). For our study purpose, intra-assay and inter-assay precision were relevant.

For the intra-assay, 0.1 mg/mL (100 µg/mL) solution was prepared and its absorbance was measured. This was repeated 5 more times (thus N = 6 replicates). Average absorbance value was put into regression equation to determine the found concentration (C_f) whereas the actual/reference value (C_a) is 0.1 mg/mL. Therefore,

$$Accuracy = \frac{(C_f - C_a)}{C_a} \times 100$$

Precision is the coefficient of variation. If the six individual concentration are Xi, and their average is μ, then standard deviation,

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}$$

Coefficient of variation,

$$CV = \frac{\sigma}{\mu} \times 100$$

For the inter-assay, 0.0625, 0.1125 and 0.1625 mg/mL vitamin E in chloroform were prepared and their absorbances were taken. This preparation and measurement of absorbance were repeated on 3 consecutive days. Thus, we have 3 values for each point. Their accuracy and precision were calculated using the above formulae.

3.3.3. Preparation and Characterization of Transferosome and Liposome

Transferosome (TSE) and liposomes (LCE) were prepared by thin-film method (79). The liposome was prepared for the purpose of comparison with transferosome preparations.

The compositions of transferosome and liposome are given in tables 3.4. and 3.5, respectively.

Table 3.4. Formulation of Transferosome (TSE)

Components	Amounts, mM
Phospholipon 90 H	82
Tween 80	15
Alpha Tocopherol	3
Glycolic acid	657
EDTA	17
PBS	q.s.

Table 3.5. Formulation of Liposome (LCE)

Components	Amounts, mM
Phospholipon 90 H	82
Cholesterol	15
Alpha Tocopherol	3
Glycolic acid	657
EDTA	17
PBS	q.s.

In the preparation of Vitamin E loaded transferosome, hydrogenated phospholipid (Phospholipon 90H), Tween 80 and vitamin E were dissolved in a 2:1 mixture of chloroform and methanol in a suitable round bottomed flask. The solvent mixture was evaporated in a rotavapor to form a thin film with a rotation speed of 200 rpm, water bath temperature of 55˚C and gradually increasing maximum vacuum. This film was exposed to a vacuum of 1 mbar overnight in a lyophilizer to remove the residual organic solvents. Later, glycolic acid and Na-EDTA were dissolved in pH 5.5 PBS. This solution and the thin film flask were brought to 60˚C which is above the transition temperature of phospholipon (55˚C). Then, the solution was added to the thin film with continuous mixing using magnetic stirrer. These milky white multilamellar

large vesicles were ultrasonicated for 1 h to reduce vesicle size. The events that take place during the formation of thin film and hydration are schematically shown in Figure 3.1. Finally, the vesicles were extruded 10 times through each of 800, 400 and 200 nm polycarbonate filter successively.

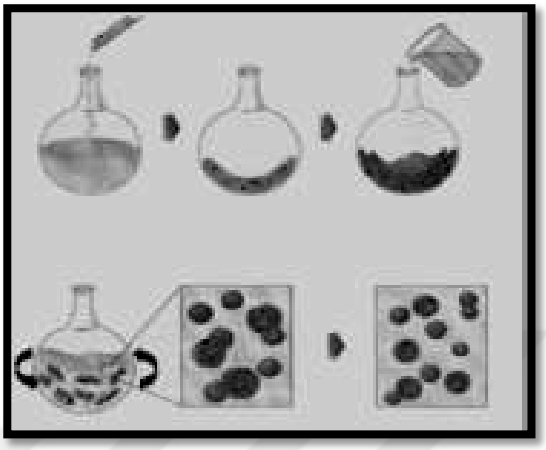

Figure 3.1. Lipid thin film formation and hydration (80)

In the preparation of Vitamin E loaded liposome, the same procedure was followed with an exception: cholesterol was used instead of Tween 80.

Vesicle size was measured by dynamic light scattering method. Zeta-potential was measured by the Technique of laser Doppler velocimetry and phase analysis light scattering (PALS). For this purpose, the vesicle suspensions were diluted with PBS so that the suspension looks only slightly turbid.

For determination of vitamin E entrapment efficiency, vesicles were purified by ultracentrifugation at 430000 g for 1 h. Supernatant was decanted and analyzed for vitamin E by the validated UV/Vis Spectrophotometric method. Deposited vesicle pellets were resuspended in PBS and analyzed for vitamin E for mass balance study. The steps in the whole process including their inclusion into polymeric hydrogel is depicted in Figure 3.2.

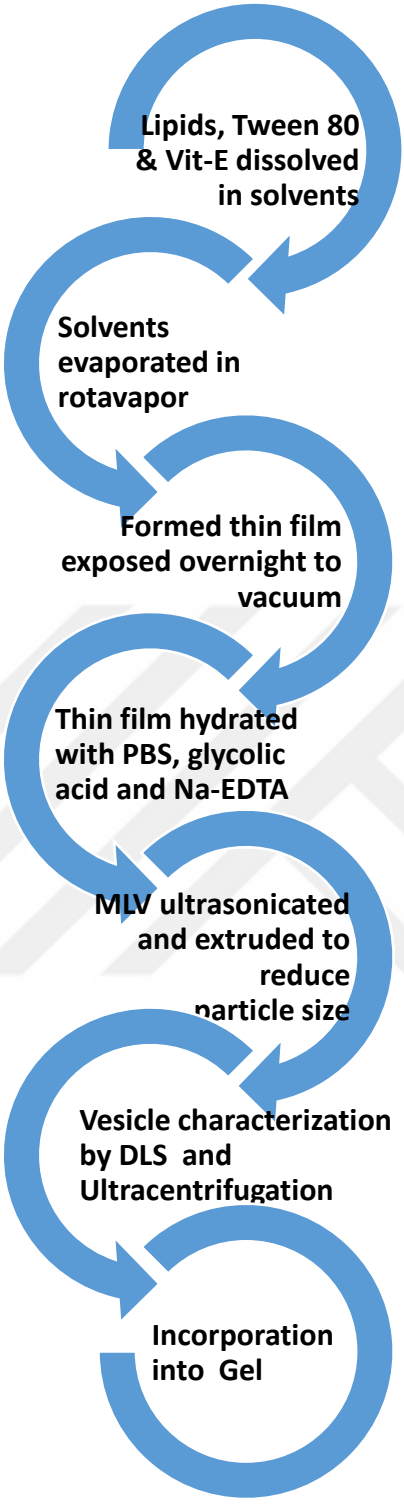

Figure 3.2. Steps in the preparation of transferosome and liposome, and their incorporation into gel.

3.3.4. Preparation of Gels with Transferosome and Liposome

The lipid vesicles were incorporated into gel formulations prepared using Carbopol Ultrez 21 and Carbopol 934 polymers. With two vesicle types and two polymer types, we have thus four formulations which are coded as GT21, GL21, GT934, and GL934. Their explanations are as follows:

GT21: Carbopol Ultrez 21 gel incorporating transferosome
GL21: Carbopol Ultrez 21 gel incorporating liposome
GT934: Carbopol 934 gel incorporating transferosome
GL934: Carbopol 934 gel incorporating liposome

The components of these gels prepared in this study are listed in Table 3.6. and Table 3.7.

Table 3.6. Gel formulation with Carbopol Ultrez 21

Components	**Amounts, %**	
	GT21	GL21
Carbopol Ultrez 21	1.50	1.50
Methyl Paraben	0.05	0.05
Propyl Paraben	0.05	0.05
Glycerin	2.50	2.50
Transferosome (TSE)	20.00	-
Liposome (LCE)	-	20.00
Triethanolamine	3.90	3.90
Water	q.s.	q.s.

Table 3.7. Gel formulation with Carbopol 934

Components	Amounts, %	
	GT934	GL934
Carbopol 934	1.50	1.50
Methyl Paraben	0.05	0.05
Propyl Paraben	0.05	0.05
Glycerin	2.50	2.50
Transferosome (TSE)	20.00	-
Liposome (LCE)	-	20.00
Triethanolamine	3.90	3.90
Water	q.s.	q.s.

In the preparation of these gels incorporating transferosome and liposome, the polymers were at first dispersed in distilled water. This is achieved within 5 min for Carbopol Ultrez 21 because of its hydrophilic nature. But Carbopol 934 needed to be added in portions to avoid nondispersible agglomeration and it was dispersed in 30 min. Preservatives were dissolved in the dispersion along with glycerin. Vesicles were added at 20% w/w concentration. Finally, triethanolamine was added to bring the pH to around 5.5 as checked with litmus paper. At this pH gel is formed and appropriate consistency is achieved.

The accurate pH values of these gels were measured with pH meter. Rheological properties were evaluated with viscometer.

4. RESULTS

4.1. Calibration and Validation Studies for Vitamin E

Absorption Spectrum

The absorption spectrum taken between 200 and 800 nm is shown in Figure 4.1. The peak absorption was found at 285 nm.

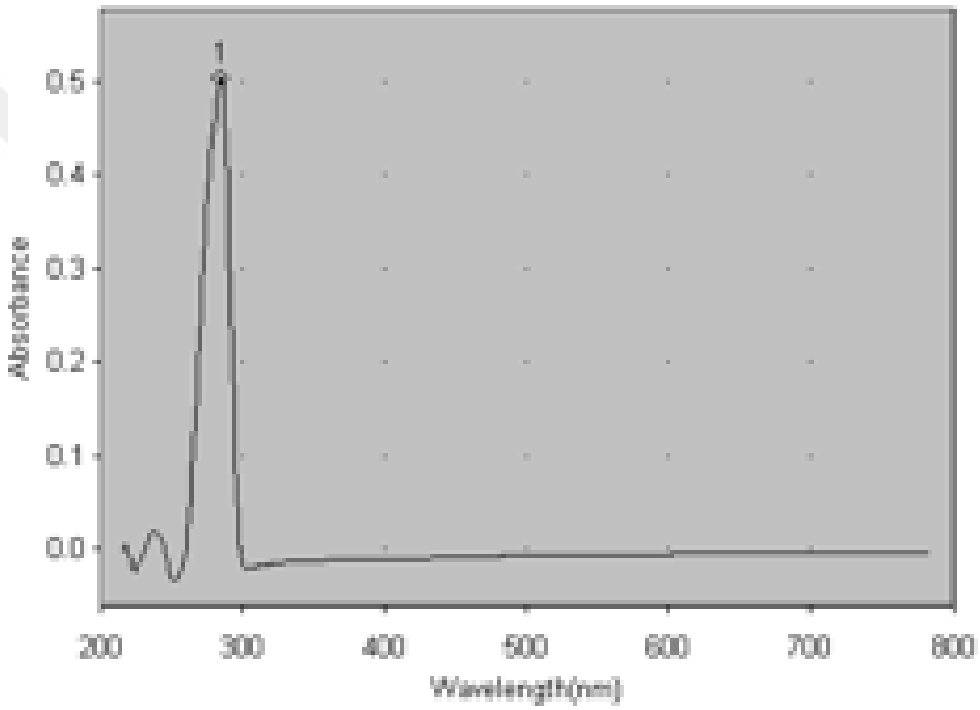

Figure 4.1. Absorption spectrum of vitamin E, λ_{max} 285 nm.

Specificity

Specificity studies was carried out as described in section 3.3.2. The results are shown in Figure 4.2.

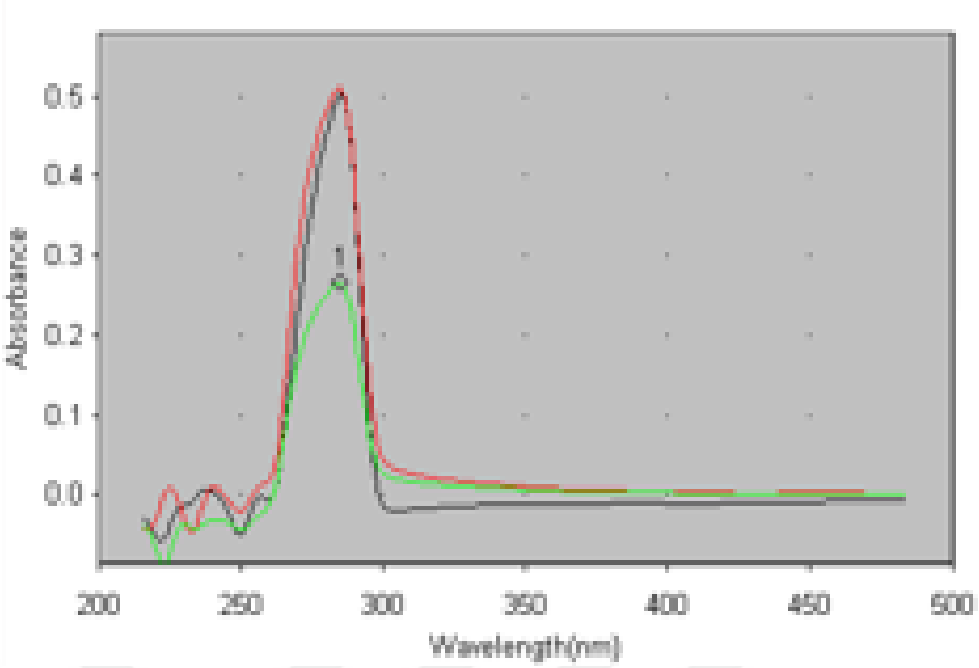

Figure 4.2. Spectra of vitamin E in chloroform (gray line), vitamin E with transferosome components in chloroform (red line) and vitamin E with liposome components in chloroform (green line).

Linearity

The absorbance values for various vitamin E concentrations are presented in Table 4.1. Average absorbance values versus concentration curve is shown in Figure 4.3.

Table 4.1. Absorbance values for linearity studies

Concentration (mg/mL)	**Absorbances**			
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
0.050	0.254	0.242	0.242	0.246
0.075	0.361	0.399	0.402	0.387
0.100	0.473	0.527	0.575	0.525
0.125	0.662	0.702	0.747	0.703
0.150	0.832	0.859	0.817	0.836
0.175	0.978	0.967	1.054	0.999

Visually, the calibration curve is clearly a straight line. Correlation coefficient (R^2) value of 0.9987 also proves the linear relationship between vitamin E concentration and absorbances. The regression line found is also shown on the graph. Therefore, unknown concentration,

$$x = \frac{Absorbance, y + 0.0644}{6.0514}$$

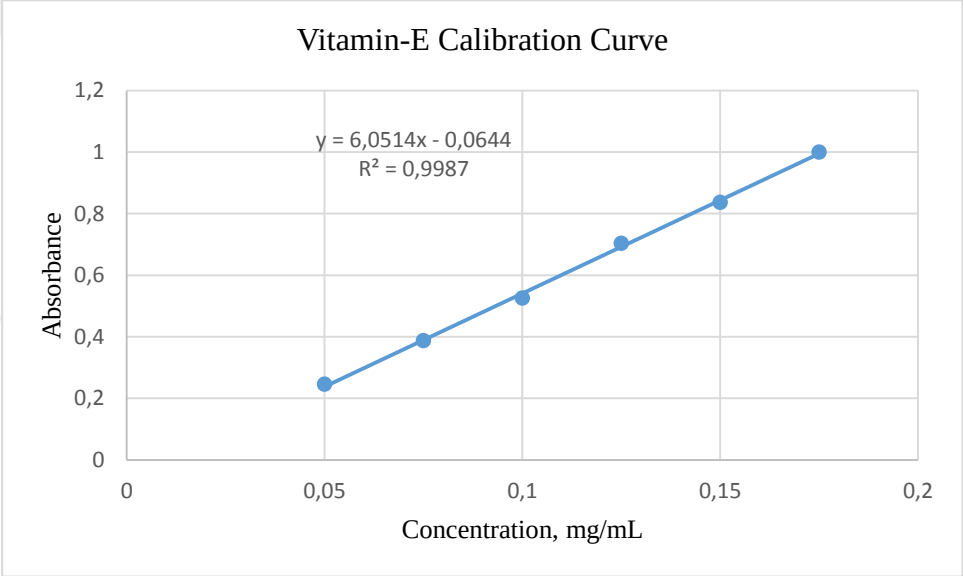

Figure 4.3. Linear relationship between vitamin E concentrations and absorbances.

Sensitivity

The sensitivity data are given in Table 4.2.

Table 4.2 Sensitivity data

	Absorbance	Concentration, mg/mL
Blank	0.003	-
Limit of Detection	0.009	0.012
Limit of Quantitation	0.030	0.015

Accuracy & Precision

Data for intra-assay precision are given in Table 4.3. Following the calculation as described in section 3.3.2, intra-assay accuracy and precision were found to be 3.43% and 4.39%, respectively.

Table 4.3. Intra-assay accuracy and precision data

Actual Concentration, mg/mL	Absorbance	Found Concentration, mg/mL	Average Concentration, mg/mL	Intra-assay Accuracy, %	Intra-assay Precision, %
0.100	0.601	0.118	0.105	3.43	4.39
0.100	0.555	0.102			
0.100	0.516	0.096			
0.100	0.560	0.103			
0.100	0.568	0.105			
0.100	0.569	0.105			

Inter-assay (inter-day) accuracy and precision data are shown in Table 4.4. Following the calculation as described in section 3.3.2, inter-assay accuracy were found to be around ±5% and precision to be less than 3%. The related precision curve are shown in Figure 4.4.

Table 4.4. Inter-assay accuracy and precision data

Actual Concentration, mg/mL	Absorbance	Found Concentration, mg/mL	Average Concentration, mg/mL	Inter-assay Accuracy, %	Inter-assay Precision, %
0.0625	0.345	0.068	0.066	5.16	2.54
0.0625	0.327	0.065			
0.0625	0.328	0.065			
0.1125	0.631	0.115	0.118	5.04	2.60
0.1125	0.668	0.121			
0.1125	0.653	0.119			
0.1625	0.876	0.155	0.157	-3,32	0.98
0.1625	0.894	0.158			
0.1625	0.889	0.158			

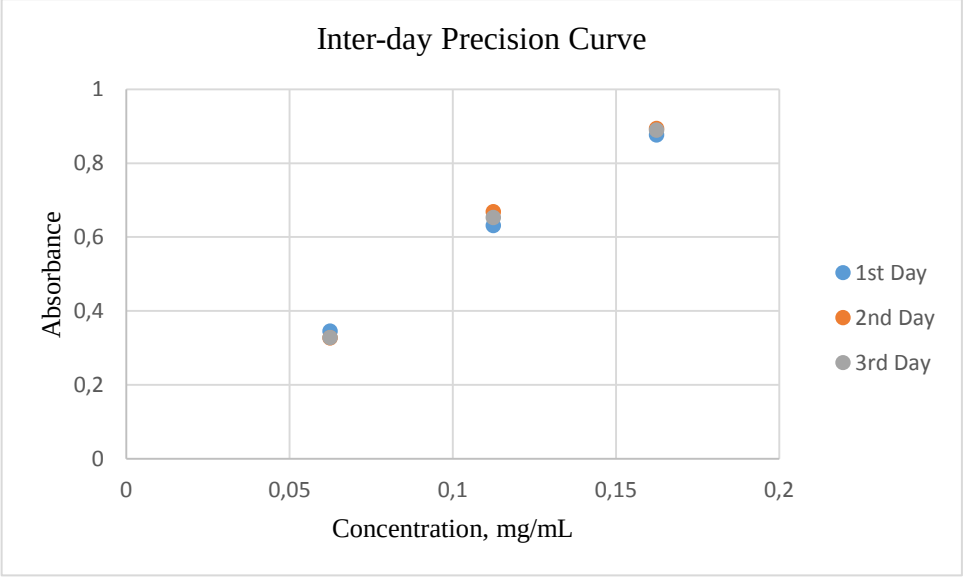

Figure 4.4. Inter-assay/interday precision curve.

4.2. Preparation and Characterization of Transferosomes and Liposomes

4.2.1 Vesicle Sizes and Zeta-potentials

The average sizes of transferosome and liposome after application of 1h ultrasonication were around 50 nm and 177 nm, respectively, as shown in Table 4.5. The zeta potentials of both the vesicle types were negligible as expected. Their size distribution are shown in Figure 4.5 and 4.6.

Table 4.5. Vesicle characteristics

Name	Size, nm Mean ± SD	Zeta Potential, mV Mean ± SD	Entrapped mM of vitamin E/100 mM lipid	Loading Efficiency, %
Transferosome, TSE	50.58 ± 21	1.02 ± 0.01	3.41 ± 0.08	96.66
Liposome, LCE	177.60 ± 36	0.9 ± 1.35	3.05 ± 0.19	86.71

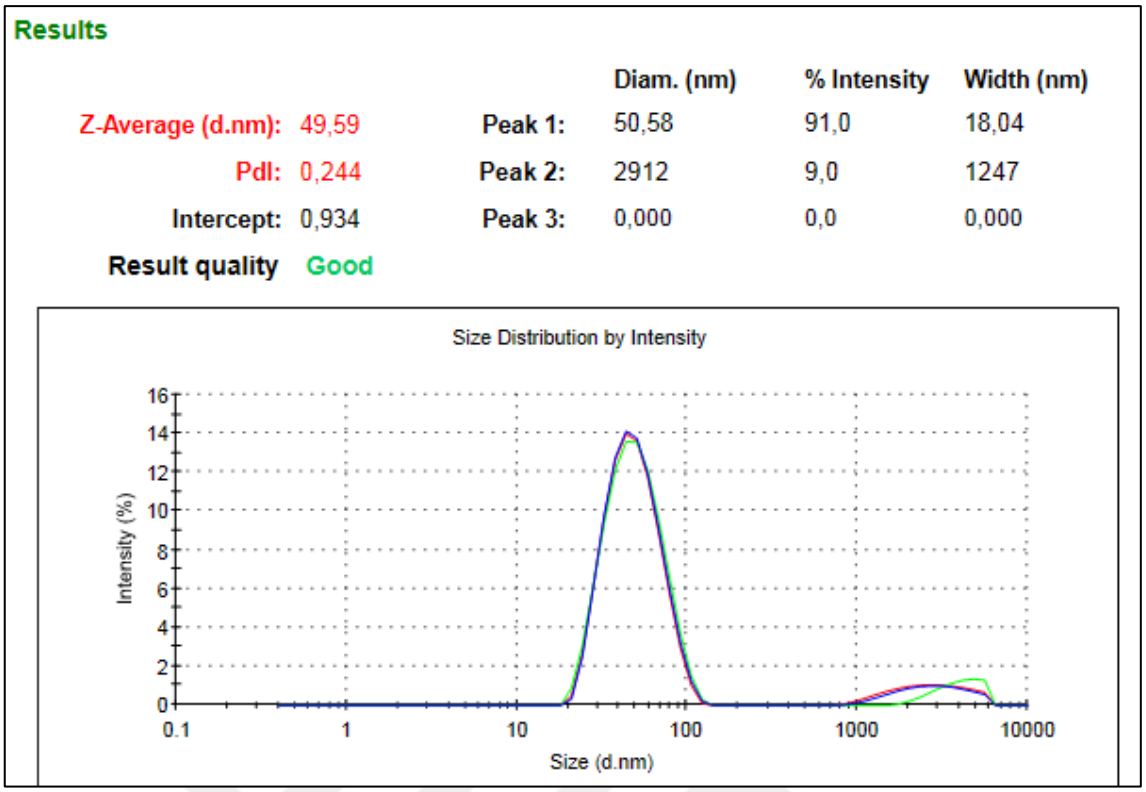

Figure 4.5. Transferosome size distribution.

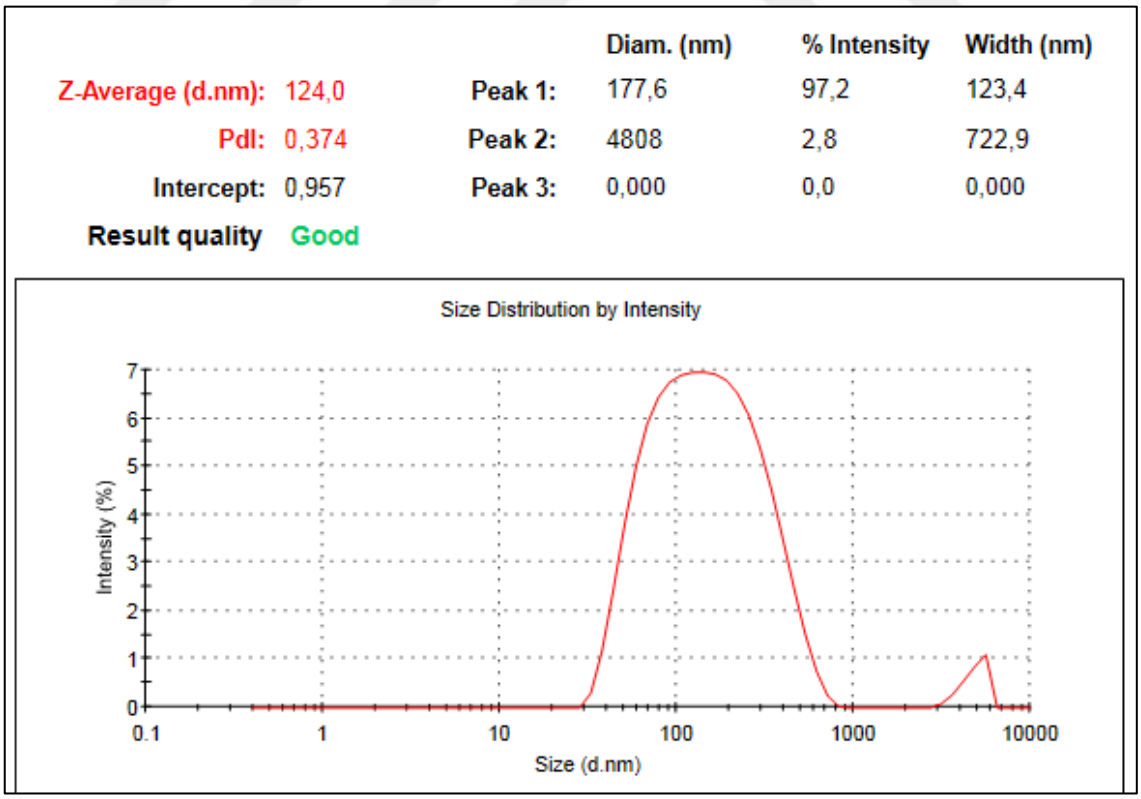

Figure 4.6. Liposome size distribution.

4.2.2 Vitamin E Entrapment Efficiency

As given in Table 4.5. 96.66% of the added vitamin-E was entrapped in transferosome. On molar basis, vitamin E to phospholipid ratio was 3.41 mole per 100 mole phospholipid. Compared to this, liposome was less efficient to entrap vitamin E. 86.71% of the added vitamin was entrapped which is equivalent to 3.05 mole per 100 mole phospholipid.

4.3 Preparation and Characterization of Gels Incorporating Vesicles

As described in 3.3.4, four different gel formulations were prepared. Their identification, pH values and viscosities are given in Table 4.6.

Table 4.6. pH and viscosity (25°C) values of gel formulation

Code	Polymer Types	Vesicle Types	Viscosity cP	pH
GT21	Carbopol Ultrez 21	Transferosome	16000-17000	5.0
GL21	Carbopol Ultrez 21	Liposome	16000-17000	6.2
GT934	Carbopol 934	Transferosome	8000-9000	5.7
GL934	Carbopol 934	Liposome	8000-9000	6.0

The viscosity, shear rate and shear stress data of these formulations are presented in Table 4.7 – 4.10. Their corresponding rheograms are given in Figure 4.7 – 4.10.

Table 4.7. Viscosity data for GT21

Shear Rate, cm^{-1}	Viscosity, cP	Shear Stress, $dyne/cm^2$
1	7250	66.5
2	5050	93.5
5	3380	158.5
10	2350	240.9
20	1460	285.5
30	1094	325.0
40	874	348.8
50	738	369.2
75	547	411.1
90	480	432.5

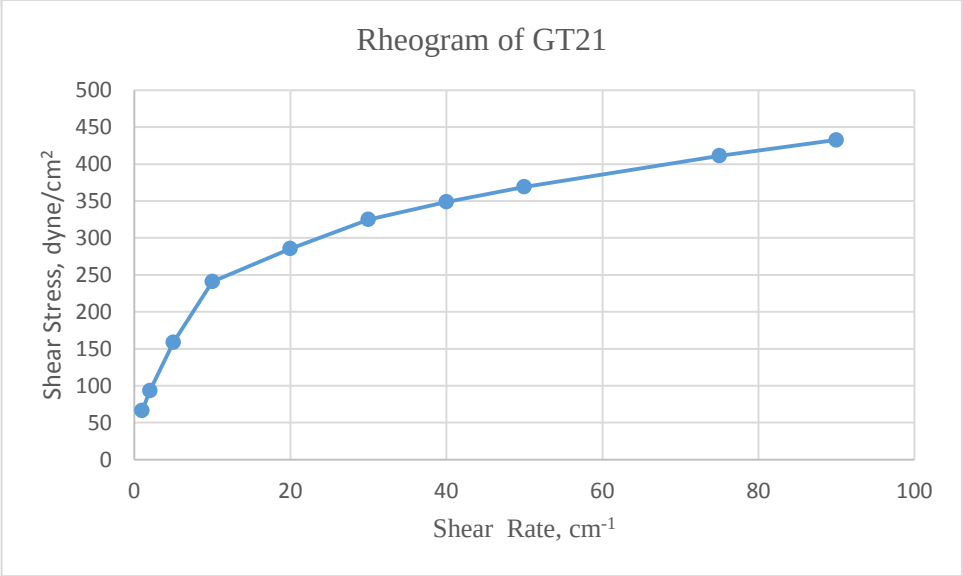

Figure 4.7. Rheogram of Carbopol Ultrez 21 Gel incorporating transferosome.

Table 4.8. Viscosity data for GL21

Shear Rate, cm^{-1}	Viscosity, cP	Shear Stress, $dyne/cm^2$
1	6480	59.9
2	4830	85.7
5	3010	148.7
10	2100	184.8
20	1248	281.3
30	983	321.0
40	749	346.9
50	713	364.3
75	489	408.8
90	352	425.7

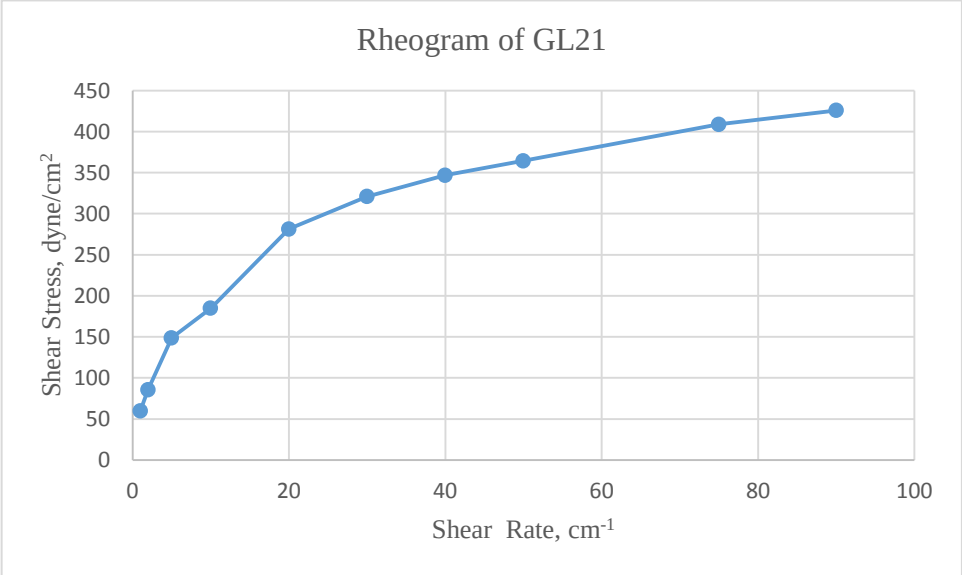

Figure 4.8. Rheogram of Carbopol Ultrez 21 Gel incorporating liposome.

Table 4.9. Viscosity data for GT934

Shear Rate, cm^{-1}	Viscosity, cP	Shear Stress, $dyne/cm^2$
1	5550	51.15
2	3150	58.59
5	1490	69.29
10	786.4	80.45
20	473.8	92.53
30	339.1	100.9
40	272.1	110.0
50	225.0	113.5
75	166.7	125.4
90	145.9	131.6

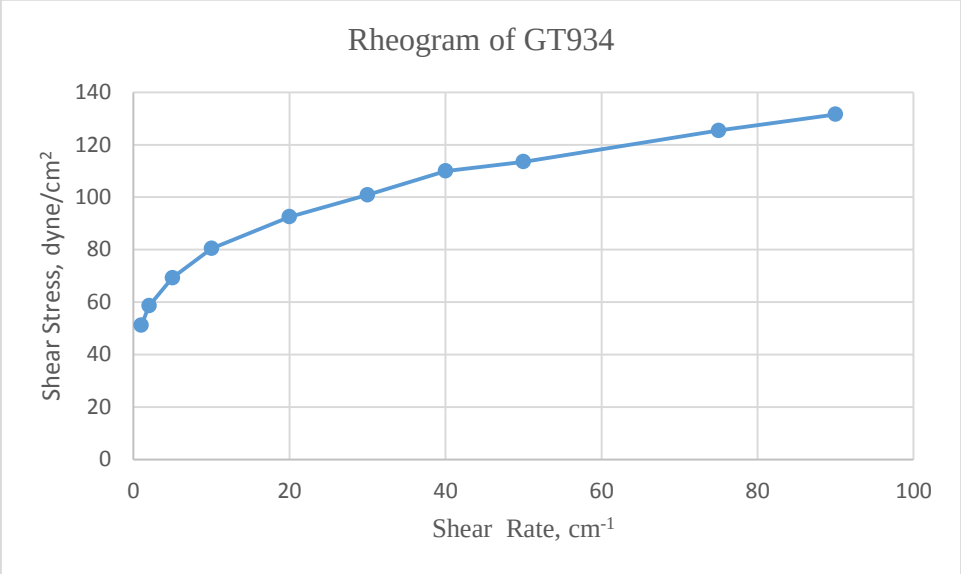

Figure 4.9. Rheogram of Carbopol 934 Gel incorporating transferosome.

Table 4.10. Viscosity data for GL934

Shear Rate, cm^{-1}	Viscosity, cP	Shear Stress, $dyne/cm^2$
1	6200	58.13
2	3550	66.03
5	1640	76.26
10	850.0	86.95
20	500.0	96.72
30	350.0	104.6
40	180.2	112.5
50	235.2	118.4
75	172.2	130.8
90	150.5	138.8

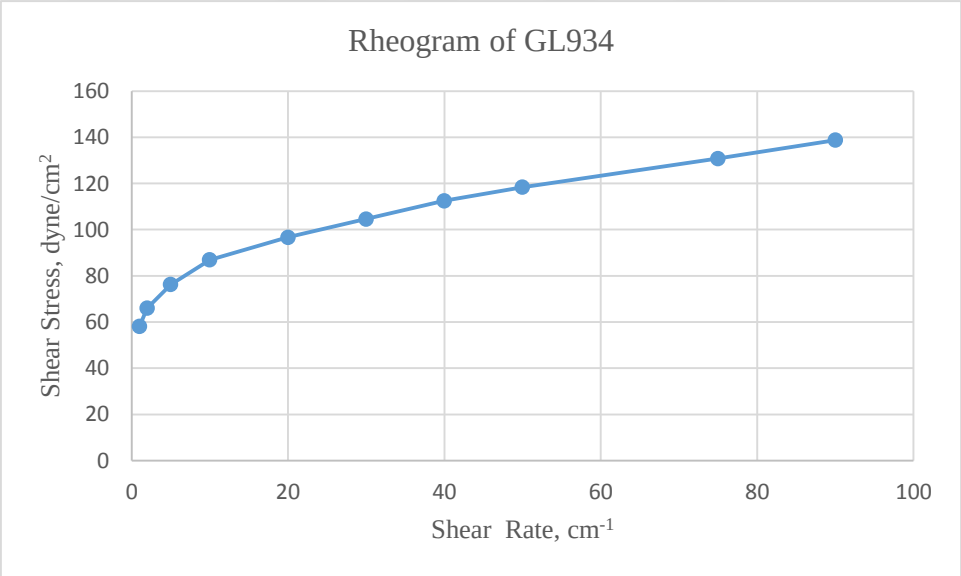

Figure 4.10. Rheogram of Carbopol 934 Gel incorporating liposome.

Linear portions of these rheograms are shown in Figure 4.11-4.14 to analyze correlation between shear stress and shear rate. Plastic viscosity are also shown on each graph as the slope of each lines.

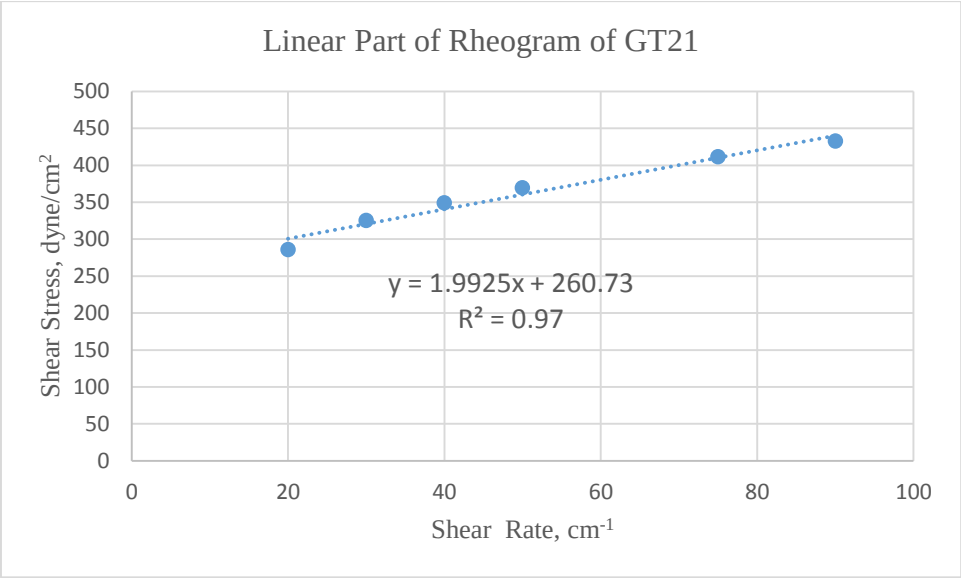

Figure 4.11. Shear stress vs shear rate correlation of linear part of rheogram of GT21.

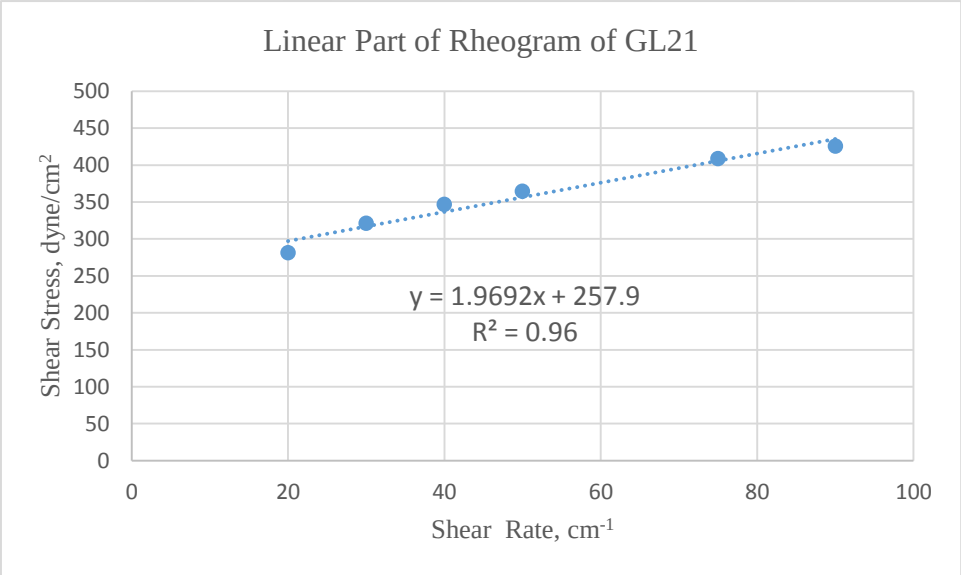

Figure 4.12. Shear stress vs shear rate correlation of linear part of rheogram of GL21.

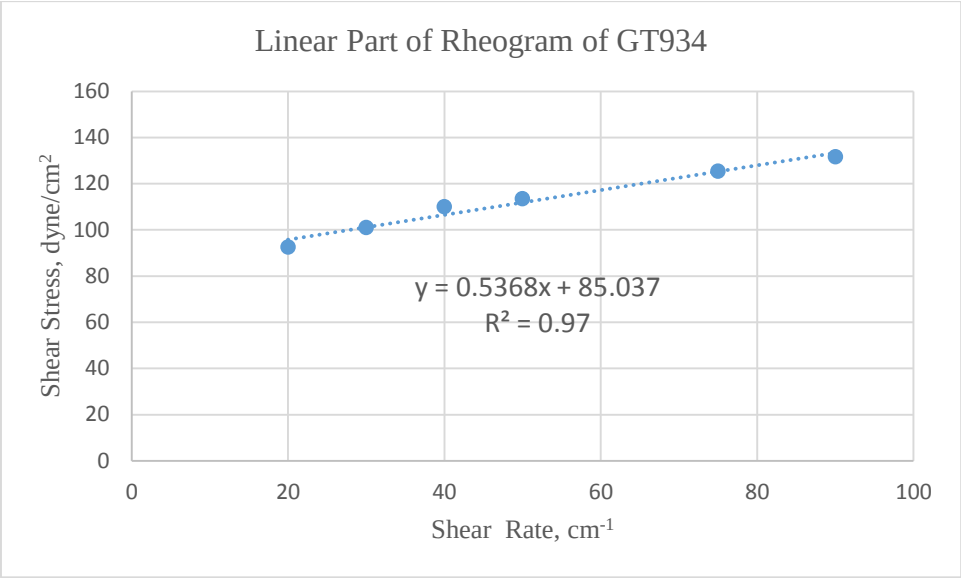

Figure 4.13. Shear stress vs shear rate correlation of linear part of rheogram of GT934.

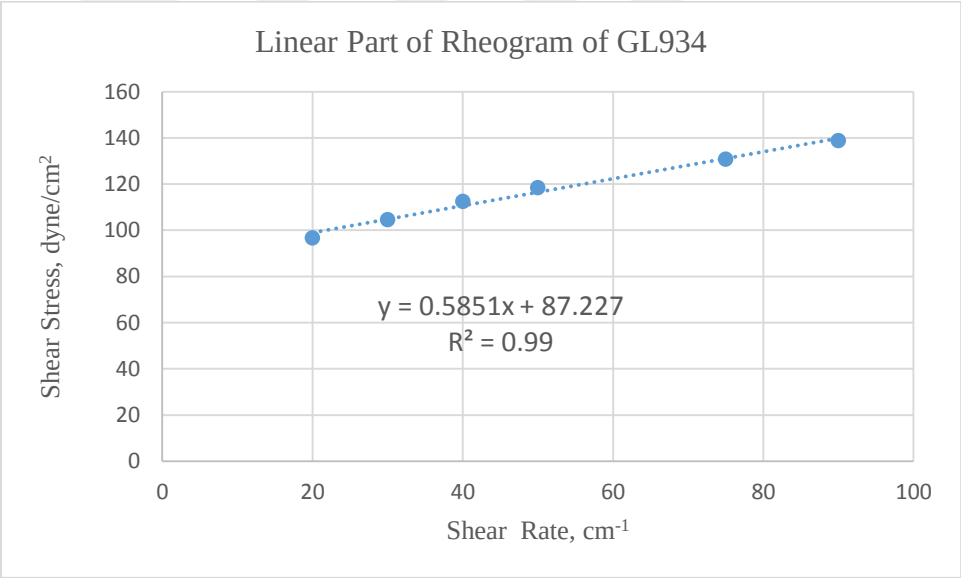

Figure 4.14. Shear stress vs shear rate correlation of linear part of rheogram of GL934.

5. DISCUSSION AND CONCLUSION

The aim of this study was to prepare and evaluate transferosome loaded with several antiwrinkle agents and incorporate them into gel formulation for easy topical application. Wrinkled skin shows higher stratum corneum pH impeding enzymatic lipid processing, degeneration of extracellular matrix primarily due to the activity of Zn & Ca dependent matrix metalloproteinases, MMP, at neutral pH. EDTA is a strong metal ion chelating broad spectrum MMP inhibitor (2). Improvement can be obtained in multiple signs of photodamaged skin by the sustained topical application of alpha-hydroxy acids, eg, glycolic acid (3). Vitamin E is essential for the stabilization of biological membranes, and the major free-radical chain-breaking antioxidant in the membrane (4). Therefore, we aimed to have a preparation containing these antiwrinkle agents with a pH values of around 5.5 which is the value for healthy skin.

The above mentioned antiwrinkle agents are not very effective when applied as conventional cosmetic formulation. This is mainly because of their inability to penetrate through the skin barrier of stratum corneum. There are several approaches to increase penetration through skin like chemical penetration enhancer, microneedles, ultrasonic or electrical penetration enhancement. With disadvantages of each of these methods, they are not suitable for regular usage. However, transferosome is an interesting delivery systems with almost problem-free characteristics.

Transferosomes are deformable lipid vesicles. Their lipid bilayer and water core allow loading of both lipid soluble materials like vitamin E and water soluble materials like glycolic acid and EDTA, respectively. They can squeeze through much smaller pores than their own size. Because of their elastic nature, the can penetrate through 10 times smaller pore size (6-7). Skin pore size is reported to be in the range of 20–40 nm (8). Therefore, a suitable limit of transferosome size should be less than 300 nm. For comparison purpose, a similar liposome formulation were also prepared and evaluated, as transferosomes are basically a type of liposome where cholesterol component is replaced by surfactant to provide elasticity.

The aqueous vesicular suspension do not have enough viscosity. So they are not suitable for topical application. Among the cosmetic forms, polymeric hydrogel is the

most suitable form for incorporation of lipid vesicles. Because their formulations are simple and does not contain any ingredient which may destabilize lipid vesicle like transferosome and liposome. Therefore, we incorporated the prepared liposome and transferosome in carbopol gel formulations.

Phosphate Buffered Saline is the most frequently used physiologically relevant salt solution. We calculated the necessary amount of acids and bases for a pH of 5.5 using the Henderson-Hasselbalch equation. The reason for selecting this pH is the fact that healthy human skin pH is 5.5. The amounts of sodium chloride, 137 mM, and potassium chloride, 2.7 mM, in PBS were similar to those found in blood plasma.

The UV absorption spectrum of vitamin E (alpha-tocopherol) was found to be 285 nm in our laboratory settings as compared to 287.5 nm reported in the literature (77). Therefore, subsequent measurements were made at 285 nm. As seen in Figure 4.2, spectra of vitamin E in chloroform, vitamin E with transferosome components (phospholipid, surfactant, EDTA, glycolic acid and PBS components) in chloroform and vitamin E with liposome components (phospholipid, cholesterol, EDTA, glycolic acid and PBS components) in chloroform were found to be similar. There was no drift of λ_{max} indicating good specificity of the analytical method. As seen in Figure 4.3, six point calibration curve, with three replicates for each point, was clearly linear with correlation coefficient (R^2) value of 0.9987 between absorbance and concentration. The concentration range selected was 0.05-0.175 mg/mL between which our expected measurement lied. The analytical method was much more sensitive; the limit of detection and limit of quantitation being 0.012 and 0.015 mg/mL. The intra-assay accuracy, the closeness of measured concentration to added concentration, was 3.43% at 0.1 mg/mL level whereas inter-assay accuracy ranged between -3.32 to 5.16% at three concentration levels between 0.0625 and 0.1625 mg/mL. The precision, the closeness to average, ranged between 0.98 and 4.39 %. As seen in Figure 4.4, the inter-assay precision values are almost inseparable for each concentration level. All these accuracy and precision values were in conformance to the usually acceptable range of ±5%.

We used phospholipon 90 H as the phospholipid component for both transferosome and liposome. It is a hydrogenated phosphatidylcholine. Its fatty acid composition is approximately 85 % stearic acid, 15 % palmitic acid. Its phase transition

temperature in hydrated form is approximately 55°C (81). On a molar basis, 15% surfactant was reported to be suitable for elasticity of lipid vesicle (82). After initial experiments, we saw that 82 mole % of lipid can load nearly 100% of 3 mole % of vitamin E. In liposomes, instead of Tween 80, equal amount (15%) of cholesterol was added. Again from literature values (2-3). We decided that 5% glycolic acid (657 mM) and 0.5% EDTA (17 mM) should be a logical level for antiwrinkle effect.

In the thin film method of preparation, lipid and lipid soluble ingredients were dissolved in chloroform-methanol mixture. It was very important to remove the residual solvents from the thin films. Exposure to very strong vacuum of 1 mbar for a prolonged period of more than 12 hours was used for this purpose, as is suggested in almost all related literature. Water soluble ingredients were dissolved in PBS and this solution was used to hydrate the thin films at 60˚C which is above the transition temperature of phospholipid. This temperature was maintained during ultrasonication and extrusion. Extrusion or sonication plays an important role in characterization and integrity of lipid vesicles. (83). Application of probe-type ultrasound for one hour for both type of lipid vesicle produced different sizes. Average size of transferosome was 50 nm with a polydispersity index of 0.244. The average size of liposomes were 177 nm with a polydispersity index of 0.374. Both the a polydispersity indexes showed that they were individually homogenous in sizes. However, the reason for different sizes, even though they were exposed to the same amount of energy, can be explained by the fact that transferosomes are deformable vesicle so high frequency sound wave easily breaks them. On the other hand, cholesterol imparts rigidity to the liposome vesicles, so their sizes remained a bit higher. However, both of the sizes were within our target range of <300 nm.

Lipid vesicles are light in nature, so it is difficult to deposit them by ordinary centrifugation. Ultracentrifugation at a very high gravity force is required, usually above 400xg (84). We used 430000x g for 1 h to separate vesicles from water. Afterwards analysis of both supernatant and deposited vesicles showed that transferosome entrapped 96.66% of the added vitamin-E (3.41 mole vitamin E/ 100 mole phospholipid). Compared to this, liposome was less efficient, 86.71% of the added amount, to entrap vitamin E. (3.05 mole/ 100 mole phospholipid).

As mentioned before, gel is the most suitable carrier for lipid vesicles. Carbopol is one of the most commonly used gelling polymer. They are high molecular weight homo- and copolymers of acrylic acid cross-linked with a polyalkenyl polyether. Various types of them are available for a wide variety of personal care products. We selected two of them: Carbopol Ultrez 21 which is easily water dispersible, and Carbopol 934, traditional type which disperses in water with difficulty.

Four gel preparation with lipid vesicles were prepared, their formulation being shown in Table 3.6 and 3.7. With initial experiments we saw that 1.5% polymer gave a consistency appropriate for topical application. Methyl- and propyl-parabens were as preservative in the usual effective concentration range. Glycerin was added as humectant. In all the preparations, vesicle suspensions (20 mM lipid) were used at 20% w/w of total gel weight. Our transferosome and liposome contained glycolic acid, as a result it pH was very low. Therefore, excess amount of triethanolamine was needed to increase the pH necessary for gel formation. Triethanolamine was added slowly until the consistency seemed to be suitable. In doing so pH left free to change. Still, pH of all the gel formulations incorporating transferosome and liposome were between 5.0 to 6.2 as seen in Table 4.6.

The viscosity, shear rate and shear stress data of the four gel formulations are presented in Table 4.7 - 4.10 with their corresponding rheograms given in Figure 4.7 - 4.10. All the rheograms showed a similar pattern but Carbopol Ultrez 21 gels (GT21 & GL21) exhibiting higher shear stress, above 400 dyne/cm^2 at 90 cm^{-1}, whereas Carbopol 934 gels (GT934 & GL934) exhibited a lower shear stress, <140 dyne/cm^2 at 90 cm^{-1}. There seemed no difference between transferosome and liposome containing gel with the same polymer. These rheogram indicates, the gels show a plastic behavior with a yield value.

From Figure 4.11-4.14, it is observed that correlation coefficients (R^2) of linear portion for rheograms GT21, GL21, GT934 and GL934 are 0.97, 0.96, 0.97 and 0.99, respectively, which confirms linearity. The plastic viscosities of these gels are 1.9925, 1.9692, 0.5368 and 0.5851 cP. These shows that Carbopol Ultrez 21 gels are more viscous than Carbopol 934 gels but with in each gel transferosome and liposome

inclusion does not make any difference. However, when practically smeared on skin, the difference in gel viscosity was not perceptible.

In conclusion, the deformable lipid vesicle, transferosome, is an appropriate delivery systems for carrying active substances, like antiwrinkle agents, deeper into the skin. Several active substances, both water soluble and lipid soluble, can be loaded into these vesicles at the same time. However, their sizes must be less than 300 nm considering the skin pore sizes of 20-40 nm. Carbopol polymeric gels are a good medium for transferosomes. These gel formulations (maximum 1.5% polymer) with transferosomes were of appropriate consistency for topical application. Thus, transferosome loaded with several antiwrinkle agents is a suitable candidate for evaluation for in vivo efficacy.

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ANNEX

CURRICULUM VITAE

Personal Information

Name	Meltem	**Surname**	Macit
Place of Birth	Iğdır	**Date of Birth**	11.09.1989
Nationality	TC	**TC Identity No**	29123290570
E-mail	meltem.macit@yeditepe.edu.tr	**Tel No**	05382401536

Educational Qualification

Degree	**Area**	**Institution of Graduation**	**Year of Graduation**
Master	Cosmetology	Yeditepe University	--
Graduation	Chemical Engineering	Yeditepe University	2013
High School	Anadolu Lisesi	Niğde Anadolu Lisesi	2007

Foreign Languages

Languages	**Foreign Language Exam Notes**
English	--

Work Experience

Duty	**Institution**	**Duration (Year-Year)**
Research Assistant	Yeditepe University	2013 -

Computer Literacy

Program	**Skill***
Word	Very Good
Excel	Very Good
PowerPoint	Very Good
Octave	Good
Fortran	Good
Matlab	Good

* Very Good, Good, Medium, or Weak

Presentation at international scientific meetings and published in the Proceedings

11th. International Symposium of Pharmaceutical Sciences (ISOPS). June 9-12, 2015. Ankara, Turkey. Oral presentation: “Assessment of the Epilepsy Knowledge of

Epileptic Patients, Non-epileptic Patients and Community Pharmacists. Macit C, Macit M, Aykut Bingol C, Clark PM.
11th. International Symposium of Pharmaceutical Sciences (ISOPS). June 9-12, 2015. Ankara, Turkey. Poster presentation: "Preparation and Characterization of Transferosomes Loaded with Multiple Antiwrinkle Agents." Macit M, Rouf MA, Aslan I, Macit C, Ozdemir S.

Others (Position in Projects / Certificates / Awards)

ISO 9001: Certificate on Quality Management Systems Yeditepe University, Faculty of Chemical Engineering (2008)
Certificate on Experimental Animal, Yeditepe University, Faculty of Medicine (2013)
Practical Training on "Drug Quality Control", Yeditepe University, Faculty of Pharmacy (2014)
Training on Dissolution Testing, Yeditepe University, Faculty of Pharmacy (2014)