T.C.

YEDITEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

THE DEPARTMENT OF COSMETOLOGY

STUDIES ON LIPOSOME, GEL AND LIPOGELOSOME FORMULATIONS CONTAINING SODIUM HYALURONATE

PHARM. İSMAİL ASLAN

ADVISORS:

PROF. DR. A. YEKTA ÖZER - ASSIST. PROF. DR. GÜLENGÜL DUMAN

ISTANBUL – 2010

Yüksek Lisans (Master) öğrencisi. İsmail ASLAN'ın çalışması jürimiz tarafından Kozmetoloji Anabilim Dalı Master tezi olarak uygun görülmüştür.

İMZA

Başkan : Prof. Dr. A.Yekta ÖZER
Üniversite : Hacettepe Üniversitesi .....

Üye : Prof. Dr. Özgen ÖZER
Üniversite : Ege Üniversitesi .....

Üye : Yard. Doç. Dr. Gülengül DUMAN
Üniversite : Yeditepe Üniversitesi .....

Üye : Yard. Doç. Dr. Alpay TARALP
Üniversite : Sabancı Üniversitesi .....

ONAY

Yukarıdaki jüri kararı Enstitü Yönetim Kurulu'nun 07.10.2010 tarih ve 32-2 sayılı kararı ile onaylanmıştır.

Prof. Dr. Selçuk YILMAZ
Müdür

DEDICATION

To my devoted family and to the memory of my grandmother.

ACKNOWLEDGEMENT

I am infinitely grateful to many people whose combined efforts helped me to make this study possible.

First and foremost, I would like to thank to my thesis advisors Prof. Dr. A. Yekta Özer (Hacettepe University) and Assist. Prof. Dr. Gülengül Duman (Yeditepe University) for their knowledge, wisdom and patient motivation afforded me for freedom to explore my intellectual interests.
I would like to express my deep appreciation for all of the advice, guidance and academic counsel to Prof.Dr. Dilek Erol (Yeditepe University).

I offer my sincerest gratitude Assist. Prof. Dr. Alpay Taralp (Sabancı University) for his support and enthusiasm for my research.

I would like to express my deep appreciation and sincere gratitude to M. Sci. Physicist İbrahim İnanç (Sabancı University) for his willingness and friendship to motivate me by contributing tremendously to this thesis.

I extend my gratitude to Prof. Dr. Hülya Akgün (Yeditepe University) for providing many opportunities for me.

I am deeply indebted to Ferhad Farshi (Ph.D), Chem. Eng. Mine Uz and Chem. Tansel Aktaş (Abdi Ibrahim İlaç San. ve Tic A.Ş.) for their support at dissolution studies.

Many thanks Dr. Axel Wolfhart (Lipoid GmbH. Ludwigshafen Germany) and Rauf Ongay (SEM Lab.) for their technical support.

I wish to extend my thanks to Prof. Dr. A. Cüneyt TAŞ, Assoc. Prof. Dr. Mustafa Çulha, Chem. Mehmet Kahraman (Yeditepe University) and Ph. D Burçin Yıldız (Sabancı University).

I also wish to extend my thanks to Nurcihan Kaplan, Elçin Yenigül Tulunay, Mahir Tulunay, Yiğit Sarıoğlu, Sümeyra Atakçı, Hattap Kütükde, all our assistants and technicians for their kind help.

Last but not least, none of this would have been possible without the love and patience of my family. I have to give a special mention for the support given by my parents and my sister.

CONTENTS

TABLES

FIGURES

ABBREVIATIONS

3D	Three dimensional
AFM	Atomic Force Microscope
BDDE	Butanediol diglycidyl ether
C 940	Carbopol 940
CHOL	Cholesterol
CP	Commercial product
CV	Coefficient of variation
DLS	Dynamic Light Scattering
DMPC	Dimyrstoylphosphatidyl choline
DMPG	1, 2-dimyristoyl-sn-glycero-3-phosphatidylglycerol
DNA	Deoxyribo Nucleic Acid
DOTAP	1, 2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DOTMA	Dioleoyl-propyl-trimethylammonium
DPPC	Dipalmitoylphosphatidylcholine
DRV	Dehydration-Rehydration Vesicle
DSC	Differential Scanning Calorimetry
EHT	Electron High Tension
FRV	Freeze-dried Rehydration Vesicle
FT-IR	Fourier Transform Infrared
GUV	Giant Unilamellar Vesicle
GV	Giant Vesicle
HA	Hyaluronic Acid
IUV	Intermediate-Sized Unilamellar Vesicle
KX	Thousand times.
LOV	Large Oligolamellar Vesicle
LUV	Large Unilamellar Vesicle
MLV	Multi Lamellar Vesicle
MVV	Multi Vesicular Vesicle
Na-HA	Sodium hyaluronate
Na-HA-D	Digested Sodyum Hyaluronate

NASHA	Nonanimal Stabilized Sodium Hyaluronate
OA	Osteoarthritis
OLV	Oligo Lamellar Vesicle
PC	Phosphatidylcholine
PEO	Polyethyleneoxide
PG	Phoshatidylglycerol
PL 100H	Phospholipon 100H
PL 20	Phosholipon 20
PL 30	Phospholipon 30
PL 85G	Phospholipon 85G
PLM	Polarized Light Microscope
PPO	Polypropyleneoxide
PXM	Poloxamer
RA	Rhomatoid Artiritis
RSD	Relative standard deviation
SE	Standard Error
SEM	Scanning Electron Microscope
SOV	Small Oligolamellar Vesicle
SUV	Small Unilamellar Vesicle
TEWL	Trans Epidermal Water Loss
U 21	Ultrez 21
ULV	Unilamellar Vesicle
UV	Ultraviolet
WD	Working Distance
X-RD	X-Ray Diffraction
$\bar{x}$	Arithmetic mean

ÖZET

Sodyum hyalüronat (Na-HA) mukopolisakkarit yapısında doğal bir polimer olup cilde esneklik sağlar. Na-HA rekombinant deoksiribonükleik asit (DNA) teknolojisi ile Streptococcus zooepidemicus isimli bakteriden üretilmektedir. Bu tez çalışmasında, Na-HA nın lipozomal jel formülasyonları şeklinde cilt üzerine topikal uygulandığında etkinliği araştırılmıştır. Bu amaçla dimyristoylphosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylglycerol(DMPG),dipalmitoylphosphatidylcholine (DPPC) and phospholipon 100H (P 100H) gibi farklı fosfolipidlerle Na-HA (45 kilo dalton) içeren ve içermeyen 16 değişik lipozom formülasyonları hazırlanmıştır. Ortalama partikül büyüklüğü dağılımı ve zeta potansiyel ölçümü yapılarak lipozomların karakteristiği değerlendirilmiştir. Ayrıca Taramalı elektron mikroskop (SEM), Atomik kuvvet mikroskop (AFM), Polarize ışık mikroskop (PLM) çalışmaları ile lipozom formülasyonlarının morfolojik özellikleri araştırlmıştır ve Na-HA nın lipozom içerisine hapsedilme kapasitesi tayin edilmiştir. Lipozom içeren jel formülasyonları hazırlamak için jel yapıcı ajan olarak Poloxamer (PXM 188 ve PXM 407) ve Carbopol türevleri (C 940 and U 21) kullanılmıştır. Na-HA'in lipozom, lipojelozom ve Na-HA nın ticari formülasyonundan in vitro salım profili "flow through cell" metoduyla çalışılmıştır. Bu çalışmalar sonucunda Na-HA içeren Phospholipon 100H: SA: CHOL (7:1:2) optimum formülasyon olarak seçilmiştir (L 37). MLV tipi olan L 37 formülasyonunun ortalama partikül büyüklüğü 1899 nm ± 13.01, zeta potansiyeli -20.9 mV ± 0.46 ve hapsetme kapasitesi % 14.95 olarak bulunmuştur. L 37 formülü U 21 jel formülü ile 1:1 oranında karıştırılarak optimum jel formülasyon LG 8 hazırlanmıştır. In vivo amaçla yapılan ön çalışma, 15 bayan gönüllü üzerinde gerçekleştirilmiştir. Optimum formülasyonlardan Na-HA içeren ve Na-HA içermeyen lipozom, lipojelozom formülasyonları ile ticari formülasyon, iki hafta boyunca günde iki kez olmak üzere gönüllülere uygulanmıştır. Cilt yüzeyindeki pH seviyesi, sebum içeriği ve nem seviyesi değişimi gibi tüm parametreler cilt analiz cihazı kullanılarak tayin edilmiş ve değerlendirilmiştir. Ayrıca cilt yüzeyindeki kırışıklık derinliklerindeki değişim cilt analiz cihazı kullanılarak ölçümlenmiştir. LG 8 formülasyonu için nem artış oranı % 30.36 ± 0.12 olarak gözlemlenmiştir. Sonuç olarak hazırladığımız LG 8 formülasyonu, konvansiyonel benzerleriyle karşılaştırıldığında derinin nemliliği ve hasta uyumu bakımından üstün bulunmuştur.

Aslan I. Sodyum hyaluronat İçeren Lipozom, Jel ve Lipojelozom Formülasyonları Üzerine Çalışmalar, Yeditepe Üniversitesi Eczacılık Fakültesi, Sağlık Bilimleri Enstitüsü Kozmetoloji Yüksek Lisans Tezi, İstanbul, 2010.

Anahtar Kelimeler: Sodyum Hyalüronat, DPPC, DMPC, PL 100H, Poloxamer, Carbopol, Jel, Na-HA Lipozomlar, Na-HA Lipojelozomlar, Stabilite, *İn vitro* Salım, Gönüllü çalışmaları.

ABSTRACT

Sodium hyaluronate (Na-HA) is a natural polymer with mucopolysaccharide structure which provides viscoelasticity to the skin. Na-HA is produced by recombinant deoxyribonucleic acid technology (DNA) from *Streptococcus zooepidemicus*. In this thesis study, the moisturizing effectiveness of Na-HA on skin was investigated in liposome, gel and lipogelosome formulations. For this purpose, 16 different liposome formulations were prepared by thin film technique with using Na-HA (45 kDa) and without Na-HA in various type of phospholipids; dimyristoylphosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoracglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC) and phospholipon 100H (PL 100H). The liposomes prepared by film technique were characterized by mean particle size and size distribution, entrapment capacity, zeta potential, phospholipid efficiency, liposome type determinations. Polarized light microscopy (PLM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) techniques were employed for obtaining size distribution and surface appearance and lamellarity. Poloxamer (PXM 188 and PXM 407) and Carbopol derivatives (C 940 and U 21) were used as gelling agents to prepare liposome loaded gel formulations. *In vitro* release of Na-HA from liposome, Na-HA lipogelosome and marketed formulation of Na-HA was studied by flow through cell method. The optimum formulation was Phospholipon 100H: SA: CHOL (7:1:2) containing Na-HA (L 37). The mean particle size, zeta potential and entrapment capacity were found as 1899 nm ± 13.01, -20.9 mV ± 0,46 and 14.95 %, respectively for the formulation L 37 . Then L 37 formulation was incorporated into the U 21 gel with 1:1 (w/w) ratio (LG 8). The preliminary *in vivo* studies were performed on 15 female volunteers. With or without Na-HA liposome, lipogelosome and marketed formulation were applied to volunteers for two weeks twice a day. All parameters as changes in humidity levels, sebum content and the pH-level on the skin surface were determined and evaluated by using the Multiprobe Adapter Systems (Corneometer, sebumeter, pH- meter, respectively). The changes of deepness of wrinkles on the skin surface were measured by Skin-visiometer. The increment of moisture level was found 30.36 ± 0.12 % for LG 8 formulation. As a result, LG 8 formulation has improved skin hydration and better compliance than the conventional formulations.

Aslan I. Studies on liposome, gel and lipogelosome formulations containing sodium hyaluronate, Yeditepe University, Faculty of Pharmacy, Institute of Health Sciences, Cosmetology Master Thesis, İstanbul, 2010.

Key Words: Sodium Hyaluronate; DPPC; DMPC; PL 100H; Poloxamer; Carbopol; Gel; Na-HA Liposomes; Na-HA Lipogelosomes; Stability; *In vitro* Release; Volunteer Studies

1. INTRODUCTION

1.1. LIPOSOMES

Liposomes are spherical microscobic dispersions with thin layer of lipid like membrane that are mainly made of phospholipids. Phospholipid is an amphipilic character which has a hydrophilic head and a lipophilic tail. In aqueus solutions, they look like artificial cell membranes. The fatty acid tails which are nonpolar alligned towards the interior of the membrane, whereas hydrophilic heads orient outward of the membrane (1,2). The basic structure of liposome is presented in Figure 1.

Liposomes are firstly discovered by Bangham in 1960s. After his intense studies, liposomes are applied to drug delivery and pharmaceutical industry. The first application and substantial improvements come from the Christian Dior with the name of Capture in cosmetic area (3).

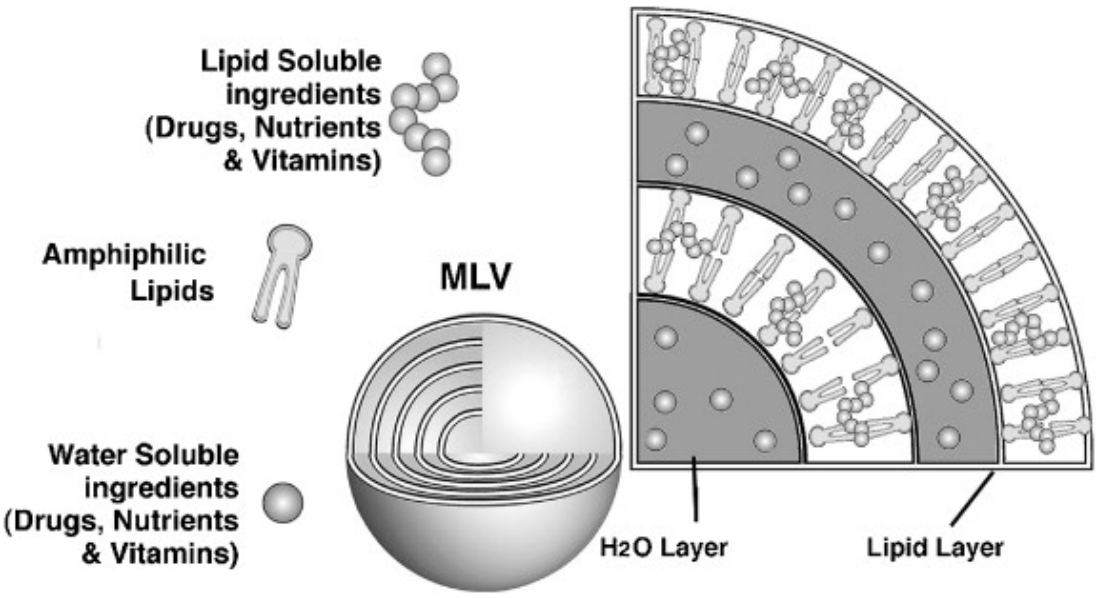


Figure 1. Cross-section of a liposome (4).

Liposomes contain phospholipids, CHOL, water, drug, electrolytes, and occasionally antioxidants, preservatives, and viscosity-inducing agents. Liposomes can be coated with PEG and immunologic fragments can be bound and so, immunoliposomes can be prepared. Figure 2 illustrates immunoliposome structure.

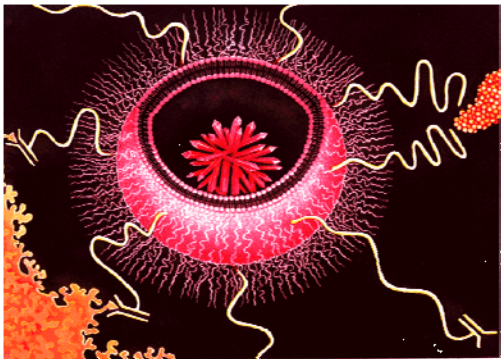


Figure 2. Structure of immunoliposome (3).

1.1.1. DEFINITION, MECHANISM of FORMATION and CLASSIFICATION of LIPOSOMES

The main source of liposome obtained from PC, lecithin of egg or vegetable origin. In research studies, synthetic forms of phospholipids such as DPPC can be used, but it is not cost-effective to use those synthetic liposomes in scale-up production. Because liposome composition and structure is very similar to cell membrane, they have very high biocompatibility and biodegradability. And their amphiphilic nature consisting of lipophilic tails and hydrophilic heads, almost any type of drug can be encapsulated easily (5).

CHOL is generally added in the formulation to make the bilayer stable and to decrease the leaking out of the encapsulated hydrophilic drug. Electrolytes are used to increase the lipid bilayer order and to supply isotonic balance. Antioxidant and preservative agents are the components of liposome formulations as well (1).

There are many types of liposomes such as MLV, SUV, LUV, FRV and they can be prepared by different preparation methods (6).

Liposomes can have negative, positive or neutral surface charge related to their consistency. They are different type of charge inducers. Stearylamine and phosphatidic acid constituents provide positive and negative surface charge, respectively. Likewise, lecithin can contribute liposomes with a neutral surface. Numerous types of liposome containing products can be formed related to method of preparation, kind of the encapsulation agent and lipid composition (6).

Liposomes are formed of small vesicles of a bilayer structure of phospholipid encapsulating aqueous dispersions and their particle size in between 20 nm-10 micrometers (2). They can be administered parenterally, topically, by inhalation or possibly by other

routes of administration. Most recently attainable products are administered parenterally (7, 8).

Table 1. Types of liposomes (3).

Type	Abbreviations	Size
Unilamellar vesicles	UV	All size range
Oligolamellar vesicles	OLV	0.1-1 µm
Multilamellar vesicles	MLV	>0.5 µm
Small unilamellar vesicles	SUV	20-40 nm
Medium sized unilamellar vesicles	MUV	40-80 nm
Large unilamellar vesicles	LUV	>100 nm
Giant unilamellar vesicles	GUV	vesicles with diameter>1 µm
Multi-vesicular vesicles	MVV	usually larger>1 µm

Although many configurations are suitable for spherical or cylindric liposomes, the frequent description is given as LUVs, SUVs and MLVs. The smaller liposomes usually range in size between 20-200 nm and the large vesicles in between 0.2-10 µm diameters. The MLVs have an onion type structure of lamellar layers (9).

Lipid vesicles are dispersed in an aqueous space even if they can be viewed as a heterogenous liquid. Liposomes are classified as SUVs, LUVs, MLVs, SOVs, LOVs, or GVs according to their particle size and the number of lipid layers (2, 10).

The ideal drug candidates for liposomal encapsulation are those that have potent pharmacological activity and are highly lipid or water-soluble. If a drug is water -soluble it will be encapsulated within the aqueous compartment and the drug concentration in the liposomal product will depend on the volume of the entrapped water and the solubility of that drug in the encapsulated water (11).

The lipophilic drug is mostly dissolved in the lipid phase. A lipophilic drug more probably will remain encapsulated longer during storage than a hydrophilic one. Because of its partition coefficient, the lipophilic drug is connected with the lipid bilayers, preferably than existing strain to the outside water phase. Generally the encapsulation capacity of is higher for lipophilic drugs than for hydrophilic drugs (1).

Multilamelar Vesicles

MLVs have multi lipid bilayers separated by aqueous layers and have a diameter of 300 nm to 15 μm. Multilamellar vesicles formed of just a few concentric lamels that are sometimes called oligolamellar liposomes. Generally oligolamellar liposomes are considered to be 2-5 bilayers and range in size from 50 to 250 nm (10,11). SOVs are formed from two or three lipid bilayers and are less than 200 nm in diameter. LOVs contain two or three lipid bilayers and are 300 –2000 nm in size. GVs are MLVs with diameters larger than 10 μm (7).

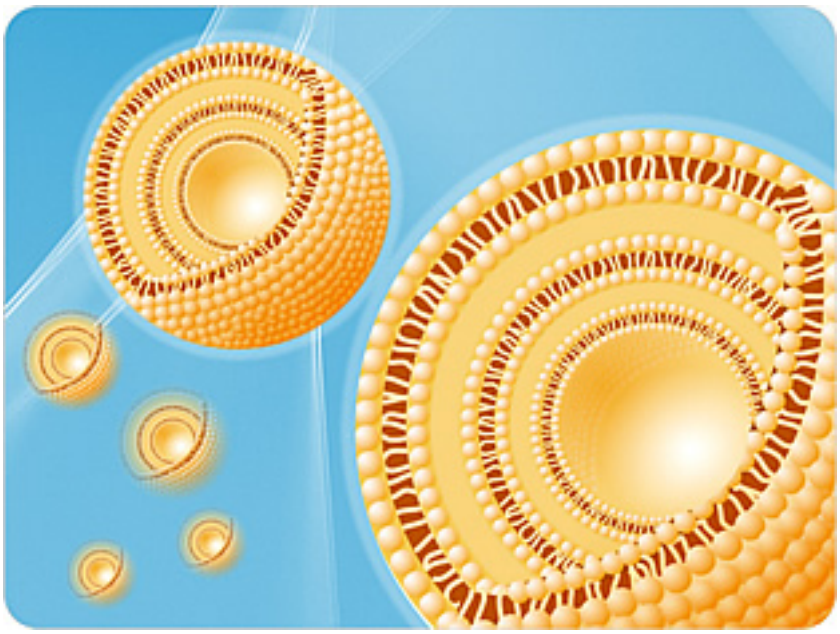


Figure 3. Schematic representation of MLVs (13).

Small Unilamellar Vesicles

Among all liposome types, Unilamellar (i.e.single bilayer) liposomes at the lowest limit of size possible for phosholipid vesicles. This limit varies slightly according to the ionic strength of the aqueous medium and the lipid composition of the membrane, but is about 15 nm for pure egg lecithin in normal saline, and 25 nm for DPPC liposomes. Since, according to recommendations, these liposomes are at or close to the lower size limit, they will be a relatively homogeneous population in terms of size (13). SUVs are composed of one lipid bilayer and one aqueous compartment. Their size ranges between 20 and 200 nm (7).

Intermediate-Sized and Large Unilamellar Vesicles

These have diameters of the order of magnitude of 100 nm, and are called LUVs if the size is >100 nm and they consist of a single bilayer. For unilamellar vesicles, the phospholipid content is related to the surface area e.g photolysis, to form inter-chain cross links to stabilize the bilayers. LUVs have one lipid bilayer closing one aqueous compartment. Their size can vary in between 300 and 2000 nm. They are particularly useful for entrapping water-soluble drugs owing to their high water encapsulation efficiency (20–68%) (14).

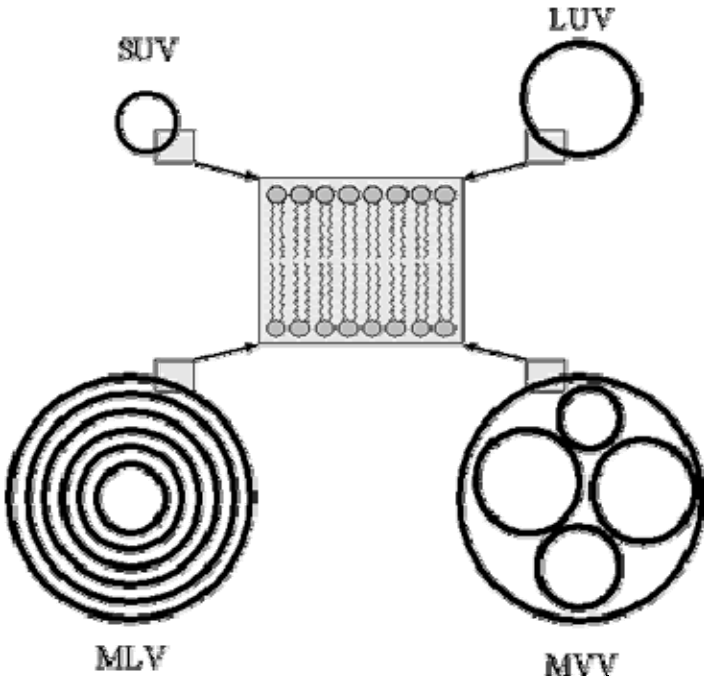


Figure 4. Types of Liposomes; SUV: Small Unilamellar Vesicles, LUV: Large Unilamellar Vesicles, MLV: Multilamellar Vesicles, MVV: Multivesicular Vesicles (3).

1.1.2. METHODS of LIPOSOME PREPARATION

Liposomes are composed by open hydration of lipid molecules, normally lipids are hydrated from dry state (thin or thick lipid film, spray dried powder), and stacks of crystalline bilayers become fluid and swell myelin-long in hydrated condition. Then thin cylinders grow and upon churning separate into self closed large, multilameller liposomes, it is because of elimination of disfavorable interactions at the edges. As soon as the large particles are composed, they can be either broken by mechanical treatment into smaller bilayered compartments and they close into smaller liposomes (12, 13).

In order to produce liposomes, they must be introduced into an aqueous space. lt is an accepted view that dry lipid films form randomly large multilamellar vesicles upon addition of an aqueous phase. When dry lipid films are hydrated the thin layer swell and grow into thin lipid tubules but in general do not disengage from the support. Only mechanical agitation provided by shaking, swirling, pipetting, or vortexing causes the thin lipid tubules to break and reseal the exposed hydrophobic edges resulting in the formation of liposomes. In order to produce smaller and less lamellar liposomes, additional energy has to be applied into the system. In the original procedure a thin lipid film is spread on the walls of a round-bottomed flask and shaken in excess of aqueous phase. The advantages of the hand-shaken method are that it provides an easy and quick way of preparing liposomes, and the vesicles prepared are relatively stable on storage. However, one of the disadvantages associated with this method that the liposome entrapment capacity is low (10, 11).

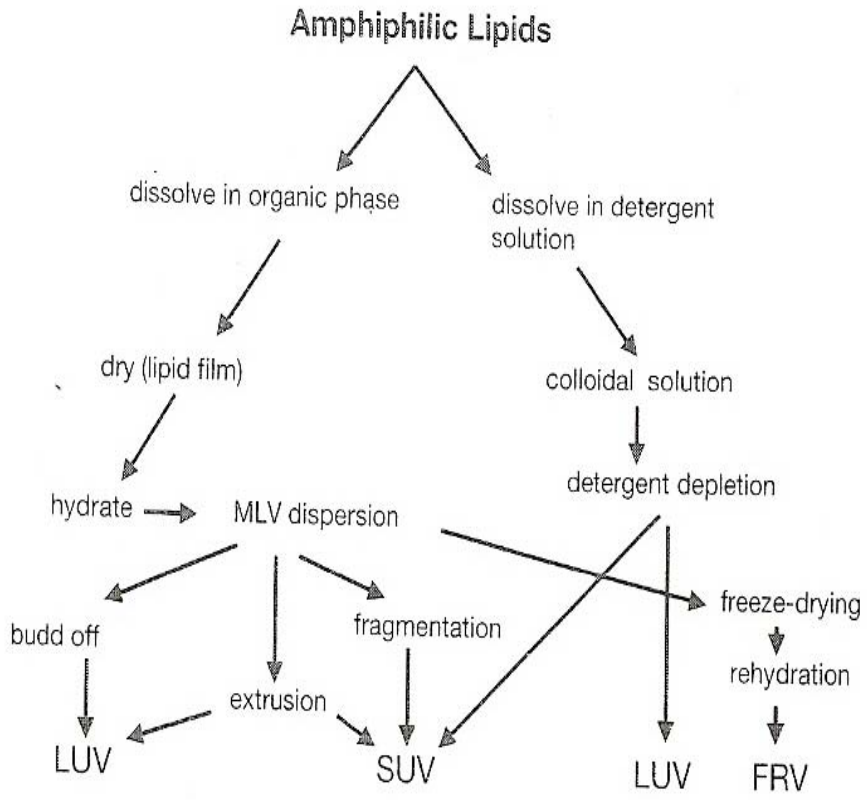


Figure 5. General classification of various liposome preparation methods. SUV , small unilamellar vesicles ; MLV , multilamellar large vesicles ; LUV , large unilamellar vesicles ; FRV freeze-drying rehydration vesicles (13).

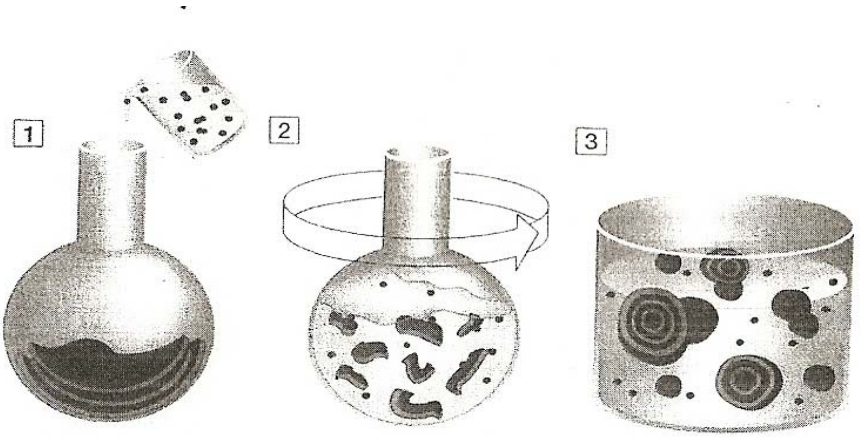


Figure 6. Schematic representation of the three stages of formation of MLVs. 1: Addition of an aqueous phase to a dry thin lipid film. 2: Swelling and peeling of the lipid film under vigorous agitation. 3: Milky suspension of equilibrated MLVs (13).

Disruption of MLV suspensions using sonic energy typically produces SUVs with diameters in the range of 15-50 nm. Sonication of various aqueous phospholipid dispersions was among the first mechanical treatments of amphiphilic lipids. The sample has not to be warmed above the phase transition temperature because of local heating and high enery input (12).

There are two techniques: Tip sonication and bath type sonication.

Tip sonication is still probably the most widely used method for the preparation of SUVs on a small scale. This method is the one with the highest enery input into lipid dispersions and can be applied directly to MLVs. The dissipation of energy at the tip results in local overheating. Therefore, the vessel must be immersed into an ice/water-bath. However, during sonication up to 1 hour more than 5% of the lipid can be destroyed. The other technique is bath sonication. Traditional laboratory bath sonicators normally do not give enough energy to liposomes to reduce vesicle sizes; only 'cup-horn' type sonicators are powerful enough for liposome preparation (9, 13).

FRVs are composed from preformed vesicles. Very high entrapment capacities, even for macromolecules, can be obtained Dehydration is best performed by freeze-drying.

REVs are mainly unilamellar, though some vesicles in each preparation may compose of several concentric bilayers, therefore composing oligolamellar vesicles. The size of REVs depends on the lipid type and its solubility in the organic solvent, the surface tension between aqueous buffer and organic solvent and on the relative amounts of water phase, organic solvent, and lipid. For REV liposomes composed of PG/PC/CHOL (1:4:5) in phosphate buffer a size range between 200–1000 nm has been described. Filtration of this preparation through a 200 nm filter gives a more uniform vesicle population of between 120-300 nm with no loss of lipid. Because of the unstable and non-homogeneous size distribution of REV liposomes anywhere between 100-1000 nm of the mean vesicle size of such preparations should be obtained for each special batch (10, 11).

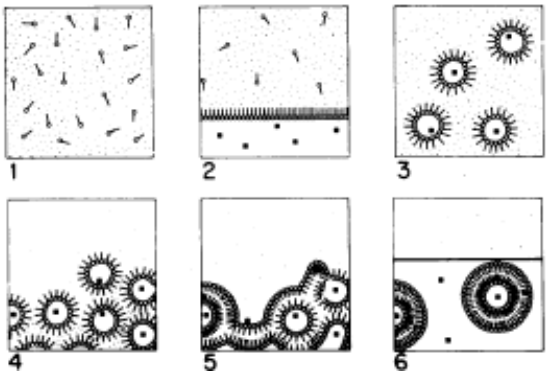


Figure 7. Diagram of the formation of REV. Dissolved lipids in appropriate solvents, lipids indicated by lollipop structures; addition of aqueous phase containing compound to be encapsulated, indicated by filled square (3).

1.1.3. APPLICATIONS of LIPOSOMES

1.1.3.1.TOPICAL APPLICATIONS of LIPOSOMES

Generally topical drug applications are safer and less strict than the intravenous applications. Thus, there are many liposomal cosmetic products on the market. First liposomal cosmetic product was produced by Christian Dior (Capture) in 1987. They are used as a replacement for creams, gels containing various herbal complexes or essential oils, moisturizing agents, antibiotics, and complex products containing recombinant proteins for wound healing (3).

One of the most important liposomal products is anti-aging creams. Otherwise, there are sunscreens, perfumes, hair products, balsams and similar products on the market. Liposomal skin care products have more than 10 % of the over $10 billion market. Table 2 shows some of these products. As in the case of topical delivery in medical applications, the workers in the field do not agree on the mechanism of action. Mostly they claim that liposomes are noninteractive, skin-nonirritating, water based matrix (without alcohols, detergents, oils and other non-natural solubilizers) for the active ingredients.

Some of these liposomes can be produced easily by mixing and homogenizing aqueous solutions with melted surfactants. These liposomes can be more stable than their natural derivatives and can easily be produced in large quantities and are very cheap (2).

Table 2. Some liposomal cosmetic formulations currently available on the market (3).

Product	**Manufacturer**	**Liposomes and key ingredients**
Capture	Cristian Dior	Liposomes in gel with ingredients
Efect du Soleil	L'Or´eal	Tanning agents in liposomes
Niosomes	Lancome (L'Or´eal)	Glyceropolyether with moisturizers
Nactosomes	Lancome (L'Or´eal)	Vitamins
Formule Liposome Gel	Payot (Ferdinand Muehlens)	Thymoxin, hyaluronic acid
Future Perfect Skin Gel	Estee Lauder TMF	TMF, vitamins E, A palmitate, cerebroside
Symphatic 2000	Biopharm GmbH	Thymus extract, vitamin A palmitate
Natipide II	Nattermann PL	Liposomal gel for do-it-yourself cosmetics
Flawless finish	Elizabeth Arden	Liquid make-up
Inovita	Pharm/Apotheke	Thymus extract, hyaluronic acid, vitamin E
Eye Perfector	Avon	Soothing cream to reduce eye irritation
Aquasome LA	Nikko Chemical Co.	Liposomes with humectant

According to the manufacturers, liposomes may transport moisturizing agent and a novel supply of lipid molecules to skin tissue better than the conventional formulations. In addition they can entrap a variety of active molecules for skin creams, creams, balsams, sun screen products and decorative cosmetics.

The topical route of liposomal drug administration can have two aspects: to generate local effects for treating skin disorders or to generate systemic effects. The science of topical dosage formulation is still attempting to design new vesicles or use drug carriers to make sure sufficient penetration or localization, prolonging release time of the drug within the specific organ although enhancing local concentration and minimizing systemic effects (15).

Several liposome and niosome formulations containing urea as their moisturizing factor were prepared by Mazda et al (15). Their *in vitro* characterizations were studied. The efficacy of these vesicles as a topical system for the delivery of water-soluble drug for the skin moisturizing was evaluated on rat skin by using urea. For this purpose ^{99m}Tc-labelled liposome and noisome formulations were used. In addition, these formulations were evaluated after application to human volunteers (15).

Liposomes are important as cosmetic products in the marketplace. Cosmetic products (such as Capture) based on liposomes actually preceded the pharmaceutical products and liposomes are now a routine ingredient in elite cosmetic products (16).

Niosomes have a structure which is similar to that of liposomes, but they are formed by synthetic nonionic surfactants. The niosomes overcome the problems which were encountered with the liposomes, namely stability and leakage of the encapsulated components. The first cosmetic product based on niosomes appeared on the market shortly after the first liposome product (by L'Oreal), and now they are used in many products, with or without encapsulated active ingredients (17).

Alain Rainberg et al evaluated Noctos (Noctosome®) formulation which was containing niosomes made with non-ionic surfactants leading to microspheres with glycopeptides in aqueus compartment of the vesicle. These niosomes penetrated and adhered well to the cells of the horny layer in both normal and dry skin (18).

Liposomes have been used in the derma-cosmetological field because of their restoring and moisturizing action. Moreover, the capability of liposomes to deliver active principles into the skin, releasing them in the deep layers, has consistently widened its action. Liposomes are currently used in antiaging, anti stretch marks, moisturizing and anticellulitis products (19).

1.1.3.2. OTHER APPLICATIONS of LIPOSOMES

Application of liposomes in basic sciences

Lipid membranes are two dimensional surfaces floating in three dimensional surfaces. In the simplest models, they can be characterized only by their flexibility which is related to their bending elasticity. A number of new theoretical concepts were developed to understand their conformational behavior. On the other hand, they can be used as a model in order to understand the topology, shape fluctuations, phase behavior, permeability, fission and fusion of biological membranes. Their aggregation leads to fractal clusters. In addition they can serve as a model to study vesiculation, including vesicle shedding and endo and exo-cytosis, of living cells (13).

Application of liposomes in medicine

Applications of liposome in pharmacology and medicine can be divided into therapeutic and diagnostic applications of liposome containing drugs or various markers, and their use as a model, tool, or reagent in the basic studies of cell interactions, recognition processes, and of the mode of action of certain substances.

Some pharmaceutical substances have a very narrow therapeutic index, meaning that the therapeutic concentration is not much lower than the toxic one. In several cases the toxicity can be diminished or the efficacy enhanced by the use of suitable drug carrier which changes the temporal and spatial distribution of the drug.

The liposome formulations for example anticancer drugs were shown to be less toxic than the free drug. Other example is anthracycline drugs. Anthracyclines prevent the growth of dividing cells by intercalating into the DNA and therefore kill quickly dividing cells. These cells are in tumors, but also in mucosa, hair, blood cells and therefore this type of drugs is very toxic. The most studied active substance is Adriamycin. Many different formulations were tried. In most cases, the toxicity was reduced about 50 %. This includes both, short term and chronic toxicities because liposome encapsulation reduces the distribution of the drug molecules towards those tissues. In some cases, such as systemic lymphoma, the effect of liposome encapsulation showed enhanced efficacy due to the sustained release effect, i.e. longer presence of therapeutic concentrations in the circulation while in several other cases the sequestration of the drug into tissues of mononuclear phagocyte system actually reduced its efficacy. Applications in man showed in general diminished toxicity, better tolerability of administration with not too encouraging efficacy. Several different formulations are in clinical studies show different results (13).

Small sized liposomes composed of lipids with long and saturated hydrocarbon chains in mixtures with CHOL were shown to deliver at the sites of inflammations so they were used for diagnostic purposes (13). Liposome formulations can also deliver anti inflammatory drugs.

Liposome formulation can be used also to deliver drugs into the lung. This is most often done by inhalation of liposome aerosols. This liposome formulation can be used either for the treatment of various lung disorders, infections, asthma, or using lungs as a drug depot for the systemic delivery.

Liposome can be applied also as a cream, gel, or tincture. In addition to subcutaneous or intramuscular drug depot these liposome formulations can be applied externally. Several researchers claim increased penetration of lipid and drug molecules into the skin. These data, as well as possible mechanisms, are, however, still a matter of controversy (3).

Oral applications of liposome are at present rather limited due to the very liposomicidal space in stomach and normally the administration of free or liposome

encapsulated drug shows generally no differences. Gastrointestinal administration, but, shows that liposome enhance the systemic bioavailability of certain water insoluble drugs and vitamins. Several designs to stabilize liposome in low pH, enzyme, and bile salts containing environments are being examined. They include liposome formed from bilayer with different chemical stability and with programmable degradation kinetics, liposome encapsulated in biodegradable gels or capsules, polymer coated liposome, and similar (13).

The parenteral, topical, inhalation can be possible routes of administration for liposomes. Current products on the market are administered parenterally (for example: Ambisome and Amphotec are Amphotericin B liposome for injection). They are sterile, non-pyrogenic lyophilized products for intravenous administration (20).

Daunorubicin citrate liposome injectable preparation is an aqueous solution of daunorubicin citrate formulated as a liposome with composed of distearoyl PC and CHOL (20).

Doxorubicin hydrochloride liposome injection consists of the drug encapsulated in stealth liposomes for parenteral administration (21).

Liposomes in bioengineering

Modern genetic engineering and gene biotechnology is based on the delivery of genetic material, i.e. fragments of DNA, into various cells and microorganisms in order to alter their genetic code and force them to produce particular proteins or polypeptides.

Nucleic acids used in gene transfer are large, with molecular weights up to million Daltons, highly charged and hydrophilic and therefore not easy to transfer across cell membranes. Additionally to classical methods, such as direct injection, phosphate precipitation and others, liposomes were tried as transfection vectors. They can deliver the encapsulated or bound nucleic acid into cells predominantly in two ways: the classical approach is to encapsulate the genetic material into liposomes and liposomes act as an endocytosis enhancer while recently the phosphate precipitation was simulated by liposomes. In these cases the nucleic acid forms a complex with several cationic liposomes and the size of the complex and its adsorption on the cell surface catalyses endocytosis or, possibly, fusion. The third, still unexplored way would be to use fusogenic liposomes or cause fusion upon adsorption of the liposome on the cell surface (3, 9).

The classical approach used predominantly LUVs made from negatively charged phosphatidylserine in order to prevent interaction with DNA molecules which may contain

up to several thousand negative charges. In some cases transfection efficiencies were improved several hundred times and plant rotoplast which are very difficult to transfect were successfully genetically altered. In the mid 80's, however, electroporation showed better results and the interest for liposome diminished markedly (14).

Recently, however, transfection was successfully performed using small uni lamellar vesicles made from positively charged lipids. First studies used cationic lipid dioleoyl-propyl-trimethylammonium (DOTMA). Later studies showed beter transfection efficiencies by using some of the commercially available cationic lipids. Better transfection efficiencies at reduced toxicity were found by using liposome containing positively charged CHOL. Many novel cationic lipids are being synthetised in order to improve transfection, especially *in vivo* (13).

1.2. GELS

Gels are transparent or translucent non-greasy semisolid formulations and they are an excellent form for several routes of administration. They are useful as liquid formulations in oral, topical, vaginal, and rectal administration. Gels can be clear formulations when all of the particles completely dissolve in the dispersing medium. But this doesn't occur in all gels, and some are therefore turbid. Clear gels are preferred by patients (25).

Gels are semisolid system in which a liquid phase is constrained within a 3-D polymeric matrix (consisting of natural or synthetic gum) having a high degree of physical or chemical cross-linking. Gels are made by using substances (called gelling agents) that undergo a high degree of cross-linking or association when hydrated and dispersed in the dispersing medium or when dissolved in the dispersing medium. This cross-linking or association of the dispersed phase will alter the viscosity of the dispersing medium. The movement of the dispersing medium is restricted by the dispersed phase and the viscosity is increased (8).

If the gel contains small discrete particles, the gel is called a two-phase system. If the gel does not appear to have discrete particles, it is called as a one-phase system. Two-phase systems are thixotropic, e.g., they are semisolid on standing but liquefy when shaken. If the particle size in a two-phase system is large, the gel is referred to as magma (8).

1.2.1. GELLING AGENTS

There are many gelling agents. Some of the common ones are acacia, alginic acid, bentonite, Carbopols® (now known as carbomers), carboxymethylcellulose, ethylcellulose, gelatin, hydroxyethylcellulose, hydroxypropyl cellulose, magnesium aluminum silicate (Veegum®), methylcellulose, poloxamers (Pluronics®), polyvinyl alcohol, sodium alginate, tragacanth, and xanthan gum. Though each gelling agent has some unique properties, there are some generalizations that can be made.

Carbomer is a generic name for a family of polymers known as Carbopol®. Carbopols® were first used in the mid 1950s. As a group, they are dry powders with high bulk densities, and form acidic aqueous solutions (pH around 3.0). They thicken at higher pHs (around 5 or 6). They will also swell in aqueous solution of that pH as much as 1000 times their original volume. Their solutions range in viscosity from 0 to 80,000 centipoise (cps) (23).

Poloxamer (Pluronics®) are copolymers of polyoxyethylene (POE) and polyoxypropylene (PPO). They can form thermoreversible gels in concentration ranging from 15 % to 50 %. This means they are liquids at cool (refrigerator) temperature, but are gels at room or body temperature (22).

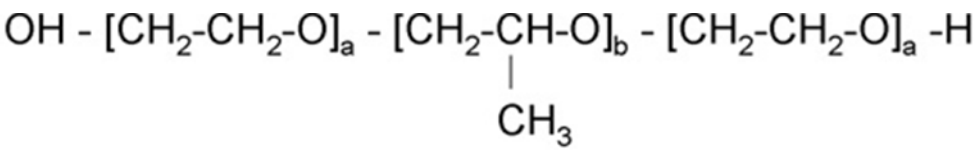
Pluronic® F-127 is often combined with a lecithin and isopropyl palmitate solution to make what is called a "PLO gel" (26).

Carbopol U 21 polymer is most versatile personal care polymer. It is a hydrophobically modified cross-linked acrylate copolymer and is designed to efficiently impart thickening, stabilizing, and suspending properties to a variety of personal care applications. The polymer incorporates patented technology, which allows it to quickly and easily self-wet.

1.2.2. PHYSICO-CHEMICAL PROPERTIES of GELS

Non-ionic surfactants are widely used in cosmetics and pharmaceuticals as solubilizers, emulsifiers, stabilizers and wetting agents. One such surfactant, poloxamer 407, is unique because its micellar rearrangement in solution will produce a change in viscosity with change in temperature.

Poloxamer 407 (PXM 407) is a nontoxic triblock copolymer comprised of polyethylene oxide (PEO) and polypropylene oxide (PPO). At sufficiently high aqueous concentrations, the poloxamer isotropic-to-gel phase transformation is realized at moderately low temperatures such as 18 ^{0}C yielding a gel a room temperature. In fact, an important characteristic of selected poloxamers is that they exhibit reversible thermal gelation. In a poloxamer-free solution, proteins are typically observed to precipitate or degrade within a relatively short period when incubated at 37 ^{0}C. In comparison, protein stability upon incubation with poloxamer 407 is reported to be significantly improved at 37 ^{0}C (26).



a: chains of poly (oxyethylene) (PEO)

b: central poly (oxypropylene) (PPO)

Figure 8. Chemical structure of PXM, a. chains of PEO, b. central PPO (25).

This PEO– PPO block copolymer has an average molecular weight of 12 500 and is commonly used in toothpaste, mouthwash and ophthalmic contact lens solution (25).

Poloxamer is a typical polymeric surfactant used in the pharmaceutical field for controlled drug release and biomedical field including burn wound covering because of its reversible thermal gelation and extremely low toxicity. However, gelation of poloxamer needs high concentration. When the gel is implanted in the body, the gel will be diluted by body fluid and finally becomes sol during implant in the body (24).

Poloxamers, especially PXM 407, have been the most commonly countered thermo sensitive material for their advantages such as easy availability, the best method for gel preparation and drug loading and good compatibility with various drug and pharmaceutical excipients (26).

Poloxamer copolymers are white, waxy granules that form clear liquids when dispersed in cold water or cooled to 0-10°C overnight (22).

Poloxamer of a series of nonionic surfactants of the polyoxypropylene-polyoxyethylene copolymer type used as surfactants, emulsifiers, stabilizers, and food additives (26).

PXM-407 contains approximately 70% PEO and 30% PPO. PXM 407 exhibits reverse thermal gelation in concentrations above 20%, and has a non-toxic nature. Due to these favorable properties, PXM- 407 has a potential use in the development of controlled drug delivery systems (27).

In many applications Carbopol U 21 polymers are more efficient than other Carbopol brand polymers. Other key benefits of Carbopol U 21 polymer include:

The unique structure of **Carbopol U 21 polymer** allows for rapid wetting and improved swelling time without the need for agitation. This processing benefit is offered without compromising the performance that the personal care industry expects from the Carbopol polymer product line.

Carbopol U 21 polymer provides high viscosity with short flow properties. This versatile product can be used when your formulations require viscosity and suspending properties. Carbopol U 21 polymer performs effectively across a broad pH range, making it a versatile ingredient for many applications.

Carbopol U 21 polymer exhibits good clarity in gel formulations, along with providing a smooth, aesthetically pleasing gel quality. In creams and lotions, it helps to create emulsions with superior skin feel and less tack (28).

1.3. LIPOGELOSOMES

Liposomes having an internal gelatinized nucleus, which are in suspension in aqueous medium and which contain low concentrations of gelatinizing substances have been patented by Hauton, who has termed them lipogelosomes. He has, in particular, developed a process for manufacturing such liposomes or lipogelosomes, which differ from conventional liposomes in that the encapsulated aqueous phase is present in semi-solid gel form and not in liquid form, and this prevents the liposomes from fusing when collisions occur. These lipogelosomes are produced entirely from natural substances, thereby minimizing the risk of intolerance. The lipogelosome consist of one bilayer interfacial phase, in the case of the unilamellar lipogelosomes, or of a multiplicity of bilayer interfacial phases, which are superimposed concentrically, in the case of the multilamellar lipogelosomes, and of a gelatinized encapsulated internal aqueous polar phase in which the gelatinized substance, which may or may not be polymerizable, is selected from polysaccharides, polypeptides or

polyacrylamides; for example, the non-polymerizable gelatinizable substance is selected from gelatin, agarose or carrageenans, and the polymerizable gelatinizable substance is selected from polyacrylamide gels. These lipogelosome possess a stability which is significantly increased as compared with the liposomes of the prior art, particularly because of the absence of interparticulate fusion during collisions (29).

Lipogelosome formulations have been prepared by the incorporation of liposomes in structured vehicles, i.e. gels. Dexamethasone sodium phosphate liposomes were mixed with 1 % Carbopol 940 gels in (1:1; w/w) ratio in order to obtain lipogelosomes having good bioadhesive properties. Good results have been reported with these lipogelosomes in the treatment of apthous stomatitis (30, 31).

Similar results have been mentioned by Türker et al with diclofenac sodium lipogelosomes carried out with rats in the treatment of inflammation by applying DPPC:SA:CHOL (7:1:2) liposomes mixed with 1 % Carbopol 940 gels in (1:1; w/w) ratio (30, 32).

1.4. SKIN

1.4.1. SKIN ANATOMY

A basic understanding of skin anatomy is important. Because, cosmeceuticals and dermacosmeceuticals are exert their functions on the skin.

Below are a few of the basic components of skin followed by a brief description their functions (33).

Stratum Corneum - This is the dead skin layer that is visible when it was looked at the skin and it is one of the layers of the epidermis. It functions to protect the living cells beneath by providing a hard barrier between the outside world and the delicate cells inside. The stratum corneum is useful for diagnosis because in some conditions the stratum corneum will become thinner than normal.

Epidermis - The epidermis covers the whole body and contains stratum corneum, stratum lucidum, stratum germinativum, stratum spinosum, stratum basale from the outer surface of the skin to the in layers, respectively. Its function is to protect the body. It produces cells that will eventually become stratum corneum cells. It contains sensory nerves specifically small diameter sensitive temperature fibers.

Dermis - The dermis is the next layer under the epidermis. The dermis contains all of the other sub-epidermal structures mentioned below. Dermis is characterized by loose, ribbon-like cells that hold dermal structures in place and serves to contain fluids (34).

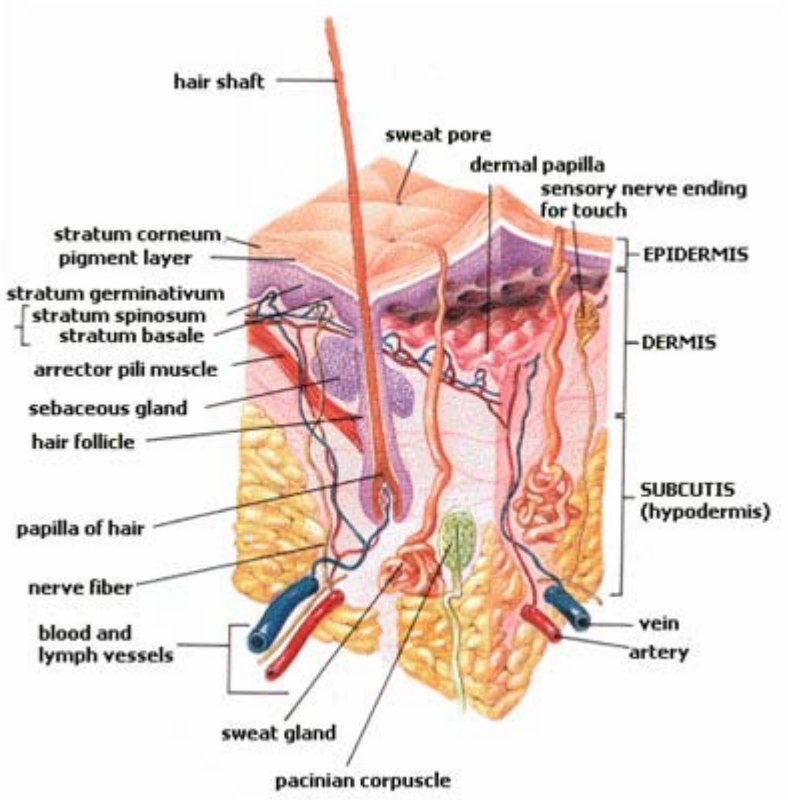


Figure 9. Skin Anatomy (34).

1.5. SODIUM HYALURONATE

Sodium hyaluronate (Na-HA) is a linear polysaccharide and a naturally produced highly viscous glycosaminoglycan. It is made from alternating units of a disaccharide of D-glucronic acid and 2-acetamido-D-glucose (N-acetyl-D-glucosamine) as a repeating unit linked at the β-(1-3)- and β -(1-4)-positions. It plays an important role in providing mechanical and transport function in body tissues (35). The chemical structure of Na-HA was determined in the 1950s in the laboratory of Karl Meyer. Na-HA is a polymer of disaccharides, themselves composed of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds. Na-HA can be 25,000 disaccharide repeats in length. Polymers of Na-HA can range in size from 5,000 to 20,000,000 Da in vivo. The average molecular weight in human synovial fluid is 3−4 million Da and Na-HA purified from human umbilical cord is 3,140,000 Da (36).

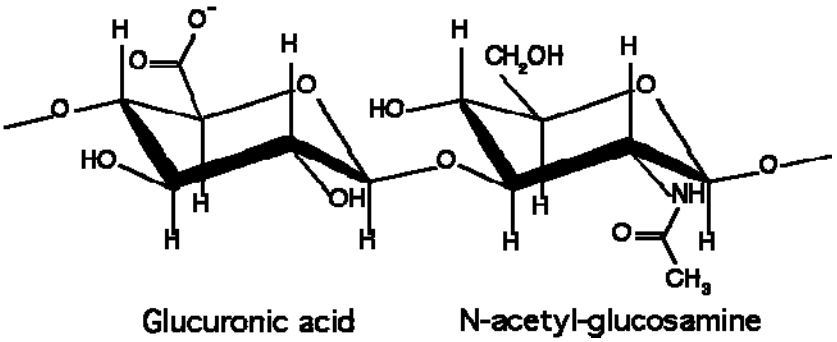


Figure 10. The Chemical Structure of Na-HA (37).

The average of 70 kg (154 lbs) person has roughly 15 grams of hyaluronan in their body, one-third of which is turned over (degraded and synthesised) every day. Na-HA is an anionic, non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues (38).

In all tissues, Na-HA is an essential part of the extracellular matrix. Very high concentrations are found in tissues such as the umbilical cord (4 $mg.g^{-1}$), synovial fluid (3-4 $mg.mL^{-1}$), and vitreous humor (0.1-0.4 $mg.g^{-1}$). The mean concentration of Na-HA is 200 $mg.\ kg^{-1}$ (0.02 %). A human body weighing approximately 60 kg contains about 12 g of Na-HA (38).

Table 3. The mean concentration of Na-HA in human body (38).

Tissue	**Sodium hyaluronate ($mg.mL^{-1}$)**
Synovial fluid	3500
Vitreus humor	200
Extracellular space	**Sodium hyaluronate ($mg.kg^{1}$)**
Cartilage	1200
Skin	200
Lung	150
Others	**Sodium hyaluronate ($mg.L^{-1}$)**
Serum	0.05
Intracellular	Absent
All tissues	200

Although a higher Na-HA concentration is found in connective tissues, the greater part of it (56.7 %) is found in the skin. In its normal state in tissues, the Na-HA is present as a free polymer, although in some tissues such as cartilage, it is linked to different structural glycoproteins (proteoglycans) or in other tissues to specific cell receptors (38).

Na-HA serves important biological functions in bacteria and higher animals including humans. Naturally occurring Na-HA may be found in the tissues of higher animals, in particular as intercellular space filler. It is found in greatest concentrations in the vitreous humour of the eye and in the synovial fluid of articular joints (39). In gram positive streptococci it appears as a mucoid capsule surrounding the bacterium.

Since its discovery in human tissue, Na-HA and its derivatives has been largely studied and applied in the biomedical arena. The appeal of this polymer has been accentuated by its high level of biocompatility. It has been used in viscosurgery to allow surgeons to safely create space between tissues. As a microcapsule it can be used for targeted drug delivery. Viscosurgical implants are constructed from Na-HA (38). Its viscoelastic character has been used to supplement the lubrication in arthritic joints. Finally, because of its high water retention capacity, this EPS (extracellular polysaccharide) also occupies a niche in the lucrative cosmetics market.

Na-HA has been traditionally extracted from rooster combs and bovine vitreous humor. However, it is difficult to isolate high molecular weight Na-HA economically from these sources; because it forms a complex with proteoglycans found in animal tissues and the extraction and purification processes result in a significant reduction in molecular weight of the polymer (39). In addition, the use of animal-derived biochemicals for human and animal therapeutics is not currently viewed as socially acceptable.

Therefore, industry has focused on bacterial fermentation processes with the hope of obtaining a commercially available sodium hyaluronate. With bacterial fermentation, extracellular polysaccharide is added to a growth medium, and control of the polymer characteristics and product yields can be manipulated. Large quantities of Na-HA can be produced in this manner.

1.5.1. DISCOVERY

Na-HA, a high molecular mass polysaccharide, was discovered by Meyer and Palmer in 1934 in the vitreous humor of cattle eyes (41). In 1932, Meyer and his assistant John Palmer isolated a novel, high molecular weight polysaccharide and reported that it was composed of "an uronic acid, an amino sugar, and possibly a pentose." (The last is incorrect.) They proposed "for convenience, the name sodium hyaluronate, from hyaloid (vitreous) uronic acid." Nearly 25 years of work were required to establish the structure of the repeating disaccharide that is the basic unit of the hyaluranan polymer, namely glucuronate 1, 3-N-acetylglucosamine-1, 4.

Na-HA is one member of a family of glycosaminoglycans that includes chondroitin/dermatan sulfate, keratan sulfate, and heparin/heparan sulfate each with a characteristic disaccharide-repeat structure of an amino sugar, either glucosamine or galactosamine, plus a negatively charged sugar, a carboxylate and/or a sulfate. The polymers are found as cell surface molecules and in the extracellular matrix. Glycosaminoglycans, with the exception of hyaluronan, are covalently bound to proteins to form proteoglycans. These ubiquitous and structurally diverse macromolecules are found as cell surface molecules and in the extracellular matrix (42).

Along with the changes in the intraocular lens, the discovery of Na-HA as a space maintaining and cushioning agent to protect the corneal endothelium has influenced and increased not only the safety of the cataract surgery, but also has opened the new field of viscosurgery (43).

Na-HA can be extracted from animal tissues or harvested as a product of bacterial fermentation. Nowadays, Na-HA can be produced in commercial quantities by bioprocess technology (44).

1.5.2. PHARMACOKINETICS of SODIUM HYALURONATE

Na-HA is a linear polysaccharide formed from disaccharide units containing N-acetylglucosamine and glucuronic acid. Its molecular weight is usually in the order of 10^6–10^7. Na-HA is reported to have a radical scavenger capacity. Therefore, it was incorporated into semisolid formulations in order to give UV-protection. Hence, determination of the release profile of Na-HA from semisolid formulations is important to predict its topical availability. In the literature, there are no publications up to date regarding with the

biopharmaceutical characterization of semisolid formulations containing drugs or cosmetics with higher molecular mass such as Na-HA (45).

The topical availability of the Na-HA varied from different vehicle systems. So does the availability of Na-HA and Na-HA-D with different molecular mass from the same vehicle system. The affinity of Na-HA to the O/W cream is lower than both to the amphiphilic cream (AC) and to the W/O cream. In addition, the topical availability of Na-HA-D (22 kDa) was found to be higher than the availability of Na-HA (1200 kDa). It is, therefore, more useful to use enzymatically digested Na-HA-D instead of intact Na-HA and hydrophilic creams (O/W creams) as vehicle systems.

1.5.3. SIDE-EFFECTS of Na-HA

Cross-linked or stabilized Na-HA is nowadays the most used resorbable filler. Since its first commercialization in 1996, many molecules have been marketed. These differ in the cross-linking or stabilization process, necessary to lengthen their degradation by hyaluronidase. The European laws classified it as a class III medical device. Because Na-HA is injectable, the medical procedure for its use requires a premarketing controlled clinical trial to attest the product's safety and efficacy with respect to a reference gold standard. The trial should be certified by a specific authorized agency. The producer should also shoulder the burden of the product's tracking and of device supervision regarding the potential postimplant adverse and side effects, which should always be notified to the Ministry of Health. The reference gold standard in the category of resorbable fillers is bovine collagen. A particular Na-HA , the nonanimal stabilized hyaluronic acid (NASHA), presently cited in most scientific publications, has been shown to be more durable and much safer compared to bovine collagen. The ideal filler, in fact, should be nontoxic, nonmutagenic, non-immunogenic, biocompatible, not migrating from the implant site, naturally integrating within tissues, easy to use, and with an acceptable duration with respect to costs and expectations (38). No serious side effects have been reported while administering Na-HA (46). For this reason, in the treatment of hyperpigmentation and scars, Na-HA is often preferred, even if the wrinkles do not completely disappear (38).

1.5.4. HYALURONIC ACID DERIVATIVES

The derivatization of HA and the synthesis of drug–HA bioconjugates offer several advantages over simple HA –drug admixtures (47). First, chemical modification allows the physicochemical properties of HA to be tailored according to the desired applications, and this can have a significant impact on the natural turn-over and clearance of the HA derivative. For example, HA has a half-life of 0.5–3 days in tissues, but is rapidly cleared from plasma by receptor- mediated uptake by liver endothelial cells with a half-life of several minutes. Second, the HA –drug bioconjugates may exhibit improved water solubility relative to the parent drug. Third, HA –drug hydrogels may be used to localize a slow-release formulation at a specific site in the body. Finally, the high-affinity HA receptors overexpressed in metastatic cells may provide important targeting opportunities for cell-selective delivery of anti-cancer agents. The receptor on rat liver endothelial cells has been shown to be only partially selective for HA, as HA uptake can be inhibited by co-administration of chondroitin sulfate; by contrast, rat colon cancer cells bear receptors that specifically recognize HA (48).

Table 4 shows a summary of companies and their HA or HA -derived products on the market or in clinical development (49).

Table 4. Companies with HA or HA -derived products on the market or in research (38).

Company	Product
Amgen	Blend of HA with Interleukin-1 antagonist
Anika	HA products for ophthalmic and veterinary use
BioCoat	HA surface coating
Biomatrix	HA derivatives for viscosupplementation
Collobrative laboraties	HA products in the cosmetic area: liposomes (Micasomes)
Fidia	HA ester derivatives for drug delivery
Kaken	HA products for osteoarthritis treatment
Seikagaku Corp.	HA enzyme conjugates

1.5.5. USE of Na-HA in COSMETOLOGY and DERMATOLOGY

Na-HA is a common ingredient in skin-care products. In 2003, the FDA approved hyaluronan injections for filling soft tissue defects such as facial wrinkles. Restylane is a common trade name for the product. Na-HA injections temporarily smooth wrinkles by adding volume under the skin, with effects typically lasting for six months (49).

Restylane™ (Q-med, Uppsala, Sweden) is formulated via fermentation, in the presence of sugar, in bacterial cultures of equine streptococci. Then the product is precipitated in alcohol, filtered, and dried. Next, the compound is cross-linked using a butanediol diglycidyl ether (BDDE) and then sterilized by heat. After washing and heating the modified HA, a gel-like material forms. The resulting product is a highly cross-linked gel stiffer and harder than Hylaform (50).

It is then broken down into smaller pieces in a similar fashion to Hylaform. The smallest particles are made available as Restylane Fine Line™, which is indicated for the upper or papillary dermis and, therefore, appropriate for treating small lines (eg, crow's feet and vertical lip wrinkles). The medium-sized particles are packaged as Restylane™, which is intended for the mid-dermis and is the one in the product line that received FDA approval in December 2003. The largest particles are sold as Restylane-Perlane™, which is indicated for treatment of the deep dermis. In the author's experience, Restylane results in more inflammation than that seen with Hylaform, Zyplast, and Cosmoplast. Injecting slowly may decrease the inflammation seen and some dermatologists have stated that they use systemic prednisone in patients to minimize swelling. However, in a direct comparison between Restylane and Zyplast for treatment of nasolabial folds, a randomized double-blind multicenter study found there was an increased incidence of moderate or severe bruising, redness, swelling, pain, tenderness and itching after the first treatment with Restylane compared with control (Zyplast). The FDA panel, which met in November 2003, felt that although the study did not show that Restylane was superior to Zyplast, it was recommended for approval because it was shown to be safe. The FDA went on to approve Restylane for cosmetic use in December 2003 (51).

Juvederm is abacterial Na-HA injectable filler, similar to Restylane, but differing slightly in terms of effect and longevity. It is used for lip augmentation, reduction of folds

and wrinkles and removal of scars. The effects of Juvederm treatments are also temporary, and costs are similar to those of Restylane.

The presence of HA in epithelial tissue has been shown to promote keratinocyte proliferation and increase the presence of retinoic acid, effecting skin hydration. Na-HA's interaction with CD44 drives collagen synthesis and normal skin function. Presence in the extracellular matrix of basal keratinocytes, HA is critical to the structural integrity of the dermal collagen matrix. These benefits make HA a very effective topical humectant; however, results may only be sustained as part of an ongoing treatment program (51).

A bacterial fermentation process similar to the one used in the manufacture of Restylane is used to produce the Na- HA in Juvederm™. Heat destroys the streptococci and it is eliminated by filtration. BDDE, the cross-linking agent used in Restylane, is used to cross-link the strands of HA in Juvederm. Juvederm differs from Hylaform and Restylane because, instead of being a gel broken into smaller particles, it is a homogenous gel. The homogenous gel is thought to be more biocompatible because it causes less friction with dermal tissues on injection, resulting in less inflammation. Although the author has seen minimal inflammation with this product, comparative clinical studies have not compared the inflammation seen with Juvederm to that of Hylaform and Restylane. The homogenous cell has less surface area exposed than the nonhomogenous products. This is thought to decrease the activity of hyaluronidase, resulting in a longer duration. The Juvederm line of products contains several variations that differ by the amount of HA per mL and the amount of cross-linking of the product, and the regularity of the cross-linking of the product. Currently, Juvederm 18, Juvederm 24, Juvederm 24 HV, Juvederm 30, and Juvederm 30 HV are available. The differences between these products are out of the scope of this article. At this time, none of the Juvederm products are approved by the FDA (52, 53).

2. EXPERIMENTAL

2.1. MATERIALS and METHODS

2.1.1. MATERIALS

2.1.1.1. CHEMICALS

Chemical Name	Company
Phospholipon 100H (PL 100H)	LIPOID
Dimrystoiylphosphatidylcholine (DMPC)	LIPOID
Dipalmitoylphosphatidyl choline (DPPC)	LIPOID
Phospholipon 20 (PL 20)	LIPOID
Phospholipon 30 (PL 30)	LIPOID
Phospholipon 85G (PL 85G)	LIPOID
1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP)	LIPOID
1,2-dimyristoyl-sn-glycero-3-phosphatidylglycerol (DMPG)	LIPOID
Stearylamine (SA)	SIGMA
Cholesterol (CHOL)	SIGMA
Dicetylphosphate (DCP)	SIGMA
Tris Buffer	SIGMA
Triton X 100	SIGMA
Sodium hyaluronate (Na-HA)	BIOPHILL

(All other chemicals were of analytical grade).

2.1.1.2.EQUIPMENT

Equipment Name and Model	Company
Zeta- Sizer	MALVERN ZS-501
Differential scanning calorimeter (DSC)	PHOENIX DSC 204
Scanning electron microscope (SEM)	LEO SUPRA 35 VP
Atomic force microscope (AFM)	SHIMADZU
Polarized light microscope (PLM)	NIKON
UV Spectrophotometer	AGILENT 8453
Vortex	HEIDOLPH
Lyophilizator L 2-4	CHRIST
Vacuum incubator	THERMO SCIENTIFIC
Rotavapor	HEIDOLPH
Sonicator	SONOREX
Water bath	BANDELIN
Dissolution test device USP 4	SOTAX SMART CE 7
Multi Probe Adapter Systems	COURAGE-KHAZAKA MPA5
Skin Visiometer	COURAGE-KHAZAKA SV 600
USP 4 Dissolution Apparatus	Sotax™ CE7
Lab-Scale	OHIUS
FT-IR	THERMO-FISCHER

2.1.2. METHODS

In this chapter, the details of methods and process used have been given.

2.1.2.1. PHYSICOCHEMICAL PROPERTIES of Na-HA

2.1.2.1.1. STANDARDIZATION of Na-HA

2.1.2.1.1.1. UV SPECTRUM of Na-HA

UV spectra were measured in two different solutions of Na-HA.

a) First of all, for the analysis of unencapsulated free Na-HA, UV spectra were measured in 10 mM Tris buffer (pH: 5.5) between 200-600 nm.
b) Secondly, for the determination of encapsulated Na-HA in liposomes, UV spectra were obtained in 2.5 % (w/w) Triton X-100 containing 10 mM Tris buffer.

2.1.2.1.1.2. FT-IR SPECTRUM of Na-HA

FT-IR spectra were determined in between 400-4000 cm^{-1} wavelength by using potassium bromure (KBr) discs.

2.1.2.1.1.3.DSC STUDIES of Na-HA

DSC of Na-HA was obtained by Differential Scanning Calorimeter.

2.1.2.1.1.4. SEM STUDIES of Na-HA

SEM images of Na-HA were also studied. Working distance (WD) was set to 9 mm. SE2 detector was occupied for the measurement. Electron high tension (EHT) of the instrument was 2.00 kV. Magnifications were 2.00, 5.00, 10.00, 15.00 and 50.00 KX, respectively.

2.1.2.1.1.5. X-RD STUDIES of Na-HA

X-RD method was used to determine the amorphous structure of Na-HA.

2.1.2.1.1.6. PHYSICAL STABILITY of Na-HA in DIFFERENT CONDITIONS

Stability of Na-HA in different conditions was measured by FT-IR. 1 % Na-HA containing Tris buffer solutions were freeze dried at – 80 ^{0}C, and measured by FT-IR. Then same solutions were heated up to 80 ^{0}C, freeze dried at – 80 ^{0}C, and then measured by FT-

IR. As the further step, % 1 Na-HA solutions were vortex for 1 min. Then freeze dried at – 80 ^{0}C, and measured by FT-IR. Then vortex solutions were sonicated for 15, 30, 45 and 60 minutes and measured by FT-IR, respectively.

2.1.2.1.2. CALIBRATION STUDIES of Na-HA

Two different calibration curves of Na-HA were prepared.

a) To determine unencapsulated free Na-HA, calibration curve was obtained with 10 mM Tris buffer solution (pH: 5.5),
b) Second calibration curve was prepared with using 2.5 % (w/w) Triton X-100 containing 10 mM Tris buffer (pH: 5.5).

2.1.2.1.3. SOLUBILITY STUDIES of Na-HA

10 mM Tris buffer solutions containing of 1 mg.ml^{-1}, 2.5mg.ml^{-1}, 5mg.ml^{-1}, 10 mg.ml^{-1}, 100 mg.ml^{-1} Na-HA were used with solubility studies. All solutions were kept from light. Also, these solutions were exposed to room temperature (25 ^{0}C) and 50 ^{0}C in water bath. Then, pH and absorbances of these solutions were determined.

2.1.2.2. COMPONENTS of LIPOSOMES

2.1.2.2.1. STUDIES on PHYSICOCHEMICAL CHARACTERISTICS of LIPOSOME COMPONENTS

2.1.2.2.1.1. PHOSPHOLIPIDS

2.1.2.2.1.1.1. PURITY of PHOSPHOLIPIDS

Phosphatidyl choline is the major component of phospholipids. According to the analysis certificates, percentage of phosphatiylcholine is i.e, >99 %, >99 % and >99 % for PL 100 H, DMPC and DPPC, respectively. Additionally, they do not contain lyso phosphatidyl choline according to analysis certificates.

2.1.2.2.1.1.2. IDENTIFICATION of PHOSPHOLIPIDS

For the identification of phospholipids, FT-IR method was used in between 400-4000 cm-1 wavelength by using KBr discs. Moreover, DSC was used as an analytical technique to examine the thermodynamic properties of phospholipids.

2.1.2.2.1.2.CHARGE INDUCERS

2.1.2.2.1.2.1. IDENTIFICATION of SA

Identification of SA was performed with using FT-IR method in terms of the wavelength in between 400-4000 cm^{-1} by using KBr discs.

2.1.2.2.1.2.2. IDENTIFICATION of DCP

For the identification of DCP was also determined by using FT-IR method in between 400-4000 cm-1 wavelength and KBr discs were used.

2.1.2.2.1.3. STABILIZING AGENT

2.1.2.2.1.3.1. IDENTIFICATION of CHOL

Identification for CHOL was obtained by FT-IR method studied in wavelength 400 to 4000 cm^{-1} with using KBr discs. Additionally, DSC method was used for identification of CHOL.

2.1.2.2.2. PREPARATION of LIPOSOME FORMULATIONS

Liposome dispersions were prepared by film technique (54). Briefly, liposome was prepared by dissolving the 40 μmol mL^{-1} of phospholipids in 30 mL chloroform in a round-bottom flask. The chloroform was removed using a rotary evaporator under reduced pressure to form a thin film over the wall of the flask. The dried film was then hydrated over a water bath with 10 mM Tris (pH 5.5) containing 10 mg mL^{-1} Na-HA. Free Na-HA was removed by centrifugation three times at 17,500 rpm for 45 min for each of them. The pellets obtained after the centrifugations were treated with detergent (Triton X-100 in 10 mM Tris at pH 5.5) solution and then final clear solution was determine spectrometry method for drug content at λ_{max}= 270 nm. Encapsulation efficiency was calculated as a fraction of Na-HA in the liposome pellets expressed as a percentage of total drug content (15). 47 different liposome formulations were prepared and further studies were carried out the last 16 different liposome formulations (L32-L47). All formulations and details were shown in Table 5.

Table 5. Liposome formulations and their compositions.

Code	Composition	pH	Molar Ratio	Observations	Result
L 1	PL 20:CHOL	5.5	7:2	Phase separation	Eliminated
L 2	PL 20:CHOL+1%Na-HA	5.5	7:2	Phase separation	Eliminated
L 3	PL 20:CHOL	7.4	7:2	Phase separation	Eliminated
L 4	PL 20:CHOL+1%Na-HA	7.4	7:2	Phase separation	Eliminated
L 5	PL 30:CHOL	5.5	7:2	Phase separation	Eliminated
L 6	PL 30:CHOL+1%Na-HA	5.5	7:2	Phase separation	Eliminated
L 7	PL 30:CHOL	7.4	7:2	Phase separation	Eliminated
L 8	PL 30:CHOL+1%Na-HA	7.4	7:2	Phase separation	Eliminated
L 9	PL 85G:CHOL	5.5	7:2	Phase separation	Eliminated
L 10	PL 85G:CHOL+1%Na-HA	5.5	7:2	Phase separation	Eliminated
L 11	PL 85G:CHOL	7.4	7:2	Phase separation	Eliminated
L 12	PL 85G:CHOL+1%Na-HA	7.4	7:2	Phase separation	Eliminated
L 13	PL 20:CHOL:SA	5.5	7:1:2	Poly dispersed particles	Eliminated
L 14	PL 20:CHOL+1%Na-HA	5.5	7:1:2	Poly dispersed particles	Eliminated
L 15	PL 30:CHOL:SA	5.5	7:1:2	Poly dispersed particles	Eliminated
L 16	PL 30:CHOL:SA+1%Na-HA	5.5	7:1:2	Poly dispersed particles	Eliminated
L 17	PL 85G:CHOL:SA	5.5	7:1:2	Poly dispersed particles	Eliminated

L 18	PL 85G:CHOL:SA+1%Na-HA	5.5	7:1:2	Poly dispersed particles	Eliminated
L 19	DMPC:DMPG:CHOL	5.5	3,5:3,5:3	Poly dispersed particles	Eliminated
L 20	DMPC:DMPG:CHOL+1%Na-HA	5.5	3,5:3,5:3	Poly dispersed particles	Eliminated
L 21	DOTAP:CHOL	5.5	7:2	Poly dispersed particles	Eliminated
L 22	DOTAP:CHOL+1%Na-HA	5.5	7:2	Poly dispersed particles	Eliminated
L 23	DOTAP:SA:CHOL	5.5	7:1:2	Poly dispersed particles	Eliminated
L 24	DPPC:CHOL	5.5	7:2	Poly dispersed particles	Eliminated
L 25	DPPC:CHOL+ 1%Na-HA	5.5	7:2	Poly dispersed particles	Eliminated
L 26	DMPC:DMPG:SA:CHOL	5.5	3,5:3,5:1:2	Poly dispersed particles	Eliminated
L 27	DMPC:DMPG:SA:CHOL+1%Na-HA	5.5	3,5:3,5:1:2	Poly dispersed particles	Eliminated
L 28	PL 100H: CH	5.5	7:2	Heterogeneous	Eliminated
L 29	PL 100H: CH+1%Na-HA	5.5	7:2	Heterogeneous	Eliminated
L 30	PL 100H:SA:CH	5.5	7:2:2	Heterogeneous	Eliminated
L 31	PL 100H:SA:CH+1%Na-HA	5.5	7:2:2	Heterogeneous	Eliminated
L 32	DMPC:DMPG:CHOL	5.5	9:1:10	Milky dispersion	Selected
L 33	DMPC:DMPG:CHOL+1%Na-HA	5.5	9:1:10	Milky dispersion	Selected
L 34	DMPC:DMPG:CHOL	5.5	9:1:25	Milky dispersion	Selected
L 35	DMPC:DMPG:CHOL+1%Na-HA	5.5	9:1:25	Milky dispersion	Selected
L 36	PL 100H:SA:CHOL	5.5	7:1:2	Milky dispersion	Selected
L 37	PL 100H:SA:CHOL+1%Na-HA	5.5	7:1:2	Milky dispersion	Selected

L 38	PL 100H:SA:CHOL	5.5	10:1:4	Milky dispersion	Selected
L 39	PL 100H:SA:CHOL+1%Na-HA	5.5	10:1:4	Milky dispersion	Selected
L 40	PL 100H:DCP:CHOL	5.5	7:1:2	Milky dispersion	Selected
L 41	PL 100H:DCP:CHOL+1%Na-HA	5.5	7:1:2	Milky dispersion	Selected
L 42	PL 100H:DCP:CHOL	5.5	10:1:4	Milky dispersion	Selected
L 43	PL 100H:DCP:CHOL+1%Na-HA	5.5	10:1:4	Milky dispersion	Selected
L 44	DPPC:SA:CHOL	5.5	5:2:3	Milky dispersion	Selected
L 45	DPPC:SA:CHOL+1%Na-HA	5.5	5:2:3	Milky dispersion	Selected
L 46	DPPC:DCP:CHOL	5.5	7:1:2	Milky dispersion	Selected
L 47	DPPC:DCP:CHOL+1%Na-HA	5.5	7:1:2	Milky dispersion	Selected

2.1.2.2.2.1. MEAN PARTICLE SIZE, SIZE DISTRIBUTION, POLYDISPERSITY INDEX and ZETA POTENTIAL STUDIES on LIPOSOMES

0.1 ml of liposomes dispersions were diluted with 0.9 ml of 10 mM Tris (pH 5.5) buffer after liposome preparation. The size distributions of the liposomes were measured by dynamic light scattering (DLS) using Particle Sizer. The mean particle size and size distribution and polydispersity index results were obtained as the average of 6 experiments.

Zeta (ζ) potential was measured by using a Zetasizer and each result was the mean of 10 measurements. All the measurements were performed at 25°C and at an angle of 90°.

2.1.2.2.2.2. DETERMINATION of PHOSPHOLIPID AMOUNT in LIPOSOME DISPERSIONS

Liposomal phospholipid content was determined by the colorimetric method of Rouser et al (55). Briefly, lipid samples are transferred into clean glass tubes and the solvent is completely evaporated. 0.65 ml perchloric acid is added and placed the tubes in the heated block for about 30 min or until the yellow color has disappeared. When cool, the tubes 3.3

ml water, 0.5 ml of molybdate solution and then 0.5 ml of ascorbic acid solution are added. They are agitated on a vortex after each addition. The tubes are placed in a boiling water bath for 5 min. The absorbance of cool sample (including the standards) was read at 800 nm.

2.1.2.2.2.3. CALCULATION of PHOSPHOLIPID YIELD

Phospholipid yield was calculated with respect to the known liposome value in terms of weight as gram.

2.1.2.2.2.4. DETERMINATION of Na-HA in LIPOSOME FORMULATIONS

2.1.2.2.2.4.1. DETERMINATION of FREE Na-HA in LIPOSOME FORMULATIONS

After 2.1.2.4, active ingredient determination was measured for three different supernatant. For this purpose, 1 ml dispersion was put in a tube and 1 ml diacetylmonooxime and 4 ml 70 % perchloric acid:sulphuric acid:water (22:60:240) mixture were added. Tubes were kept in boiling water for 30 minutes. Later on the tubes were cooled in the water bath. Absorbances of the mixtures were analyzed at λ =270 nm.

2.1.2.2.2.4.2. DETERMINATION of ENCAPSULATED Na-HA in LIPOSOMES

Subsequent 2.1.2.4, pellets were vortexed for 5 minutes in vortex. Then 2.5 % Triton X-100 containing solution was added into the liposome dispersions and vortexed for 5 minutes again. Afterwards, 1 ml dispersion was put in a tube and 1 ml diacetylmonooxime and 4 ml 70 % perchloric acid:sulphuric acid:water (22:60:240) mixture were added. Tubes were kept in boiling water for 30 minutes. Later on the tubes were cooled in the water bath. The measurement of the absorbances were studied at λ =270 nm for each tubes.

2.1.2.2.2.5. PLM STUDIES of LIPOSOME FORMULATIONS

The determination of vesicle types of liposome was performed by using PLM. Magnification of the microscope was 100 X the each liposome dispersion.

2.1.2.2.2.6. SEM STUDIES of LIPOSOME FORMULATI ONS

SEM images of liposomes were examined. WD was 9 mm. SE2 detector was used. EHT was 2.00 kV. Magnifications were 2.00, 5.00, 10.00, 15.00 and 50.00 KX, respectively.

2.1.2.2.2.7. AFM STUDIES of LIPOSOME FORMULATIONS

AFM studies were performed in contact mode with a commercial AFM using standard silicon nitride (Si_3N_4) tips and typical force of 5–20 nN. A drop (5–10 μl) of each of liposome dispersions was deposited on freshly cleaved mica substrates, dried in air and visualised under AFM at room temperature (56).

2.1.2.2.2.8. PHYSICAL STABILITY STUDIES on LIPOSOME FORMULATIONS

As mentioned in 2.1.2.4, ultracentrifugation was applied for three times×45 minutes at 10395 g to the liposome coded L 37. Free Na-HA and encapsulated Na-HA amounts were calculated. Afterwards ultracentrifugation, 1 ml 10 mM Tris buffer (pH 5.5) was added into liposome. Liposome was kept at both room temperature (25 ºC) and refrigerator temperature (4 ºC). Dispersions were completed to 10 ml with 10mM Tris buffer (pH 5.5) and after ultracentrifugation un encapsulated Na-HA amount was calculated at the end of 1, 3, 7, 14, 21, 28 day and 2, 3 month, respectively.

2.1.2.3. GELS and LIPOGELOSOME FORMULATIONS

In this research, 4 different types of polymers were examined. Lipogelosome formulations were prepared by incorporation of liposome in structured vehicles. C 940 and U 21 at the concentration of 0.5 % in distilled water and Na-OH at the concentration of 0.5% were used as gel forming agents because of their good bioadhesive properties. PXM derivatives forming gels were prepared on a weight basis using the cold method (57). An appropriate amount of PXM 188 and PXM 407 to yield 10% and 20% gels was slowly added to cold liposome dispersions (4°C) by maintaining constant stirring. The dispersion was kept in the refrigerator until a clear solution was formed over night (58).

For the preparation of lipogelosomes, gel formulations of C-940 and NaOH and liposomal Na-HA were mixed in (1:1) ratio on weight basis. The pH values were measured by using pH-meter. All the pH values of formulation were adjusted to pH 5.5 (28).

16 different gel formulations were designed. All formulations and details were shown at Table 6.

Table 6. Code and compositions of gel and lipogelosome formulations.

Code	**Composition**
LG 1	C 940 (0.5 %) Gel in pH:5.5 10 mM Tris Buffer
LG 2	C 940 (0.5 %) Gel + 1% Na-HA in pH:5.5 10 mM Tris Buffer
LG 3	C 940 (0.5 %) Gel+ L36 liposomes in pH:5.5 10 mM Tris Buffer
LG 4	C 940 (0.5 %) Gel+ L37 liposomes inpH:5.5 10 mM Tris Buffer
LG 5	U 21 (0.5 %) Gel in pH:5.5 10 mM Tris Buffer
LG 6	U 21 (0.5 %) Gel + 1% Na-HA in pH:5.5 10 mM Tris Buffer
LG 7	U 21 (0.5 %) Gel+ L36 liposomes in pH:5.5 10 mM Tris Buffer
LG 8	U 21 (0.5 %) Gel+ L37 liposomes inpH:5.5 10 mM Tris Buffer
LG 9	PXM 188 (10 %)+PXM 407(10 %) Gel in pH:5.5 10 mM Tris Buffer
LG 10	PXM 188 (10 %)+ PXM 407(10 %) Gel+1%HA in pH:5.5 10 mM Tris Buffer
LG 11	PXM 188 (10 %)+ PXM 407(10 %) Gel+ L36 liposomes in pH:5.5 10 mM Tris Buffer
LG 12	PXM 188 (10 %)+ PXM 407(10 %) Gel+ L37 liposomes inpH:5.5 10 mM Tris Buffer
LG 13	PXM 407(20 %) Gel in pH:5.5 10 mM Tris Buffer
LG 14	PXM 407(20 %) Gel+1%HA in pH:5.5 10 mM Tris Buffer
LG 15	PXM 407(20 %) Gel+ L36 liposomes in pH:5.5 10 mM Tris Buffer
LG 16	PXM 407(20 %) Gel+ L37 liposomes in pH:5.5 10 mM Tris Buffer

2.1.2.3.1. POLYMER IDENTIFICATION STUDIES

2.1.2.3.1.1. POLYMER IDENTIFICATION STUDIES with FT-IR

FT-IR spectrums of all polymers were recorded by using FT-IR spectrophotometer wavelength of 400 to 4000 cm^{-1} and KBr discs were used.

2.1.2.3.1.2.POLYMER IDENTIFICATION STUDIES with DSC

DSC was used as the analytical technique to examine thermodynamic properties of polymers. The temperature was optimized at 0°C. min^{-1}.

2.1.2.3.2. RHEOLOGICAL STUDIES of GELS and LIPOGELOSOMES

The rheological properties of the gels were studied using a rheometer. The measuring system used was concentric cylinders or Couette geometry, with an inner diameter of 25 mm, an outer diameter of 27 mm and 32 mm height. The cylinders are surrounded for a double jacket with an electric resistance and the whole unit can be heated. The double jacket is connected to a liquid nitrogen reservoir and the gel can be cooled. The temperature is determined by a thermocouple that was connected to the inner cylinder. 10 ml of cold polymer solutions were transferred to the cylinders (56).

To measure the linear viscoelastic properties, the instrument was used in the oscillatory mode, in which the outer cylinder performs dynamic oscillations at a given frequency. To measure the shear steady state properties, the same geometry was used; in this case, the outer cylinder rotates at a given angular velocity (ω), which produces a shear rate (γ) gradient through the gap between the two cylinders.

The linear viscoelastic properties measured were the complex moduli, $G^*(\omega)$ and the complex viscosity, $\eta^*(\omega)$. $G^*(\omega) = G' + iG''$, where G' is the storage modulus and G'' is the loss modulus. G' is related to the storage of energy during the cycle or elastic energy, while G' is related to the dissipation of energy during the cycle or viscous energy. $\eta^*(\omega) = \eta' - i\eta''$, where η' is the dynamic viscosity and η'' is the imaginary viscosity.

The shear steady state property measured was the shear rate-dependent viscosity, $\eta(\gamma)$.

2.1.2.4. *IN VITRO* RELEASE STUDIES of Na-HA from LIPOSOMES, LIPOSOGELOSOMES and COMMERCIAL FORMULATIONS

A novel dialysis adapter was designed for USP Apparatus 4 to be used in conjunction with 22.6 mm sample cells. Figure 11/A is a scheme of the dialysis adapter design and Figure11/B shows the placement of the adapter in USP Apparatus 4. The design of the dialysis adapter is a hollow cylinder and the base and top of the cylinder are made of circular Teflon with groves for O-rings seals. The top and base are supported by three metallic wires that provide the framework for the adapter. The Teflon top has an opening that can be closed with a screw. A dialysis membrane is placed over this frame and sealed with O-rings at the top and bottom. The adapter cell with a dialysis membrane was fixed on a cross shaped platform which fits the 22.6 mm USP Apparatus 4 cell dimensions. This final assembled adapter is placed in the upright position inside the USP Apparatus 4 sample cells. The apparatus 4 can be operated in both the open and closed configurations and the flow rate varied as required. The specifications of the dialysis adapter are total volume, 10 ml (59).

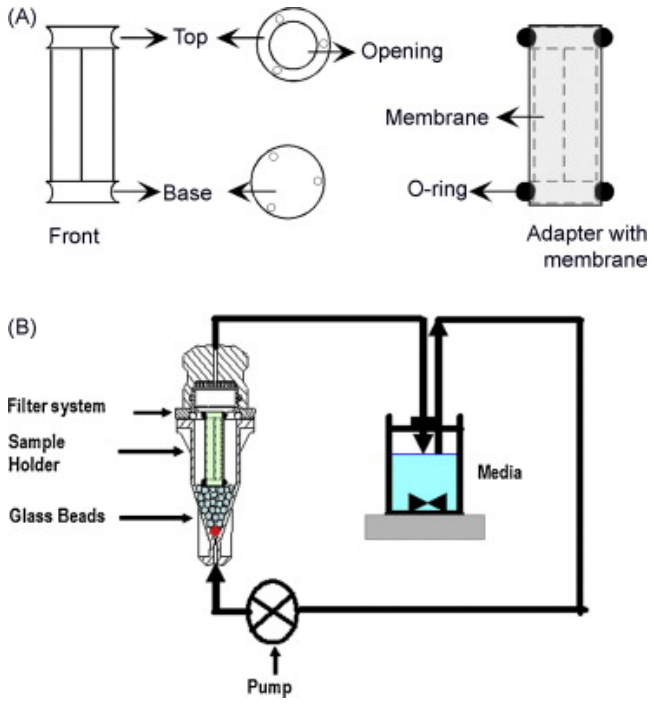


Figure 11. (A) Schematic of the dialysis adapter design. (Left) the front of the dialysis adapter, (middle) top and bottom parts, (right) adapter with dialysis membrane sealed with O-rings. (B) The placement of the adapter in USP apparatus 4 (59).

For the USP 4 method, a USP Apparatus 4 equipped with 22.6 mm diameter cells was used at 37 °C. A ruby bead (5 mm diameter) was placed at the base of the 22.6 mm sample cell and 4 g of 1 mm diameter glass beads were added to fill the bottom conical part of the sample cell. Liposome formulations were added to the dialysis adapter and the opening was sealed with a screw. For release studies, ~10ml of liposome suspensions was added to the dialysis adapter. The adapter was placed in the USP 4 sample cell as shown in Fig. 13/B for release studies. Tris buffer kept at pH 5.5 37 °C was used as release media in these studies. Liposome formulations were evaluated at flow rates of 8 ml. min^{-1}. USP Apparatus 4 release studies conducted at a flow rate of 8 ml. min^{-1} were used. At each time point, 1 ml samples were withdrawn from the media reservoir containers of the USP Apparatus 4. Na-HA was analyzed via UV spectrophotometer at 270 nm. The results were reported as mean ± SD (n = 6).

2.1.2.5. PRELIMINARY SKIN HYDRATATION STUDIES in HUMAN VOLUNTEERS

The preliminary studies were performed on 15 female volunteers. The optimum formulation with Na-HA and without Na-HA liposome, lipogelosome and commercial formulation were applied to the volunteers for two weeks twice a day. The ages of the volunteers were in between 35-50. They were all females.

The changes in moisture content, sebum levels, pH and the deepness of wrinkles were observed and determined by Multiprobe Adapter Systems (Figure 12). The measurement of sebum is based on greasy spot photometry. The tape of the sebumeter brought into contact with skin. It becomes transparent in relation to the sebum on the surface of the measurement area. Then the tape is inserted into the aperture of the device and the transparency is measured by a photocell. The light transmission represents the sebum content. The hydration measurement is based on capacitance measurement of a dielectric medium. The corneometer measures the change in the dielectric constant due to skin surface hydration changing the capacitance of a precision capacitor. The measurement of pH is based on a combined electrode, where both glass H+ ion sensitive electrode and additional reference electrode are placed in one house. It is connected to a probe handle containing the measurement electronics (60).

The changes of deepness of wrinkles on the skin surface were measured by Skin-Visiometer (Figure 13). The device features a parallel light source and a CMOS-camera with

640 * 480 pixels. The replica is placed between these. The light absorption of the blue color is known. When the light penetrates the replica, it is absorbed according to the thickness of the silicone material. The replica reproduces the heights and depths of the skin as a negative, i.e. wrinkles are higher in the replica as the silicone is thicker in this place. The amount of absorbed light is calculated by Lambert and Beer's Law. The outgoing light is proportional to the incoming light, the thickness of the material and the material constant k (61).

Statistical Analysis

Data obtained from optimum liposomal formulation (L), lipogelosome (LG) formulation, and commercial product (CP) of Na-HA were determined by Student t test analysis. *P* value less than 0.05 were considered significant.

Figure 12. Multi Probe Adapter System (MPA 5) (60).

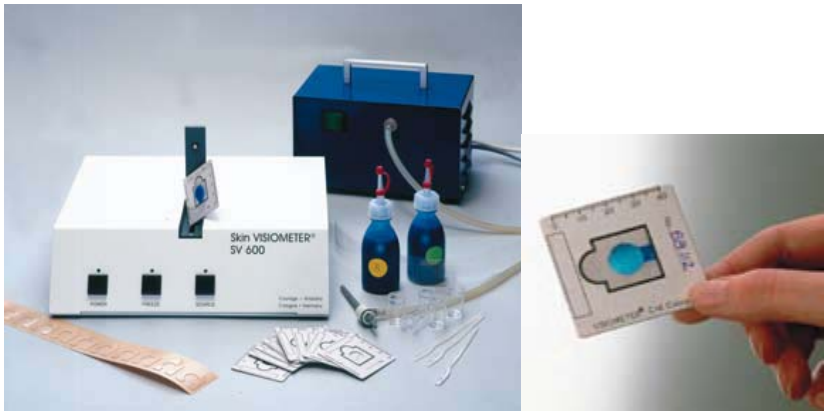


Figure 13. Skin Visiometer (SV 600) (61).

2.1.2.6.VALIDATION of ANALYTICAL METHOD

Validation of analytical method for the determination of the amount of the substance to be analyzed in terms of the reliability of the method used to indicate the intended application processes to be carried out. A measurement system is called validation if it is both accurate and precise. The required parameters provide analytical validation by following terms; linearity, accuracy, precision, range and method repeatability, reproducibility, sensitivity, specificity, stability (62).

Linearity

Linearity of the experimental findings at an analytical method is the conformity of the experimental findings with the direct or indirect substance concentration in the sample. Calibration curves were obtained as described in Chapter 2.1.2.1.2. After the preparation of stock solutions, ten different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 $mg.mL^{-1}$) of pure Na-HA were obtained. The calibration curves were plotted by performing the six replicate determinations of corresponding concentrations of Na-HA to the absorbances of solutions. Descriptive coefficients (correlation-r and determination-r^2) were calculated and linearity of equation was shown.

Accuracy and Precision Studies

The term precision is defined as the closeness of agreement between quantity values obtained by replicate measurement of quantity under specified conditions. The determination of this parameter is one of the basic steps in the process of achieving repeatability and reproducibility in method validation.

There is no numerical value for precision; standard deviation (SD), standard error (SE), or coefficient of variation (CV) is the terms of precision.

Precision of analytical method is performed by consecutively measuring the samples in the same concentrations in the statistically meaningful replicas. Arithmetic mean ($\bar{x}$), SE and CV of these measurements are calculated for the evaluation of method precision.

The accuracy of the proposed method was evaluated by performing six replica determinations of Na–HA in pure form at ten different concentrations by short term (intra day) and daily (inter day) precisions.

Repeatability

The absorbances of the solutions at the upper (1 $mg.mL^{-1}$) and lower (0.1 $mg.mL^{-1}$) concentrations obtained from stock solutions were measured spectrophotometrically at λ_{max}: 270 nm six times. $\bar{x}$, SE and CV values were calculated for the concentrations obtained from these absorbances. If the CV found smaller than 2 %, it shows the repeatability of the analytical method.

The same methodology was repeated on different days (0. days and 2. days) to show intra and inter day repeatability.

Reproducibility

The absorbances of the solutions at the upper (1 $mg.mL^{-1}$) and lower (0.1 $mg.mL^{-1}$) concentrations obtained "seperately" from stock solutions were measured spectrophotometrically at λ_{max}: 270 nm six times. $\bar{x}$, SE and CV values were calculated for the concentrations obtained from these absorbances. If the CV found smaller than 2 %, it shows the reproducibility of the analytical method.

Specificity

In order to determine whether the analytical method used covers only Na-HA or the other excipients, the spectrums of the formulation excipients were taken in between λ_{max}: 200-450 nm.

Stability

In order to investigate the stability of Na-HA in Tris buffer (pH: 5.5, 10 mM) throughout the working day; the absorbances of the solutions in 1 $mg.mL^{-1}$ and 10 $mg.mL^{-1}$ concentrations were measured at the beginning and 24^{th} h. Then, the changes in concentrations by 24 h were evaluated.

3. RESULTS

In this Chapter, the results have been given the physicochemical properties of Na-HA liposomes, gels and lipogelosomes.

3.1. RESULTS of PHYSICOCHEMICAL PROPERTIES of Na-HA

3.1.1. RESULTS of STANDARDIZATION of Na-HA

3.1.1.1.RESULTS of UV LIGHT SPECTRUM of Na-HA

a) As mentioned in 2.1.2.1.1.1, UV spectrum of Na-HA in 10 mM Tris buffer (pH:.5.5) was determined at λ_{max} =270 nm (Figure 14).

b) UV spectrum of 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH:.5.5) was determined at λ_{max} =272 nm (Figure 15).

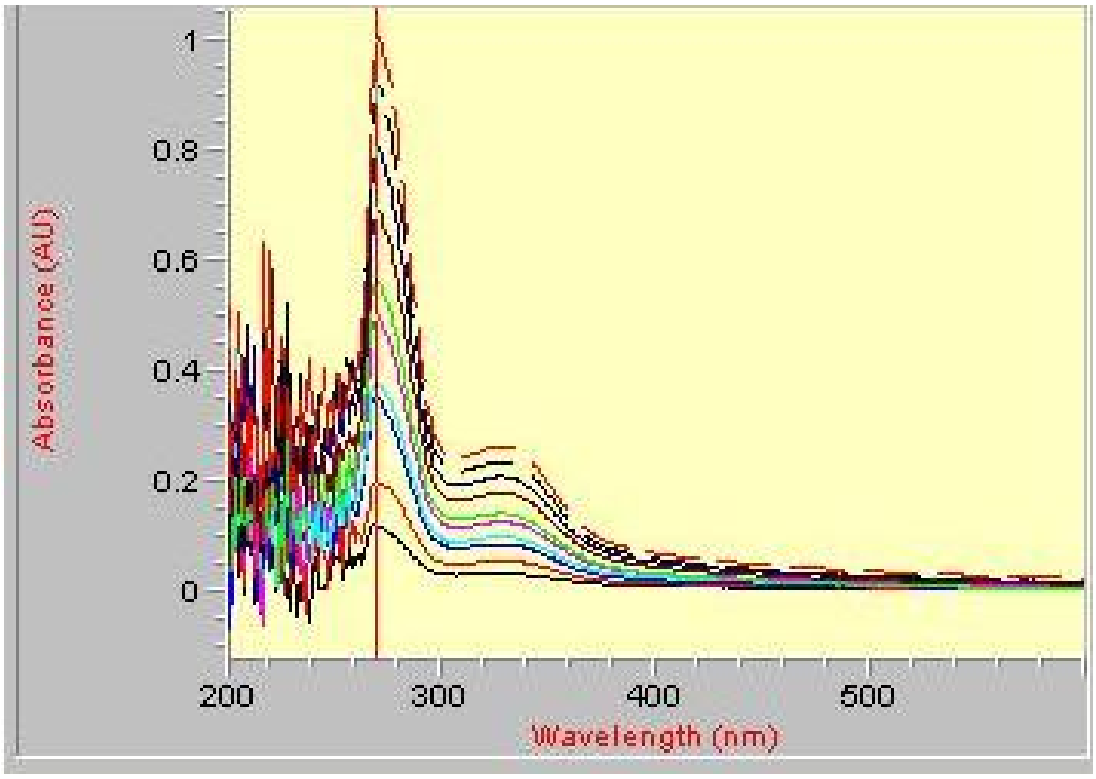


Figure 14. UV spectrum of Na-HA in 10 mM Tris buffer (pH: 5.5) at λ_{max} =270 nm. (---0.1 mg.ml^{-1}, ---0.2 mg.ml^{-1}, ---0.3 mg.ml^{-1}, ---0.4 mg.ml^{-1}, ---0.5 mg.ml^{-1}, ---0.6 mg.ml^{-1}, ---0.7 mg.ml^{-1}, ---0.8 mg.ml^{-1}, ---0.9 mg.ml^{-1}, ---1 mg.ml^{-1})

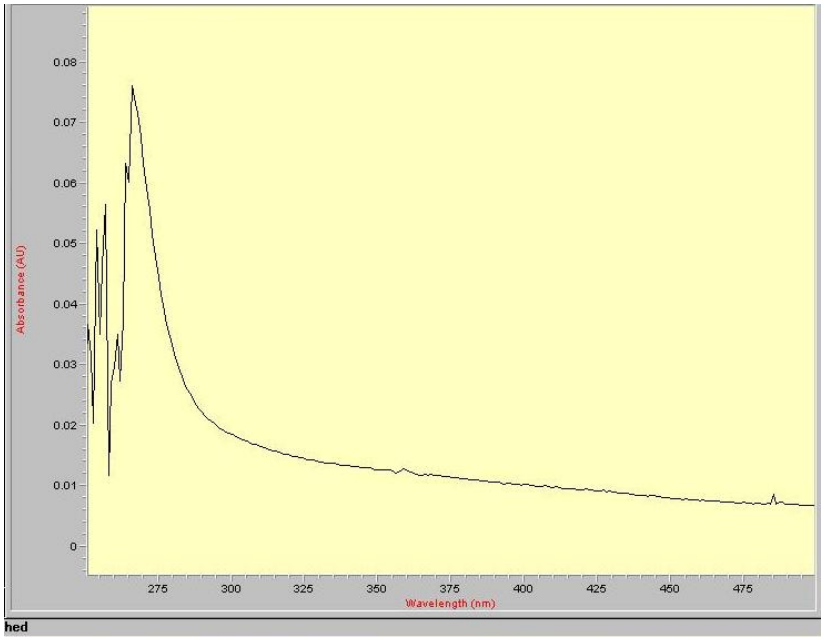


Figure 15. UV spectrum of 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH: 5.5) at λ_{max} =272 nm.

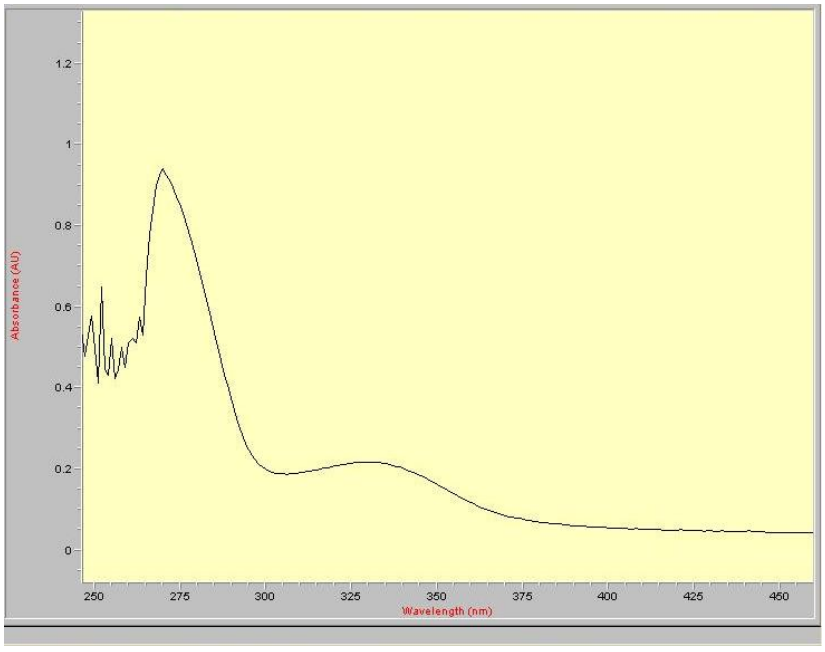


Figure 16. Determination of specific wavelength for Na-HA while Na- HA with the other ingredients of the formulations.

3.1.1.2.RESULTS of FT-IR of Na-HA

FT-IR spectra of Na-HA were determined in between 400-4000 cm^{-1} wavelength and by using potassium bromide (KBr) discs. Characteristic bands were recorded at 3271, 2360, 2341, 1617, 1559, 1540, 1507, 1405, 1031, 668 and 649, respectively.

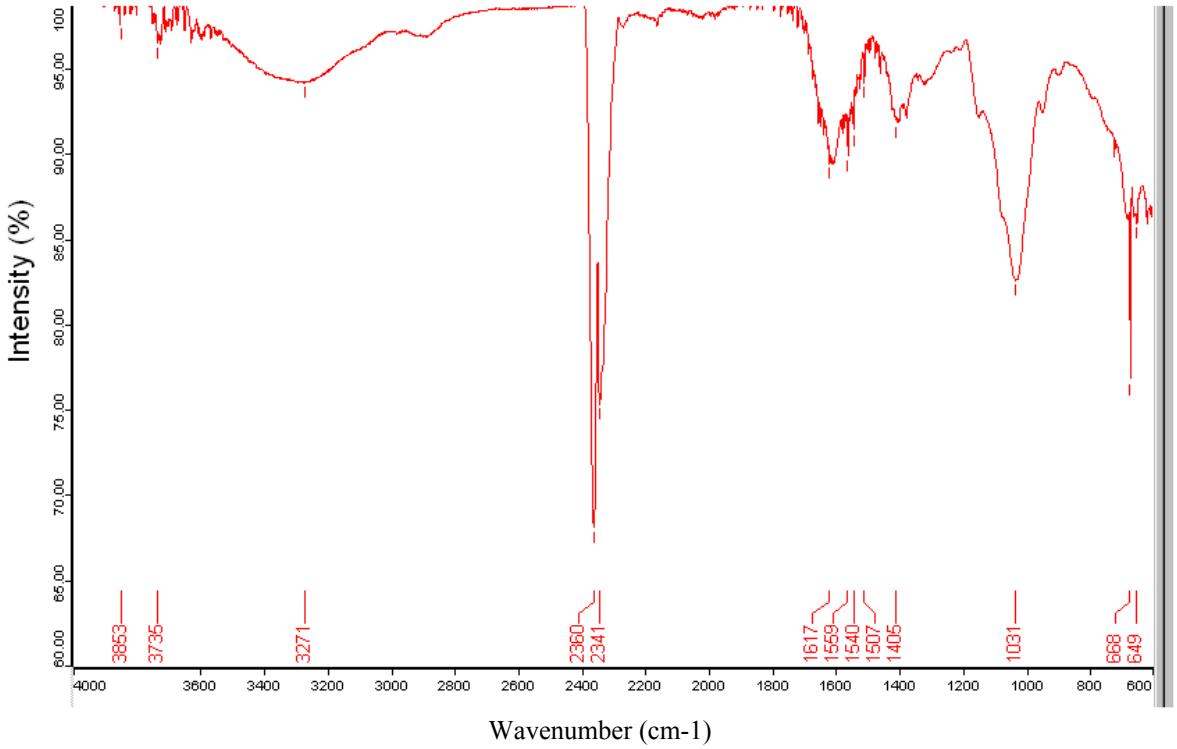


Figure 17. FT-IR Spectra of Na-HA .

3.1.1.3.RESULTS of DSC of Na-HA

DSC result of Na-HA is given in Figure 18.

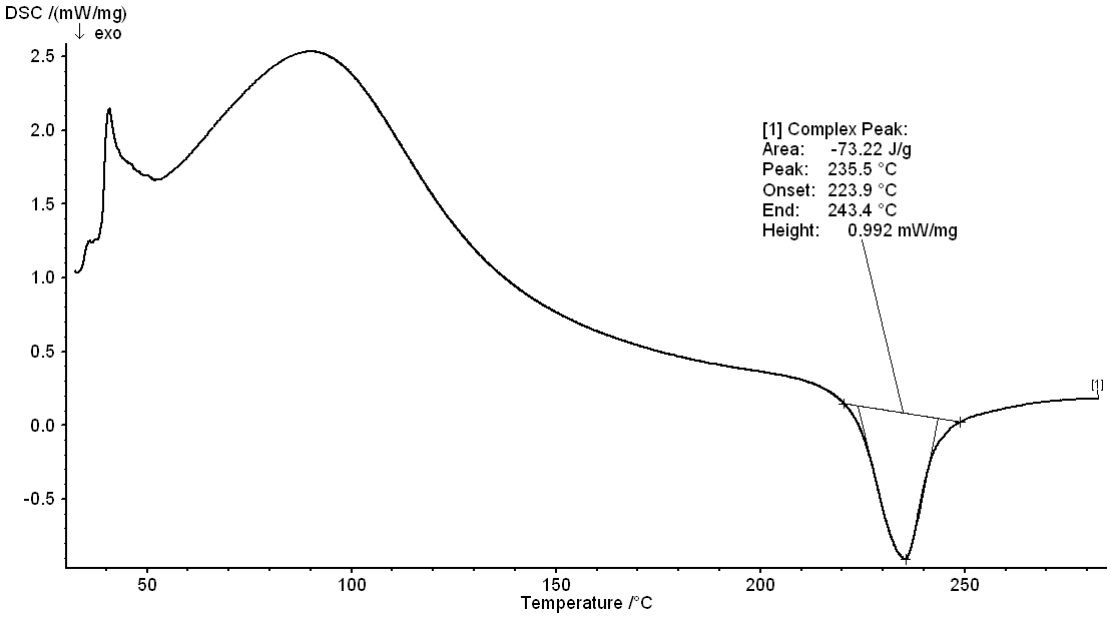
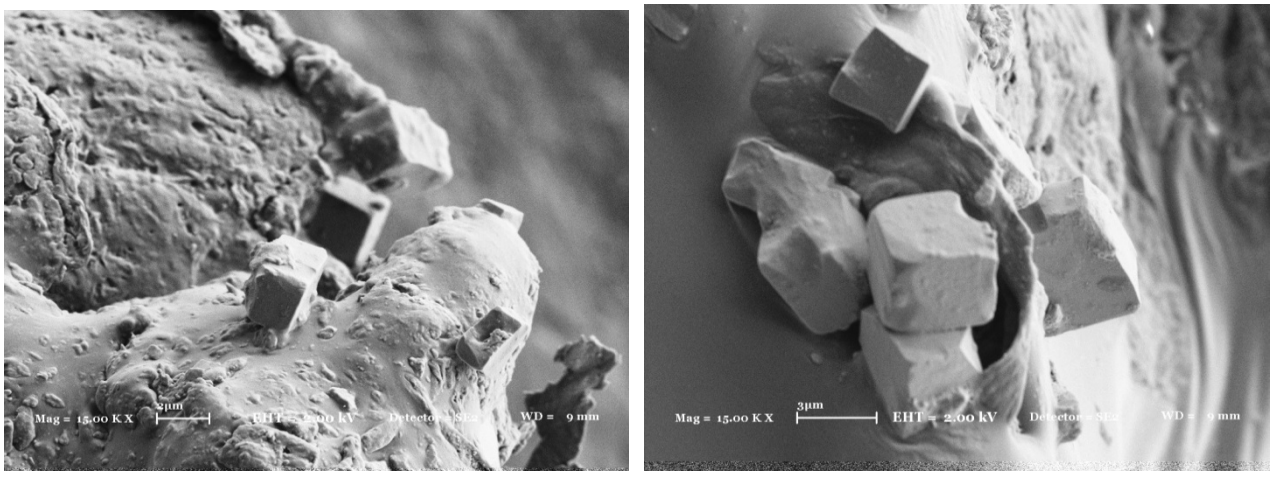


Figure 18. DSC thermogram of Na-HA .

The phase transition temperature was found as 235.5 ^{0}C.

3.1.1.4. RESULTS of SEM IMAGES of Na-HA

SEM images of Na-HA are given in Figure 19 A and B.



A **B**

Figure 19. SEM images of Na-HA (A: 15 KX, B: 15 KX).

3.1.1.5. X-RD SPECTRA of Na-HA

Amorphous structure of Na-HA is represented in Figure 20 by X-ray diffractometer.

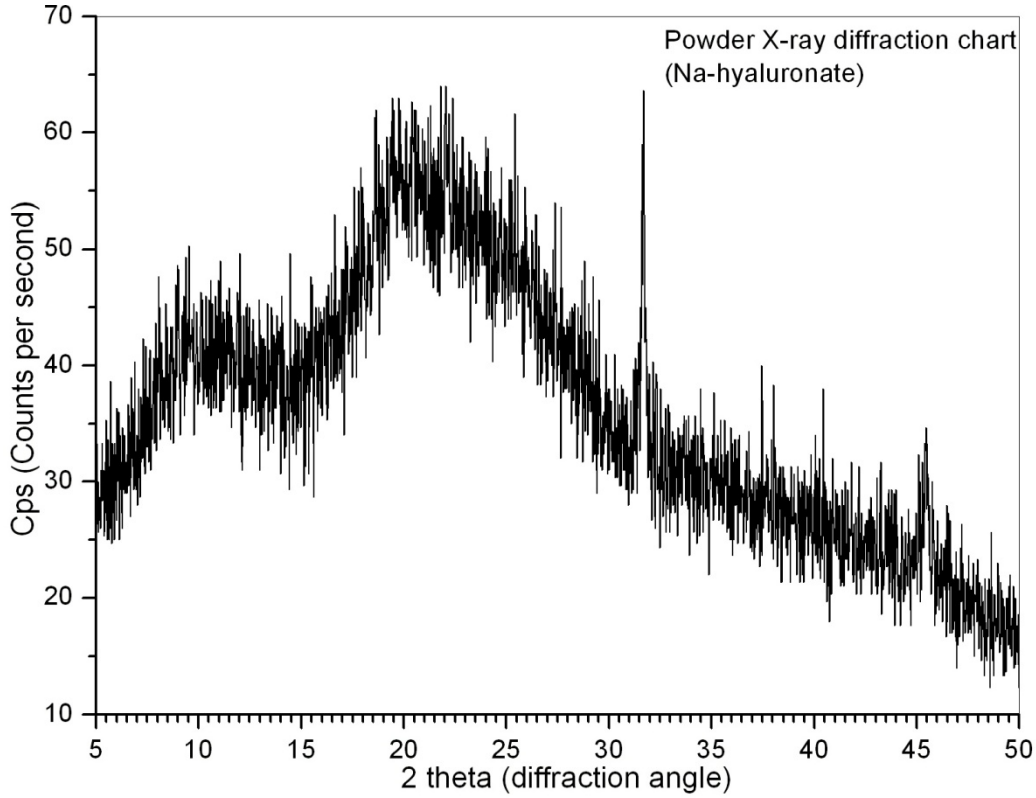


Figure 20. X-RD spectrum of Na- HA.

3.1.1.6. RESULTS of STABILITY RESULTS of Na-HA in DIFFERENT CONDITIONS

Na-HA solutions under different conditions were analyzed by FT-IR (Figure 21).

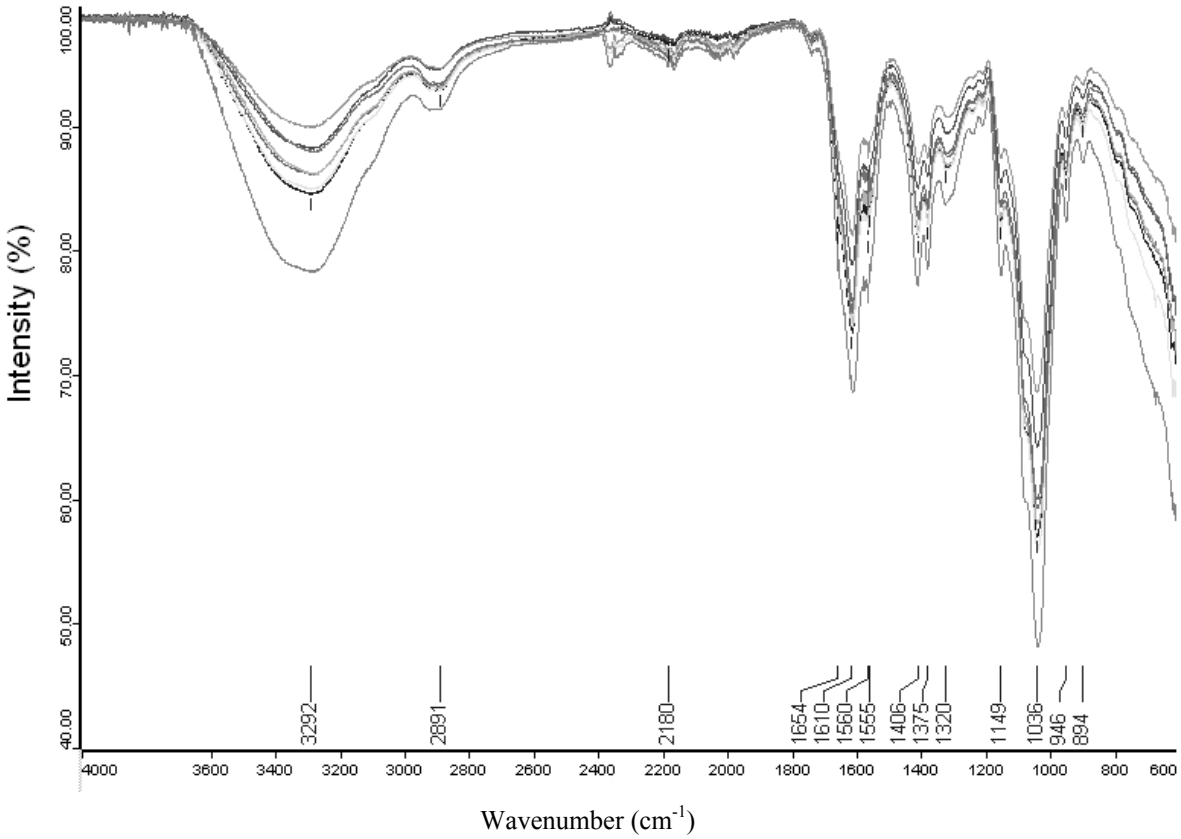


Figure 21. FT-IR spectrum of Na-HA in different conditions.

3.1.2. CALIBRATION CURVE of Na-HA

Calibration curve of Na-HA in 10 mM Tris buffer (pH: 5.5) was plotted at λ_{max}=270 nm in Figure 22 and calibration curve of 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH: 5.5) was measured at λ_{max} =272 nm shown in Figure 23.

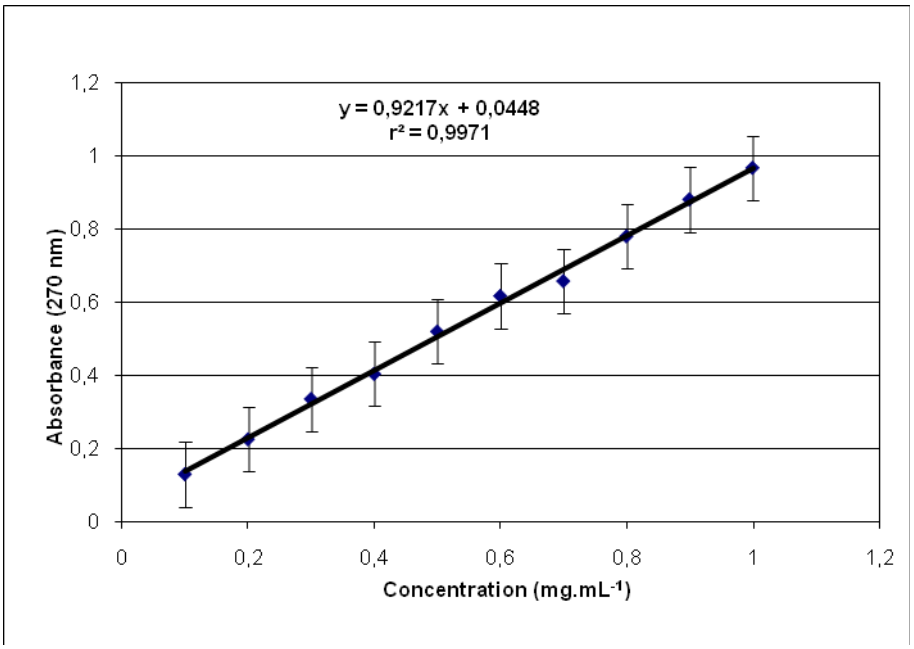


Figure 22. Calibration curve of Na-HA in 10 mM Tris buffer (pH: 5.5).

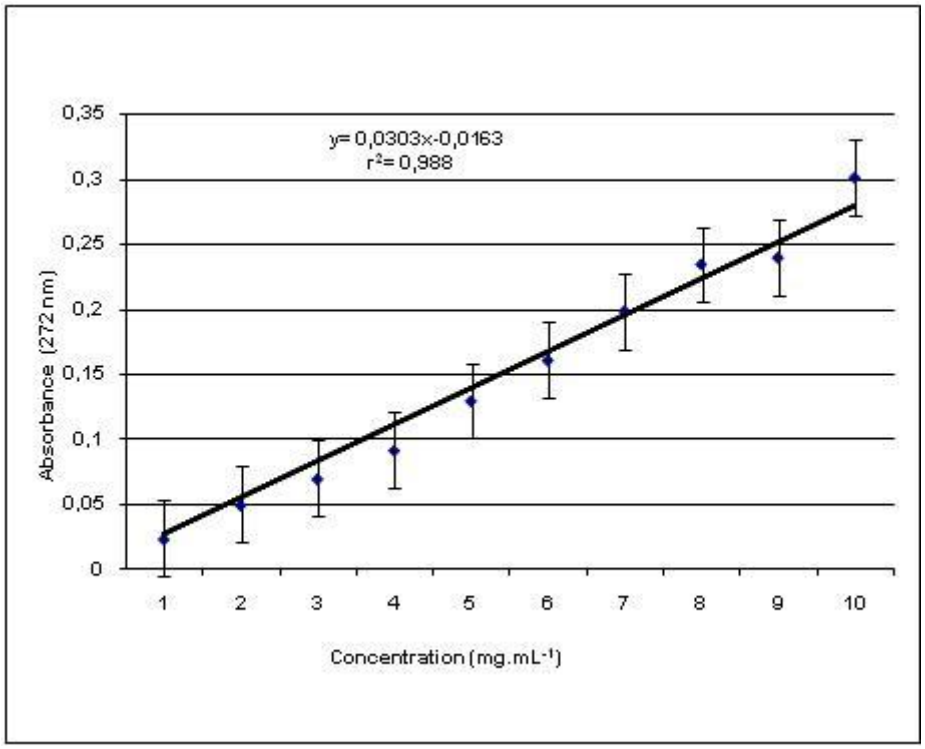


Figure 23. Calibration curve of 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH: 5.5).

3.1.2.1 RESULTS of ANALYTICAL METHOD VALIDATION

Linearity

As mentioned in Chapter 2.1.2.6, results of regression analysis were presented in Figure 22 and 23. Regression equations for both Na-HA in 10 mM Tris buffer (pH: 5.5) and 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH: 5.5) having a coefficient of determination (r^2) of 0.997 and 0.988 respectively, could be used to predict concentration from peak area.

Accuracy and precision

As mentioned in Chapter 2.1.2.6, the accuracy and precision of the calibration curve of Na-HA were evaluated measuring repeatability at the same concentrations levels by intra day and daily (inter day) measurements.

i. Repeatibility

As mentioned in Chapter 2.1.2.6, the repeatability of analytical method of Na-HA determined with two different concentrations (1 $mg.mL^{-1}$-0.1 $mg.mL^{-1}$) by six times and $\bar{x}$, SE and CV values were obtained. The CV values were found less than 2 %. The same methodology was repeated on different days (0. days and 2. days) to show intra and inter day repeatability as shown in Table 7 and Table 9.

Table 7. Repeatability results of two different concentration of Na -HA (0.1 mg.mL^{-1} 1 mg.mL^{-1})

Sample	Absorbance	Concentration	Average ± SD	CV (%)
0.1 mg.mL^{-1}	0,136 0,148 0,140 0,134 0,132 0,145	0.099 0.111 0.103 0.096 0.094 0.108	0.102 ± 0.006	0.059
1 mg.mL^{-1}	0.975 0.982 0.947 0.944 0.938 0.975	1.009 1.017 0.979 0.975 0.969 1.009	0.993 ± 0.021	0.020

ii. Reproducibility

Reproducibility of analytical method of Na-HA determined with two different concentrations (1 mg.mL^{-1}-0.1 mg.mL^{-1}) (Chapter 2.1.2.6). Data were obtained "seperately" from stock solutions and again $\bar{x}$, SE and CV values were obtained. The CV values were found less than 2 % (Table 8).

Table 8. Reproducibility results of two different concentration of Na -HA (0.1 mg.mL^{-1} 1 mg.mL^{-1})

Sample	Absorbance	Concentration	Average ± SD	CV (%)
0.1 mg.mL^{-1}	0.110 0.117 0.129 0.109 0.128 0.121	0.071 0.078 0.088 0.070 0.087 0.084	0.080 ± 0.008	0.101
1 mg.mL^{-1}	0.849 0.890 0.984 0.940 1.015 1.002	0.990 1.038 1.150 1.097 1.187 1.171	1.105 ± 0.078	0.071

Specificity

According to Chapter 2.1.2.6, to determine whether the analytical method used covers only Na-HA or the other ingredients, the spectrums of the formulation additives (PL 100H, SA, CHOL) were taken in between λ_{max}: 200-450 nm. Specificity is the ability of the method to measure the Na-HA response in the presence of other ingredients (Figure 16).

According to these results 270 nm is a specific wavelength for Na-HA and the other ingredients of the formulation are not interfering with the active substance.

Stability

The stability of Na-HA in Tris buffer (pH: 5.5, 10 mM) throughout the working day: the absorbances of the solutions in 1 mg.mL^{-1} and 10 mg.mL^{-1} concentrations were measured at the beginning and 24th h (Chapter 2.1.2.6). Then, the changes in concentrations by 24 h were evaluated statistically (Table 9). CV data were found less than 2 %.

Table 9. The stability results of Na-HA in Tris buffer (pH: 5.5 10 mM) at 0 - 24 h time intervals with two different concentrations (1-10 $mg.ml^{-1}$) measured room temperature.

Time (hour)	Concentration ($mg.ml^{-1}$)	Calculated Concentration ($\mu g.ml^{-1}$) Mean ± SD	CV(%)
0	1	1.041 ± 0.022	0.21
24	1	1.045 ± 0.006	0.57
0	10	9.791 ± 0.042	0.42
24	10	9.834 ± 0.022	0.22

3.1.3. RESULTS of SOLUBILITIY of Na-HA

As mentioned in 2.1.2.1.3., solubility of Na-HA was determined and shown in Table 10.

Table 10. Results of solubility of Na-HA (n=6).

STORAGE CONDITIONS	DISSOLVED Na-HA IN THE BEGINNING ($mg.mL^{-1}$)					MEASURED Na-HA AFTER 2 DAYS ($mg.mL^{-1}$)				
ROOM TEMPERATURE (25 ^{0}C)	1	2.5	5	10	100	0.99	2.48	4.94	9.98	99.24
REFRIGERATOR TEMPERATURE (4 ^{0} C)	1	2.5	5	10	100	0.99	2.48	4.94	9.99	98.74
1 HOUR AT 50 ^{0} C	1	2.5	5	10	100	0.99	2.48	4.95	9.97	99.20
pH	5.48	5.79	5.98	6.12	6.57	5.81	6.04	6.21	6.30	6.58

3.2. RESULTS of IDENTIFICATION of LIPOSOMES

3.2.1. RESULTS on PHYSICOCHEMICAL CHARACTERISTICS of LIPOSOME COMPONENTS

In this Chapter, results of physicochemical characteristics of liposome components are given.

3.2.1.1. STANDARDIZATION of LIPOSOME COMPONENTS

3.2.1.1.1. STANDARDIZATION of PHOSPHOLIPIDS

3.2.1.1.1.1. IDENTIFICATION of PHOSPHOLIPIDS

FT-IR spectra of PL 100H, DMPC, and DPPC were represented in Figures 24-26, respectively. The characteristic FT-IR peaks were found at 2955, 2649, 1734, 1053,817 cm^{-1} for PL 100H; at 3010, 2854, 1736, 1467, 1246, 1062 cm^{-1} for DMPC and at 2956, 2850, 2361, 1735, 1061 cm^{-1} for DPPC. In addition, DSC thermal curves of PL 100H and DPPC were represented in Figures 27-28. The phase transition temperatures were determined as 61,2 ^0C and 48,9 ^0C for PL 100H and DPPC, respectively.

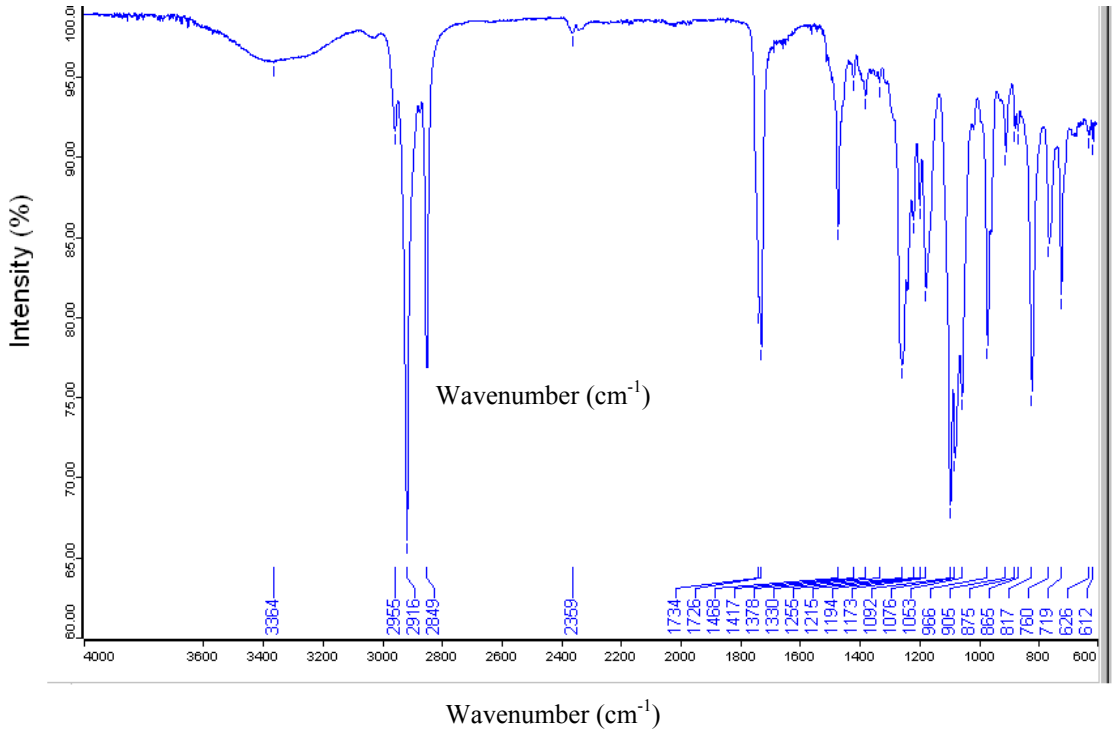


Figure 24. FT-IR spectrum of PL 100H.

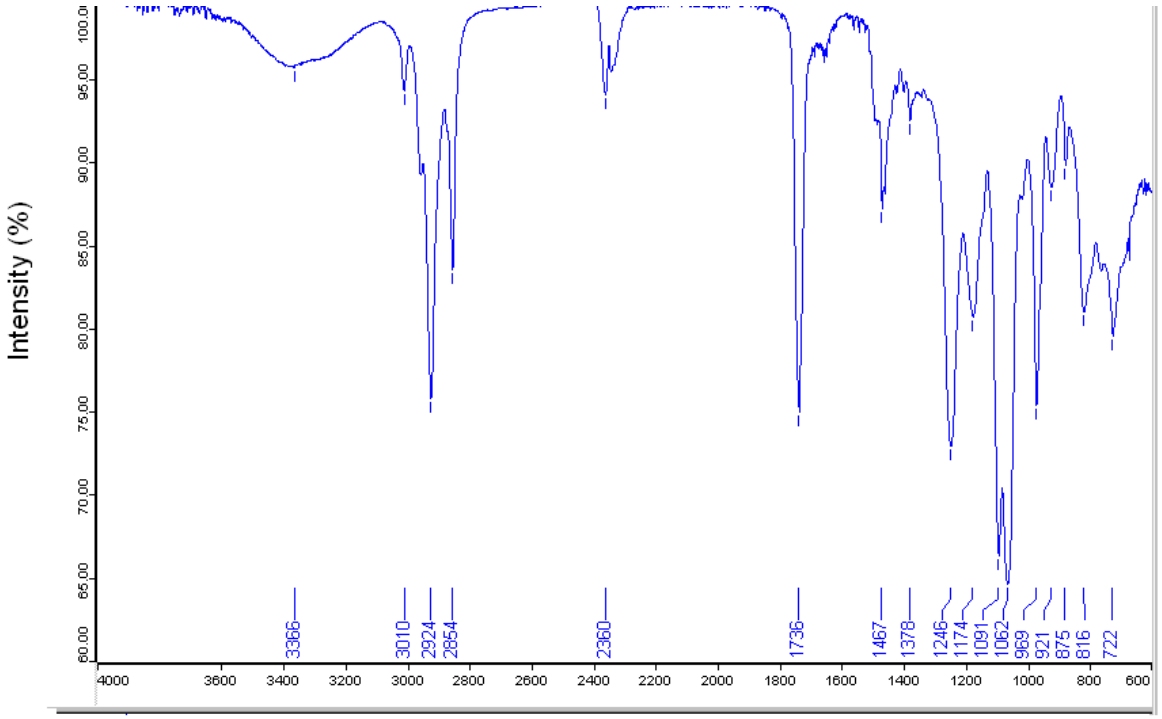


Figure 25. FT-IR spectrum of DMPC.

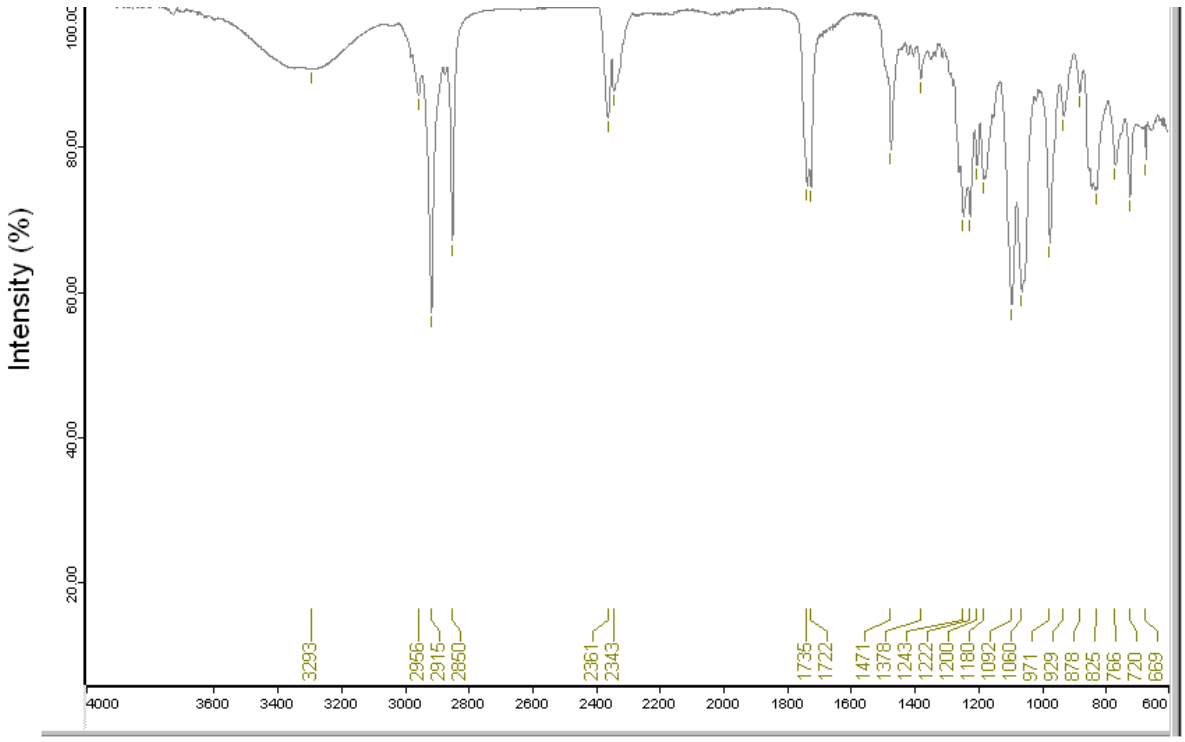


Figure 26. FT-IR spectrum of DPPC.

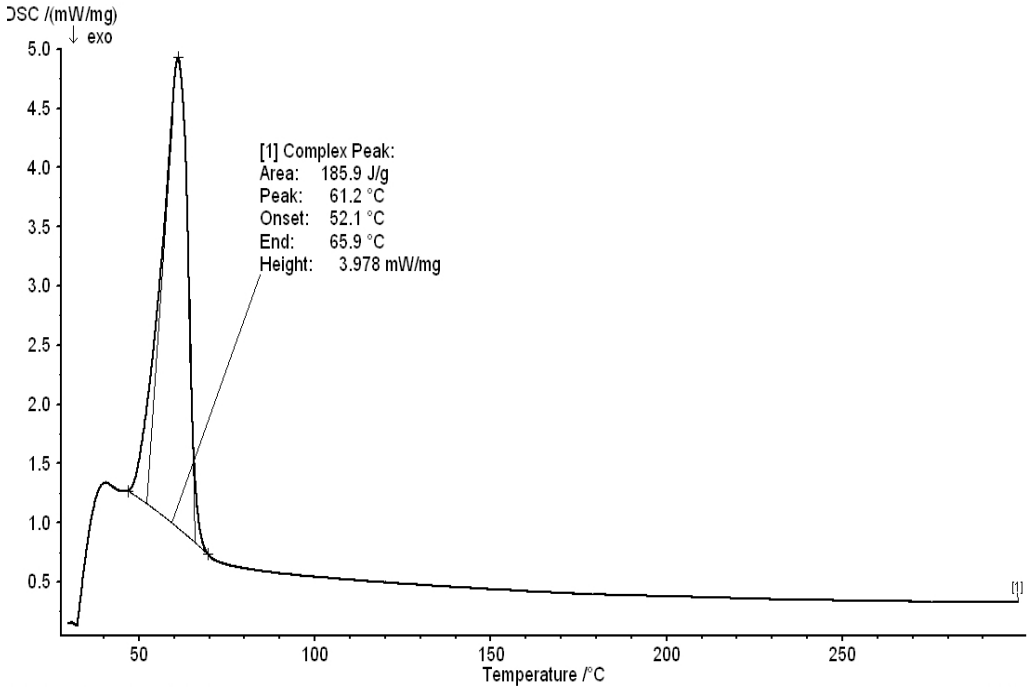


Figure 27. DSC thermogram of PL 100H.

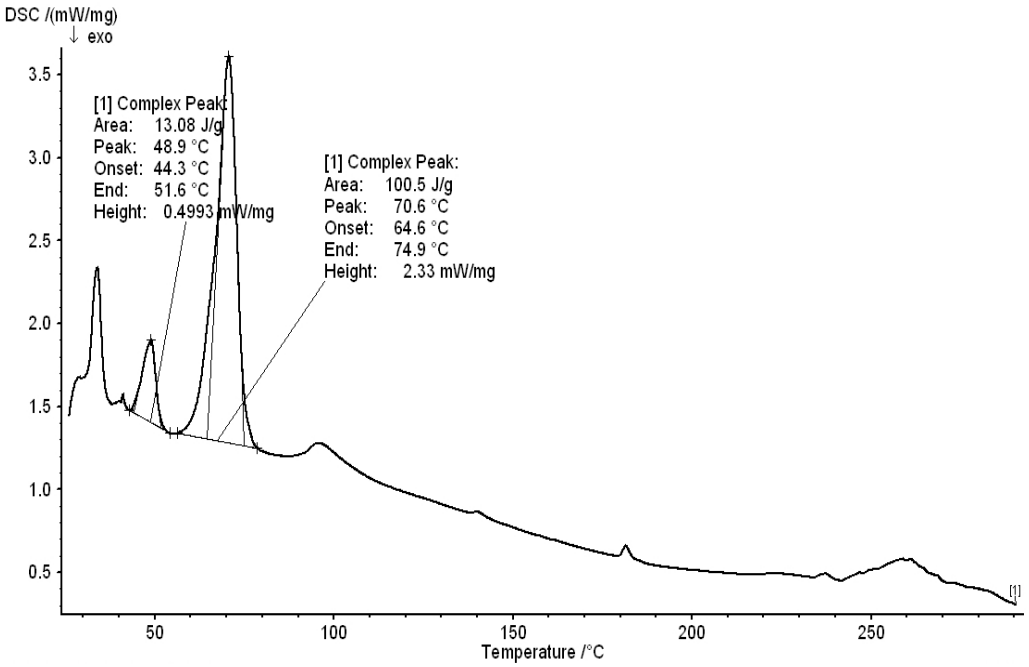


Figure 28. DSC thermogram of DPPC.

3.2.1.1.2. CHARGE INDUCERS

3.2.1.1.2.1.IDENTIFICATION SA by FT-IR METHOD

FT-IR spectrum of SA was shown in Figure 29. The characteristic FT-IR peaks were found at 2954, 2848, 1566, 1472, 1316, 1060, 816 cm^{-1} for SA.

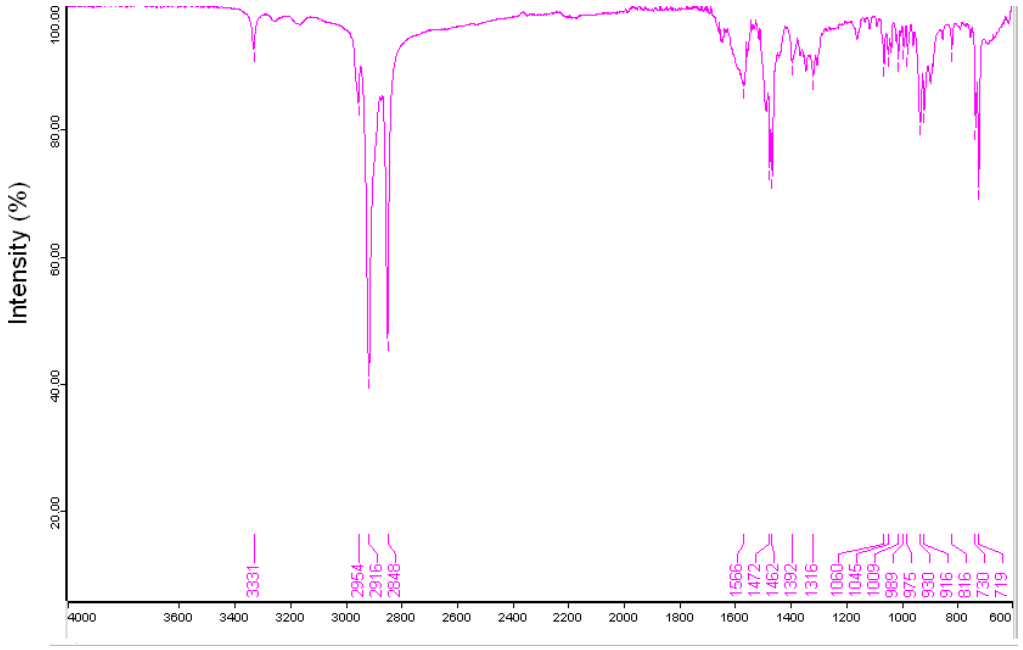


Figure 29. FT-IR spectrum of SA.

3.2.1.1.2.2.IDENTIFICATION of DCP by FT-IR METHOD

FT-IR spectrum of DCP was represented Figure 30. The characteristic FT-IR peaks were found at 2882, 2361, 1466, 1342, 1100, 962, 841 cm^{-1} for DCP.

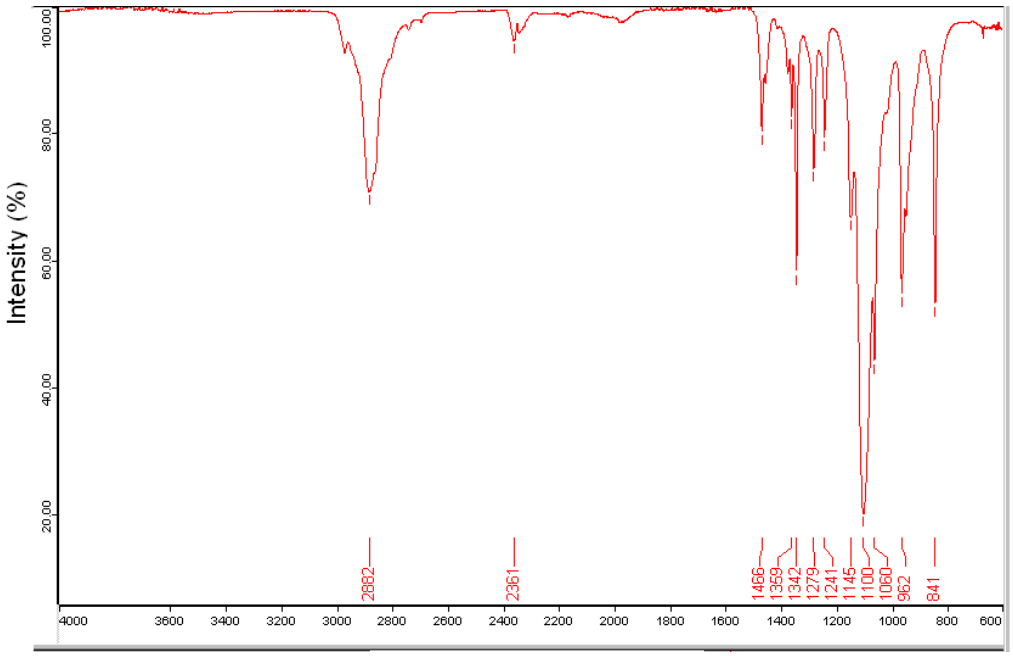


Figure 30. FT-IR spectrum of DCP.

3.2.1.1.3. STABILIZING AGENT

3.2.1.1.3.1. RESULTS of IDENTIFICATION of CHOL

FT-IR spectrum of CHOL was shown in Figure 31. The characteristic FT-IR peaks were found at 2931, 2163, 1466, 1056, 927 cm^{-1} for CHOL. DSC thermal curve of CHOL was presented in Figure 32. Melting point was found 148 ^0C.

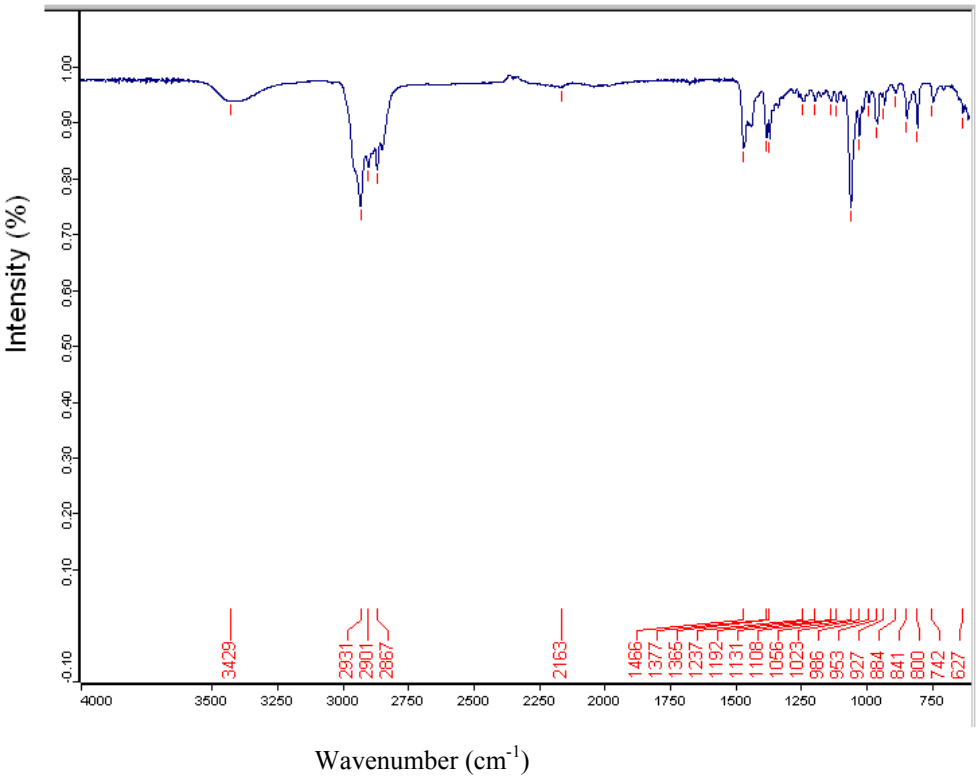


Figure 31. FT-IR spectrum of CHOL.

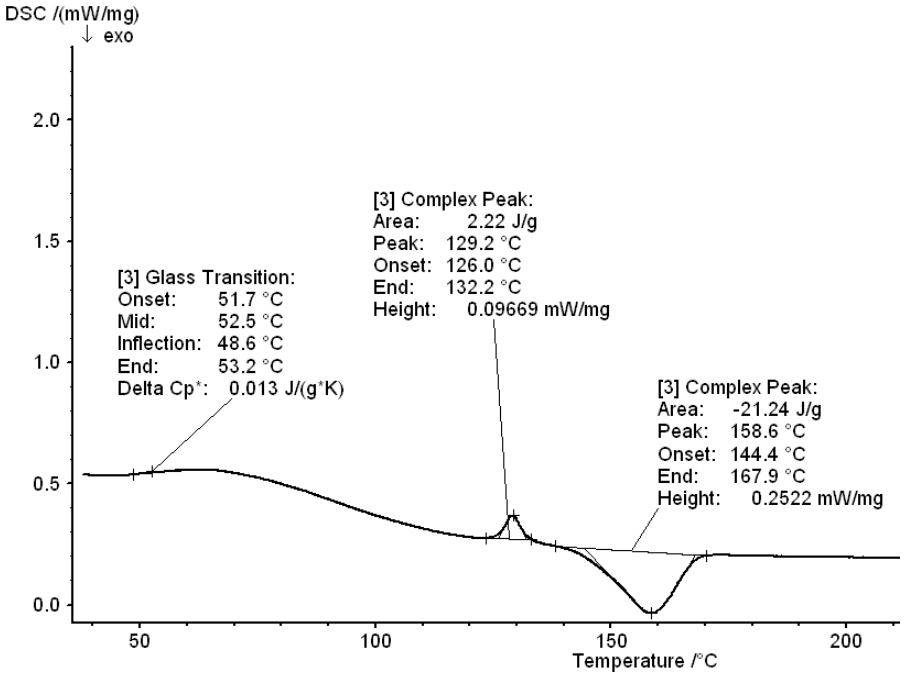


Figure 32. DSC thermogram of CHOL.

3.3. RESULTS of PREPARED LIPOSOMES

Particle size distribution, zeta potential, encapsulated Na-HA amount, free Na-HA amount in liposomes, phospholipid yield, PLM images, SEM images and AFM images of liposomes were given in this Chapter.

3.3.1. RESULTS of MEAN PARTICLE SIZE, SIZE DISTRIBUTION, POLY DISPERSITY INDEX and ZETA POTENTIAL of LIPOSOMES

Results of mean particle size and size distribution, PDI and zeta potential of liposomes were shown in Table 11.

Table 11. Mean particle size, PDI and zeta potential results of L 32-L 47 liposomes (n=3).

Code	Composition	Molar Ratio	Average diameter (nm)	PDI	Zeta potential (mV)
L 32	DMPC:DMPG:CHOL	9:1:10	624 ± 1.14	0.607 ± 0.02	-46.5 ± 1.33
L 33	DMPC:DMPG:CHOL+1%Na-HA	9:1:10	1214 ± 12.60	0.694 ± 0.02	-43.9 ± 1.91
L 34	DMPC:DMPG:CHOL	9:1:25	1426 ± 27.29	0.543 ± 0.01	-37.9 ± 1.30
L 35	DMPC:DMPG:CHOL+1%Na-HA	9:1:25	1460 ± 35.08	0.771 ± 0.03	-35.1 ± 0.91
L 36	PL 100H:SA:CHOL	7:1:2	676 ± 5.73	0.461 ± 0.02	+23.1 ± 1.04
L 37	PL 100H:SA:CHOL+1%Na-HA	7:1:2	1899 ± 13.01	0.356 ± 0.01	-20.9 ± 0.46
L 38	PL 100H:SA:CHOL	10:1:4	908 ± 24.40	0.670 ± 0.03	+42.9 ± 1.12
L 39	PL 100H:SA:CHOL+1%Na-HA	10:1:4	2370 ± 102.20	0.274 ± 0.01	-25.2 ± 0.50
L 40	PL 100H:DCP:CHOL	7:1:2	1146 ± 12.30	0.568 ± 0.02	-49.7 ± 1.11
L 41	PL 100H:DCP:CHOL+1%Na-HA	7:1:2	1572 ± 57.92	0.391 ± 0.01	-64.6 ± 3.03
L 42	PL 100H:DCP:CHOL	10:1:4	674 ± 30.48	0.323 ± 0.01	-43.7 ± 1.03
L 43	PL 100H:DCP:CHOL+1%Na-HA	10:1:4	1132 ± 30.83	0.451 ± 0.02	-51.7 ± 2.09
L 44	DPPC:SA:CHOL	5:2:3	717 ± 11.90	0.641 ± 0.03	+38.9 ± 1.61
L 45	DPPC:SA:CHOL+1%Na-HA	5:2:3	1130 ± 37.98	0.729 ± 0.02	-20.3 ± 0.91
L 46	DPPC:DCP:CHOL	7:1:2	643 ± 32.60	0.603 ± 0.03	-31.6 ± 0.72
L 47	DPPC:DCP:CHOL+1%Na-HA	7:1:2	1038 ± 2.82	0.437 ± 0.01	-19.3 ± 0.90

Table 11 a. Student's t test results of particle sizes of L 36 and L 37 formulations.

FORMULATION	L 36 and L 37
Student's t test	insignificant p>0.05

3.3.2. RESULTS of PHOSPHOLIPID YIELD in LIPOSOME DISPERSIONS

Phospholipid yields in different molar ratios were shown in Table 12.

Table 12. The yield of phospholipids in the liposome formulations.

Code	**Phospholipid amount in the beginning (µg.mL^{-1})**	**Phospholipid amount at the end of sonication and ultracentrifugation (µg.mL^{-1})**	**Phospholipid yield (%)**
L 32	13572	11389	83.92
L 33	13572	11213	82.62
L 34	13572	10511	77.45
L 35	13572	10267	75.65
L 36	10920	9943	91.05
L 37	10920	10680	97.80
L 38	15600	13507	86.58
L 39	15600	14143	90.66
L 40	10920	9213	84.37
L 41	10920	8712	79.78
L 42	15600	13228	84.79
L 43	15600	12948	83.00
L 44	7340	6719	91.54

L 45	7340	6479	88.27
L 46	10260	9031	88.02
L 47	10260	9098	88.68

3.3.3. RESULTS of DETERMINATION of Na-HA AMOUNT in LIPOSOME FORMULATIONS

3.3.3.1. ENCAPSULATED Na-HA AMOUNT in VESICLES

Encapsulated Na-HA amount in vesicles were shown in Table 13.

3.3.3.2. FREE Na-HA AMOUNT in LIPOSOME FORMULATIONS

Unencapsulated (free) Na-HA amount in liposome formulations were calculated and shown in Table 13.

Table 13. Encapsulated and free Na-HA amount in liposome formulations.

Code	1.Supernatant ($mg.mL^{-1}$)	2.Supernatant ($mg.mL^{-1}$)	3.Supernatant ($mg.mL^{-1}$)	Encapsulated Na-HA Amount ($mg.mL^{-1}$)	Na-HA Amount in the Beginning ($mg.mL^{-1}$)	Encapsulation Capacity (%)
L 33	5.544	2.772	0.046	0.924	10.000	9.24
L 35	6.936	1.734	0.029	1.156	10.000	11.56
L 37	5.980	1.869	0.037	1.495	10.000	14.95
L 39	5.860	2,197	0.073	1.465	10.000	14.65
L 41	6.169	1.919	0.013	1.371	10.000	13.71
L 43	6.088	2.283	0.015	1.522	10.000	15.22
L 45	6.633	2.412	0.060	0.603	10.000	6.03
L 47	6.571	2.605	0.065	0.650	10.000	6.50

Table 13 a. Student's t test results of encapsulation percentage of L 37 and L 43 formulations.

FORMULATION	L 37 and L 43
Student's t test	significant $p<0.05$

Table 13 b. Student's t test results of encapsulation percentage of L 37 and L 39 formulations.

FORMULATION	L 37 and L 39
Student's t test	significant $p<0.05$

3.3.4. PLM IMAGES of LIPOSOMES

The images of Na-HA containing MLV type liposomes were obtained by PLM (Figure 33-40).

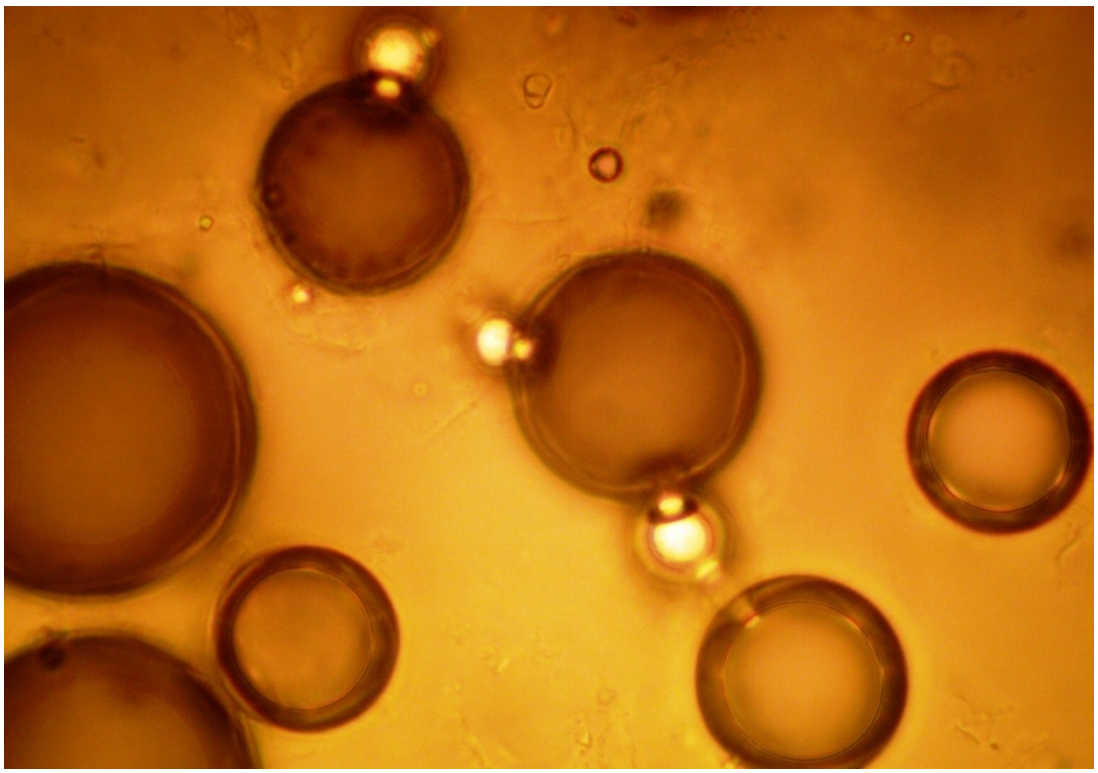


Figure 33. PLM image of L 33.

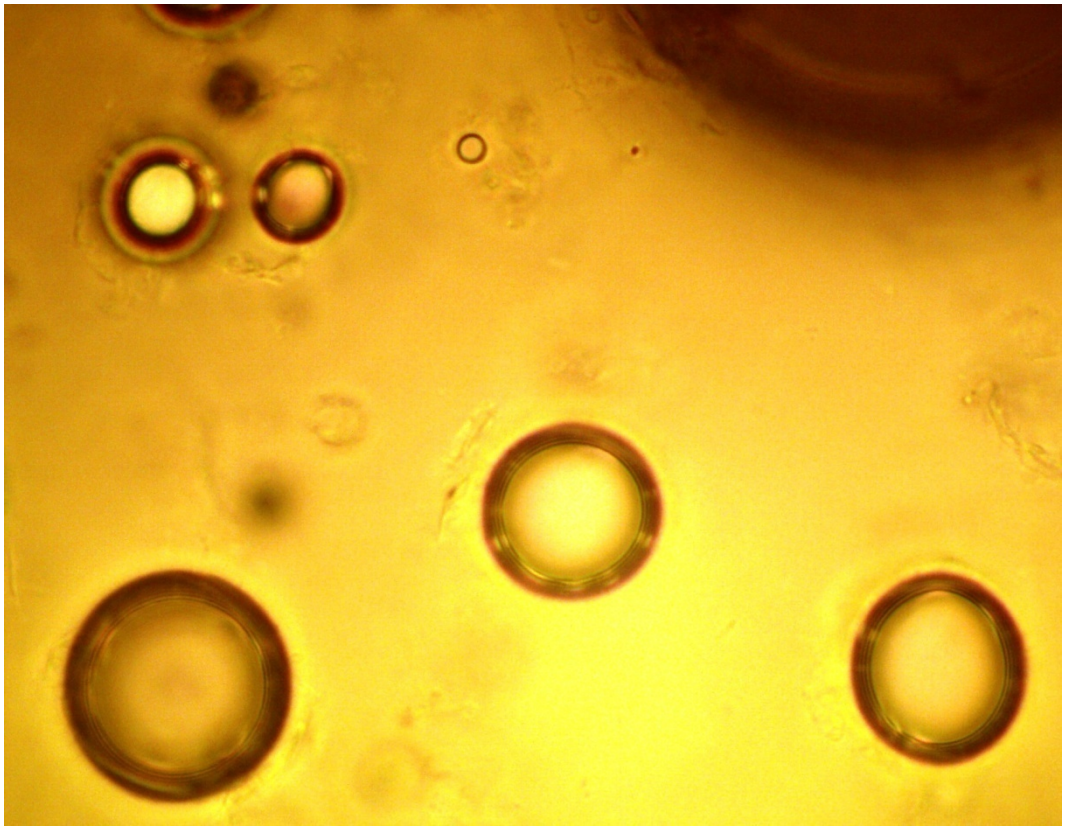


Figure 34. PLM image of L 35.

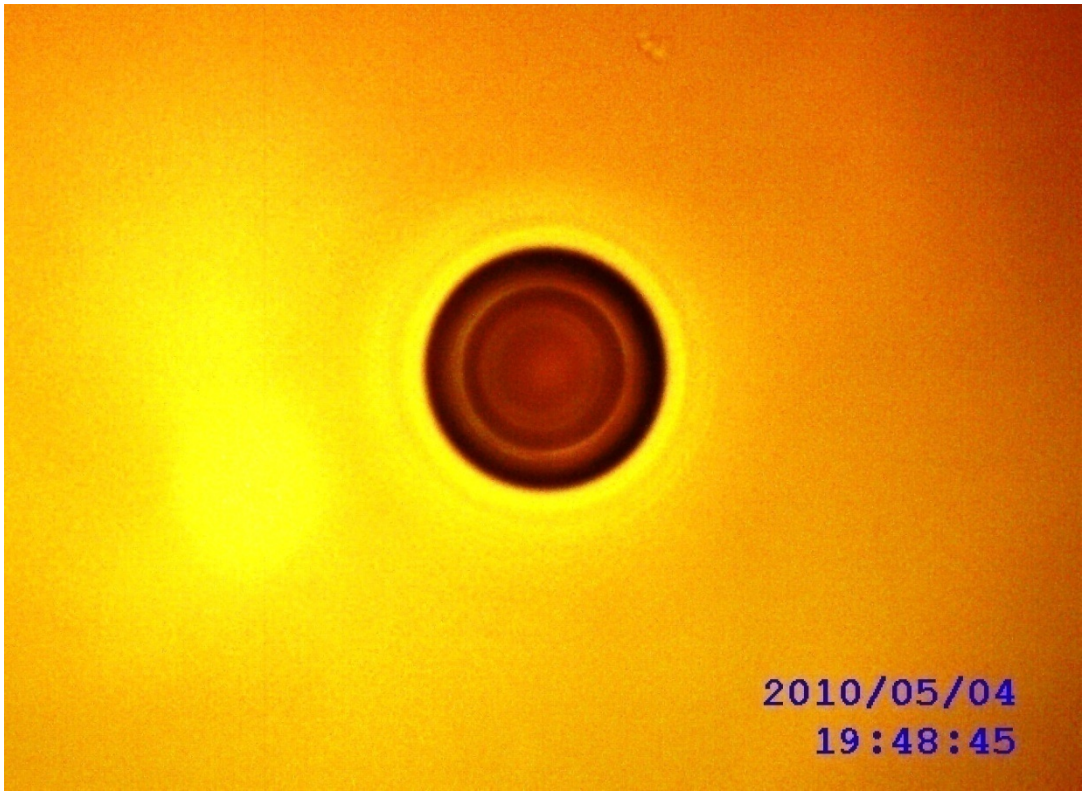


Figure 35. PLM image of L 37.

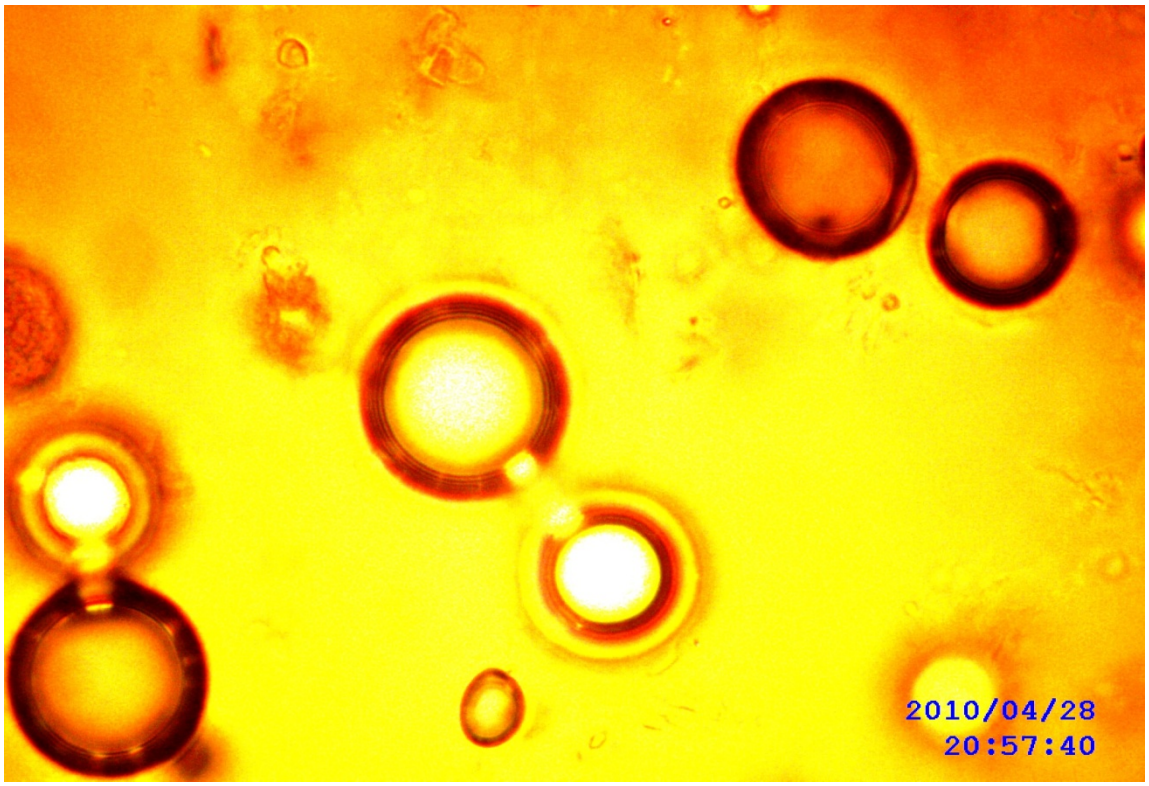


Figure 36. PLM image of L 39.

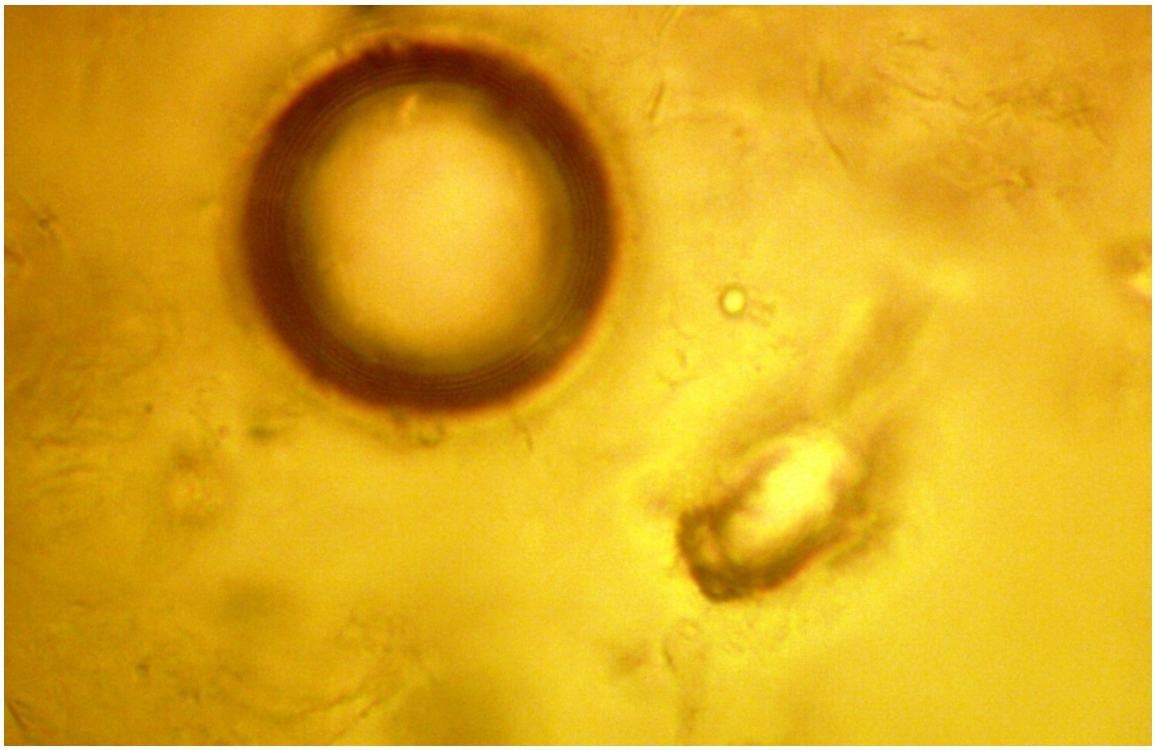


Figure 37. PLM image of L 41.

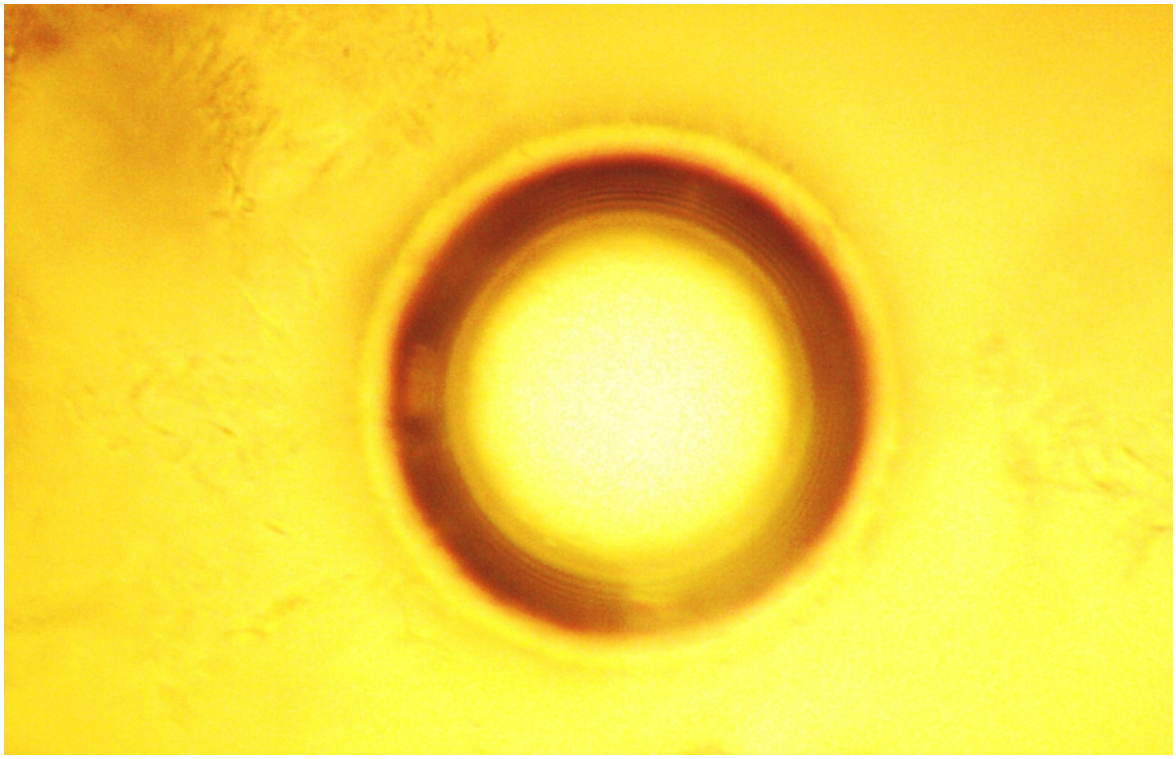


Figure 38. PLM image of L 43.

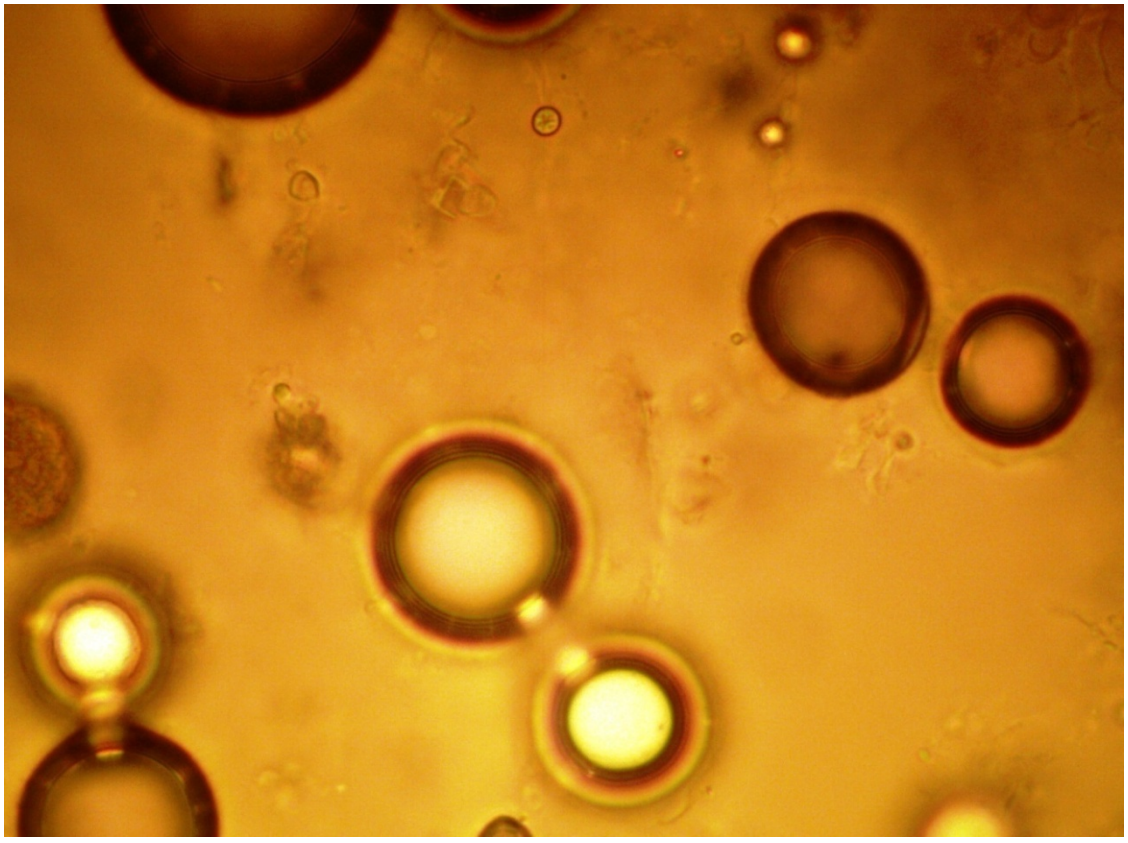


Figure 39. PLM image of L 45.

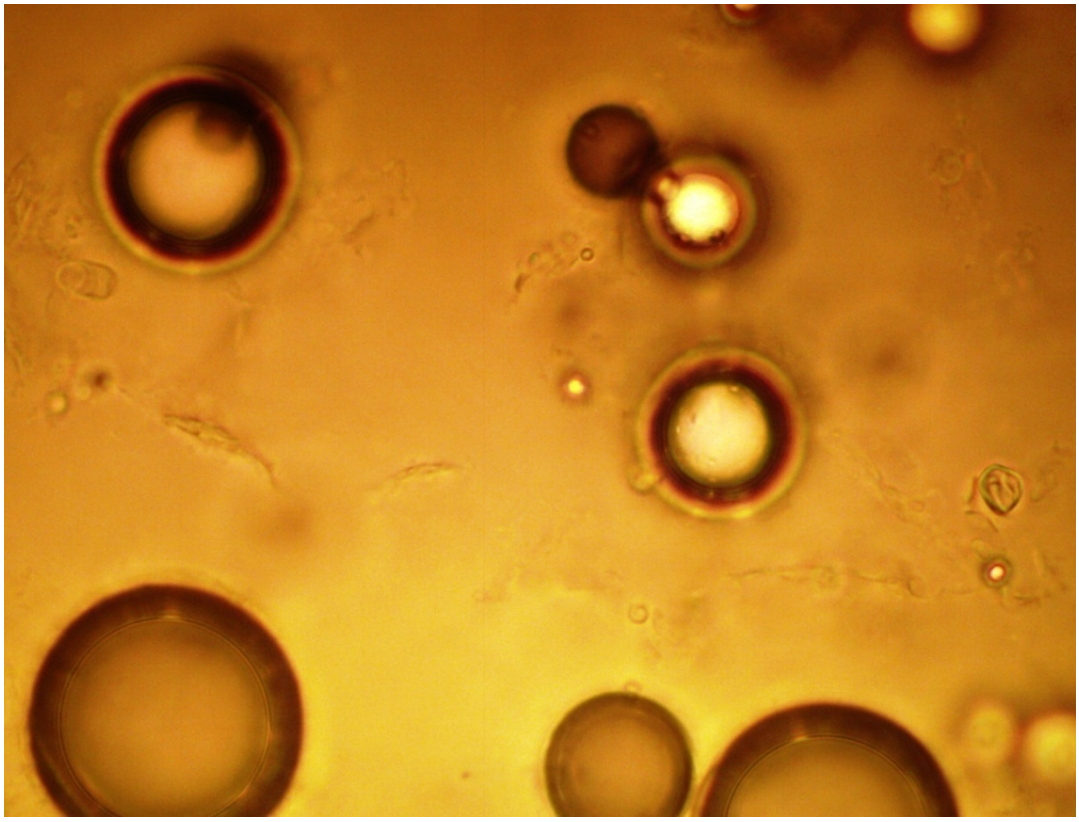
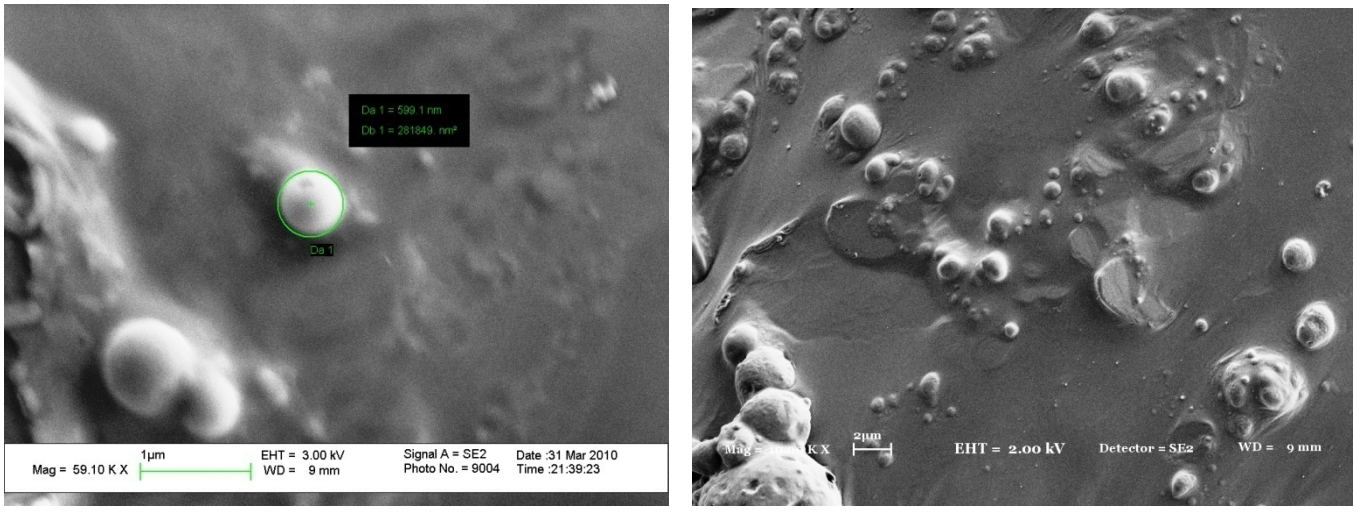


Figure 40. PLM image of L 47.

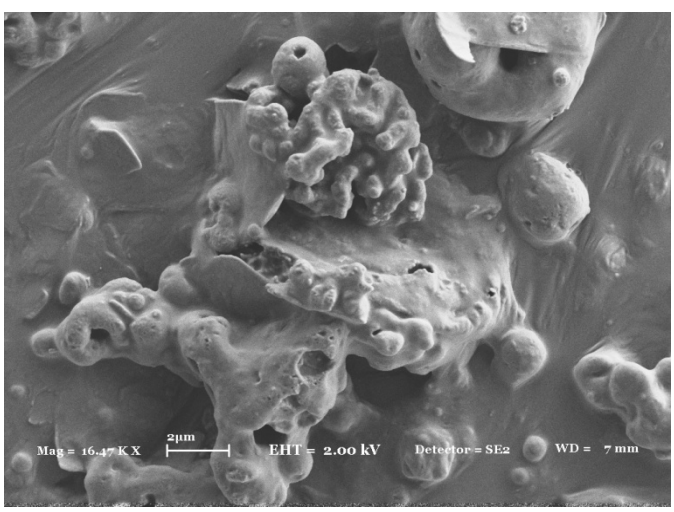
3.3.5. SEM IMAGES of LIPOSOMES

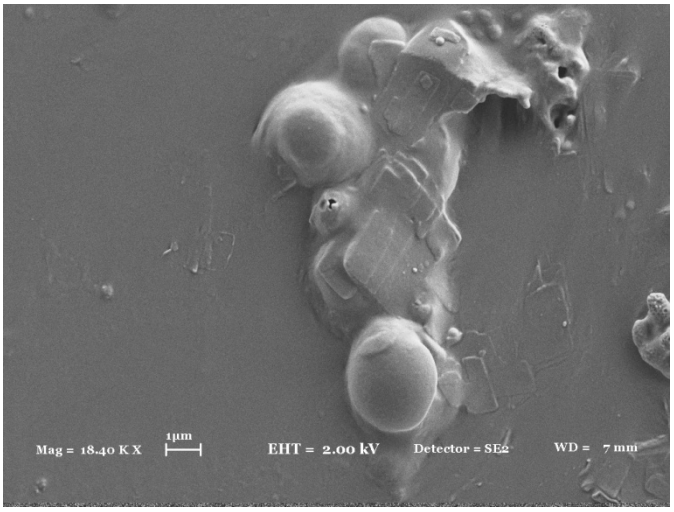
SEM images of liposomes were illustrated in Figure 41-56.



A **B**

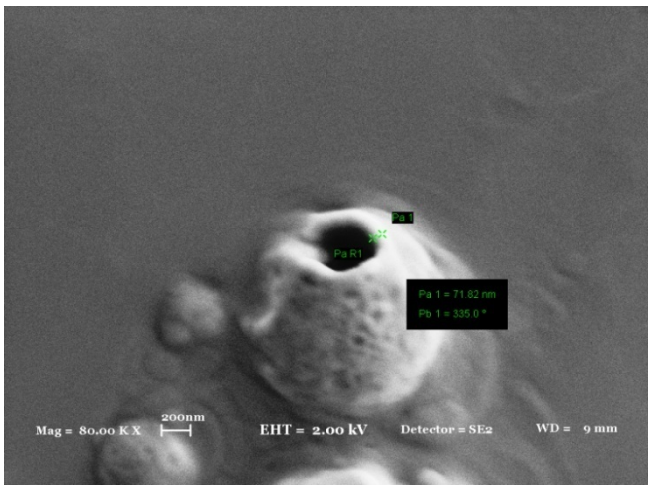
Figure 41. SEM images of L 32 (A, B).

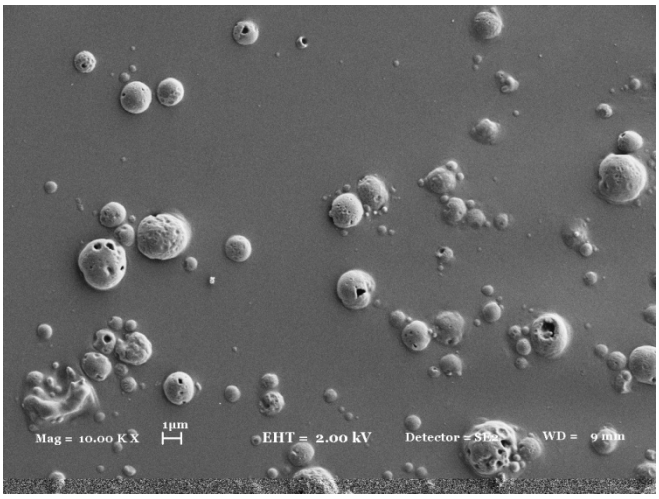




A **B**

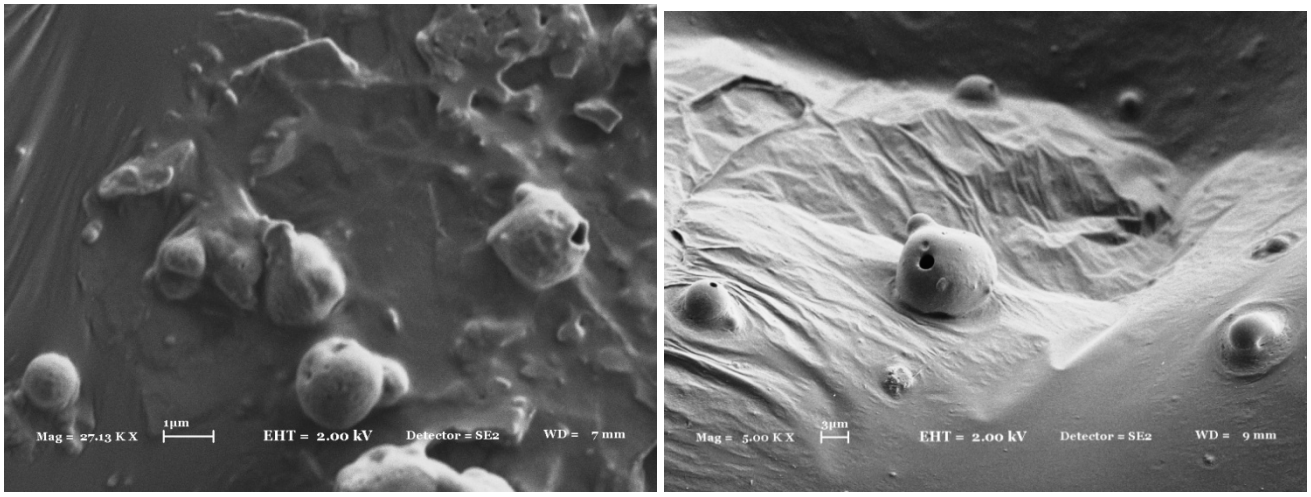
Figure 42. SEM images of L 33 (A, B).





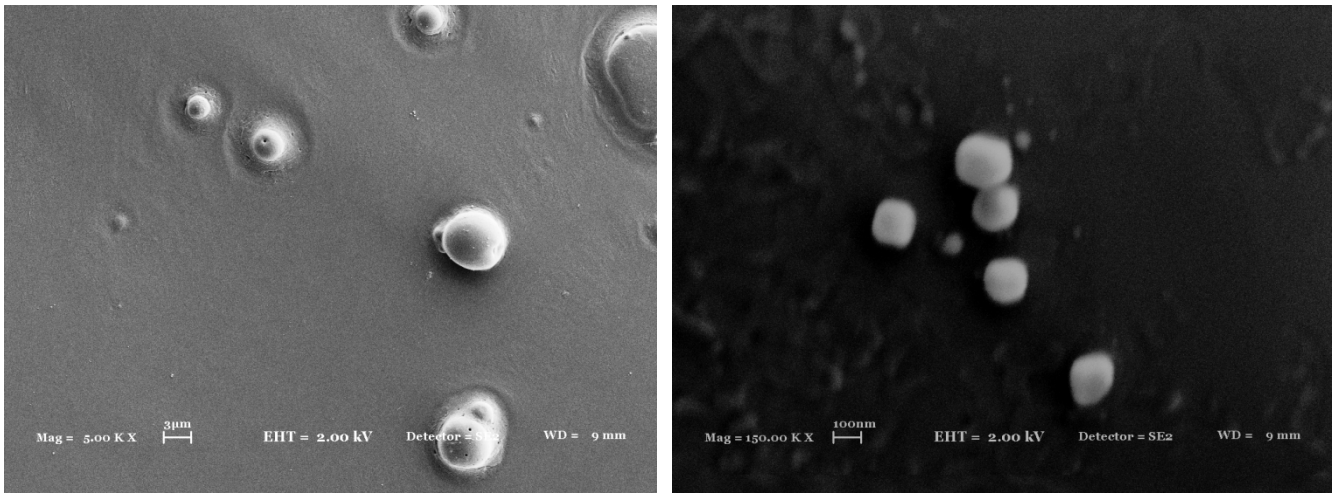
A **B**

Figure 43. SEM images of L 34 (A, B).

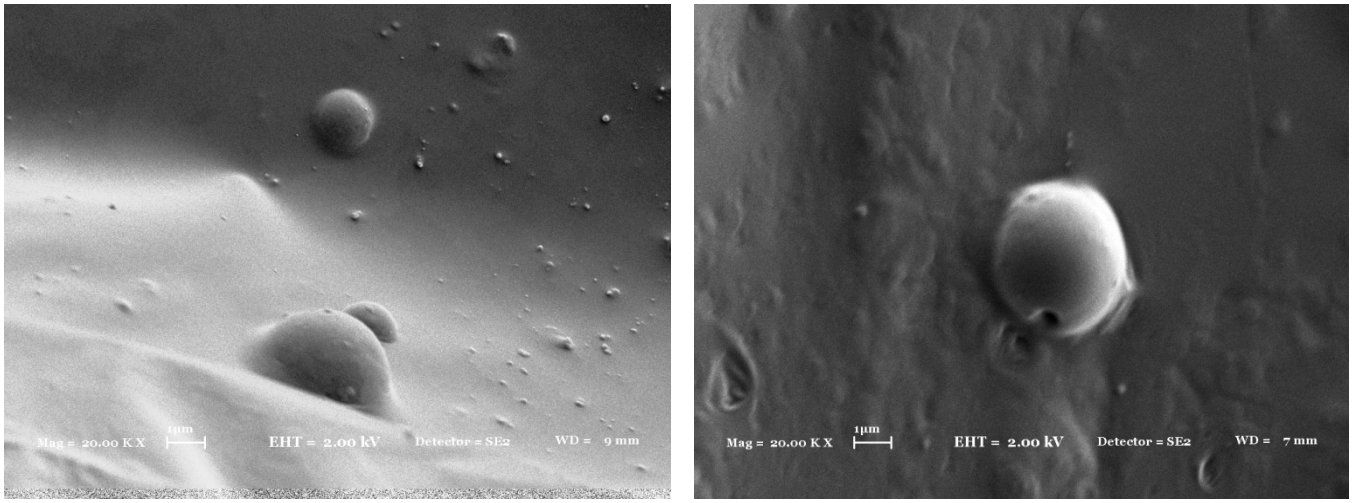


A **B**

Figure 44. SEM images of L 35 (A, B).

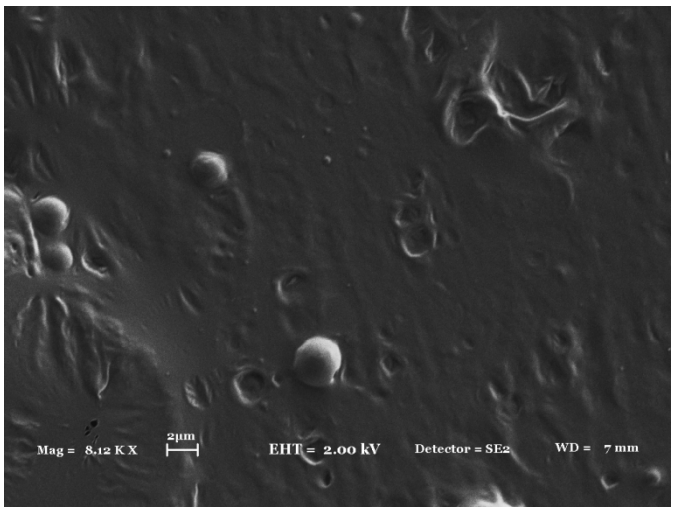


A **B**

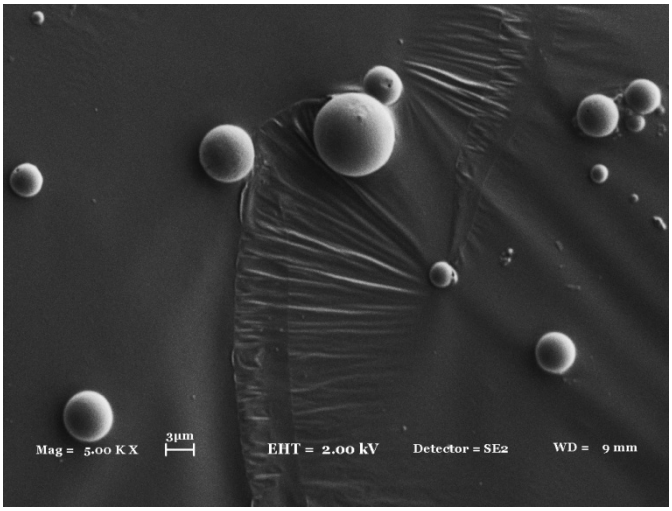


C **D**

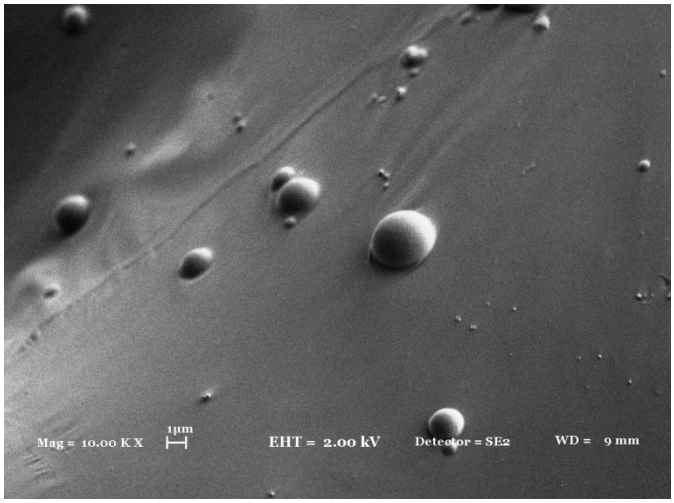
Figure 45. SEM images of L 36 (A, B, C, D).



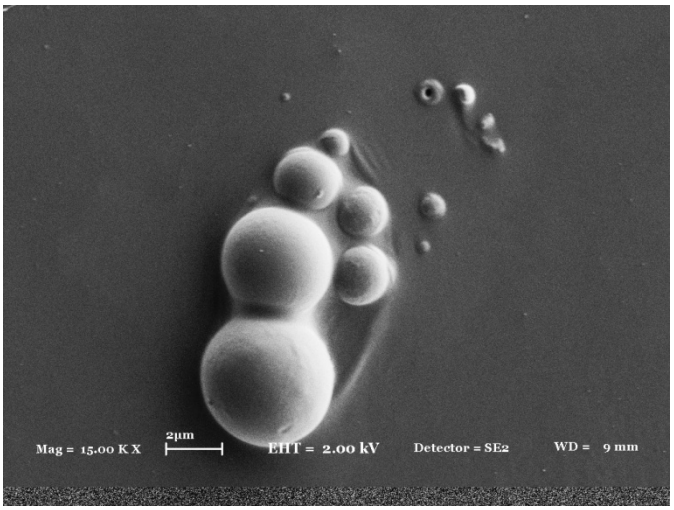
A



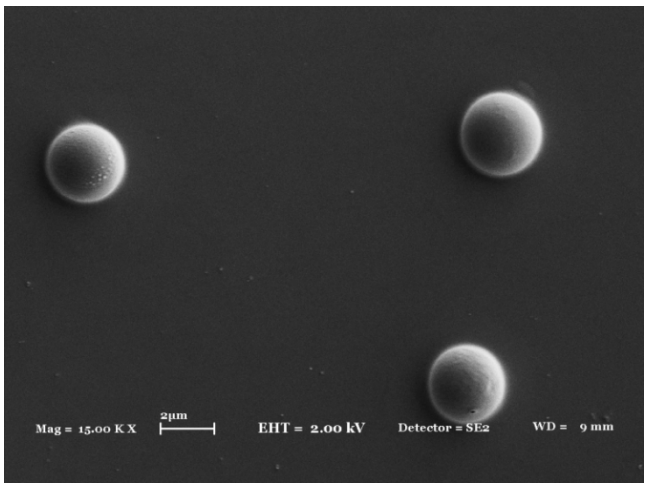
B



C

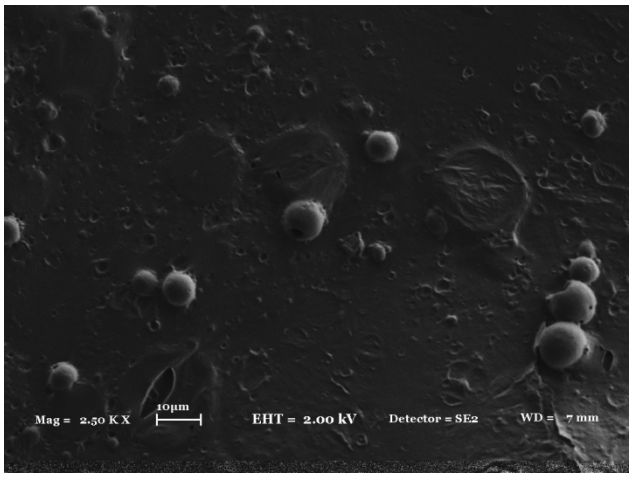


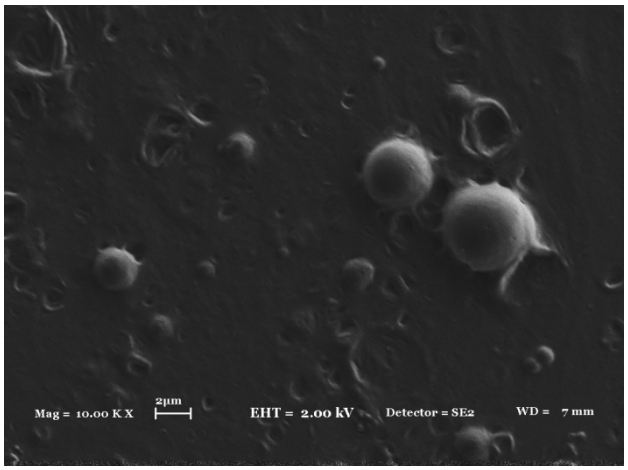
D



E

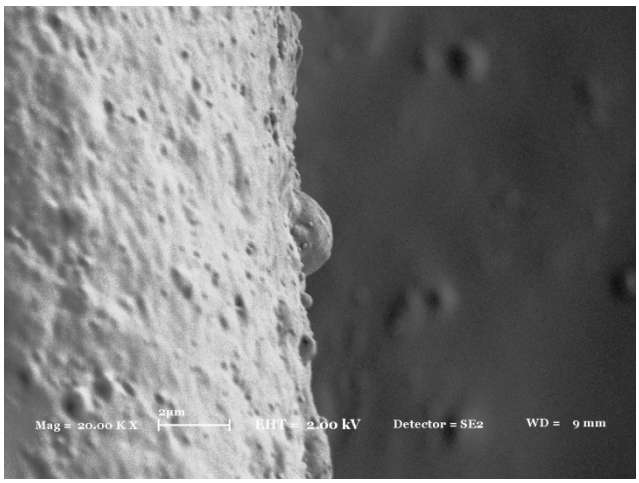
Figure 46. SEM images of L 37 (A, B, C, D, E).





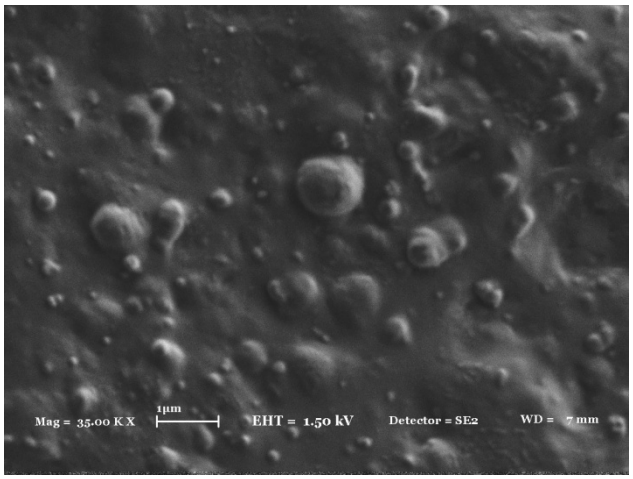
A **B**

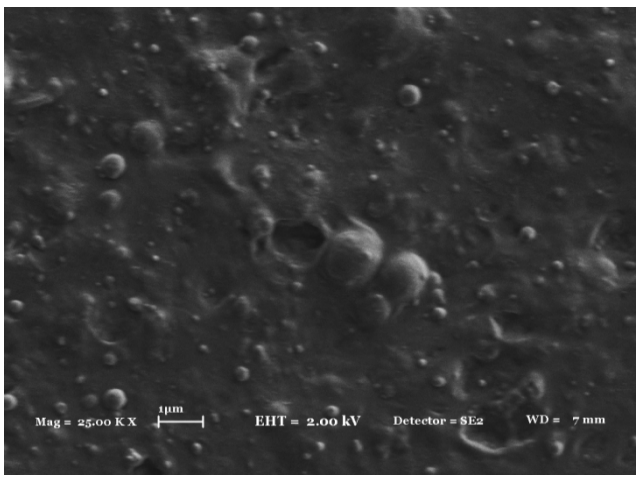
Figure 47. SEM images of L 38 (A, B, C, D, E) .



A **B**

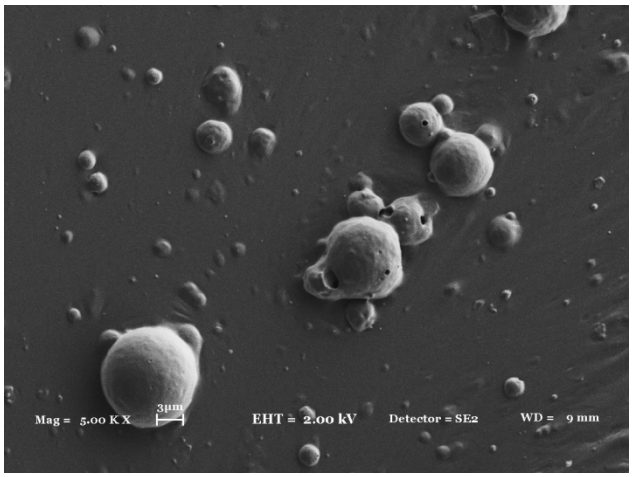
Figure 48. SEM images of L 39 (A, B).

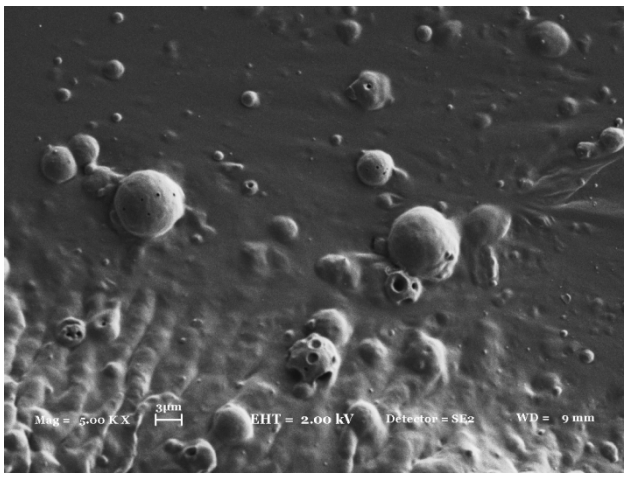




A **B**

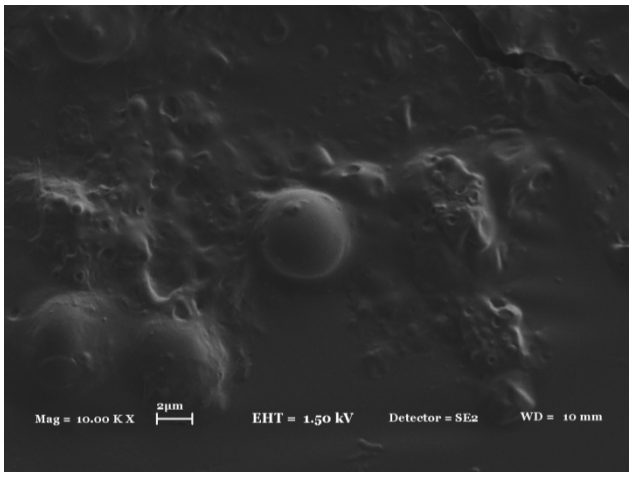
Figure 49. SEM images of L 40 (A, B).

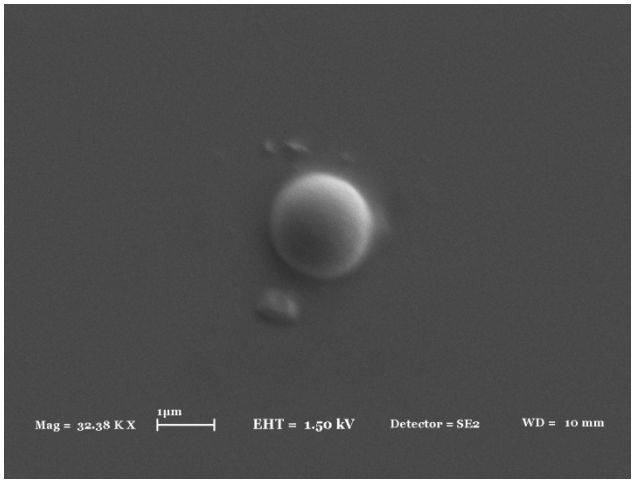




A **B**

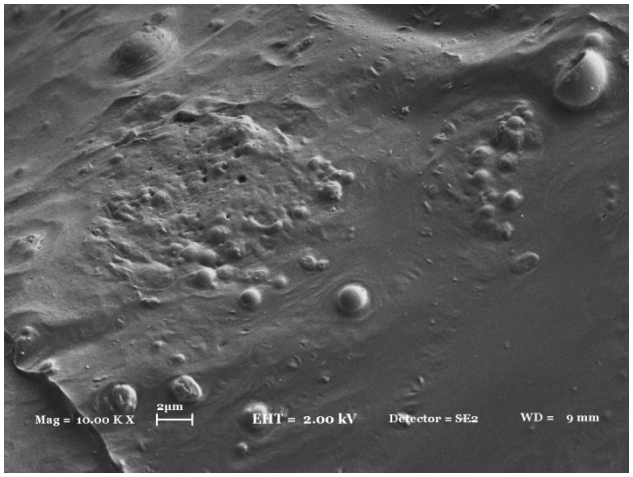
Figure 50. SEM images of L 41 (A, B).

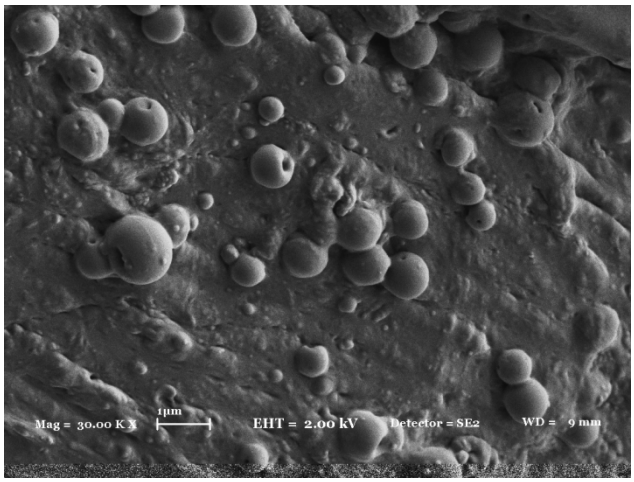




A **B**

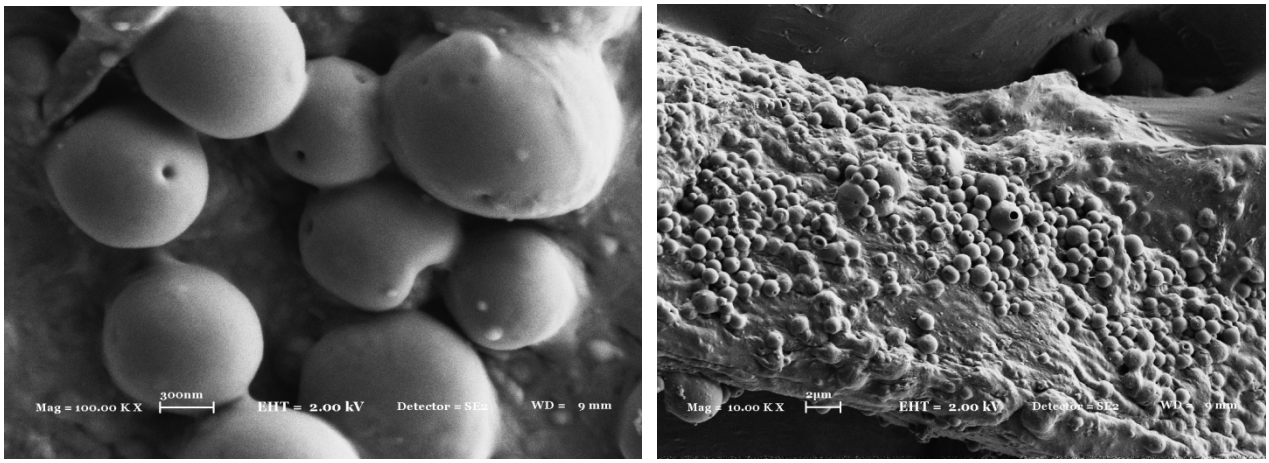
Figure 51. SEM images of L 42 (A, B).



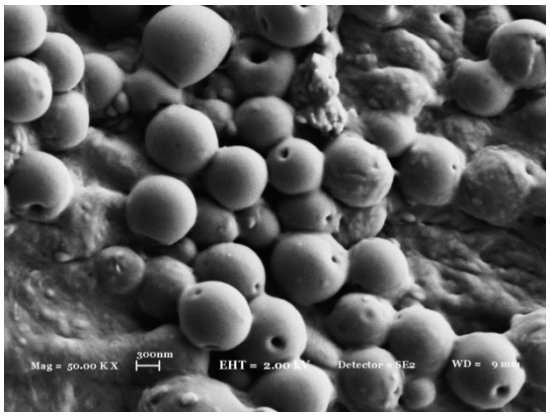


A **B**

Figure 52. SEM images of L 43 (A, B).

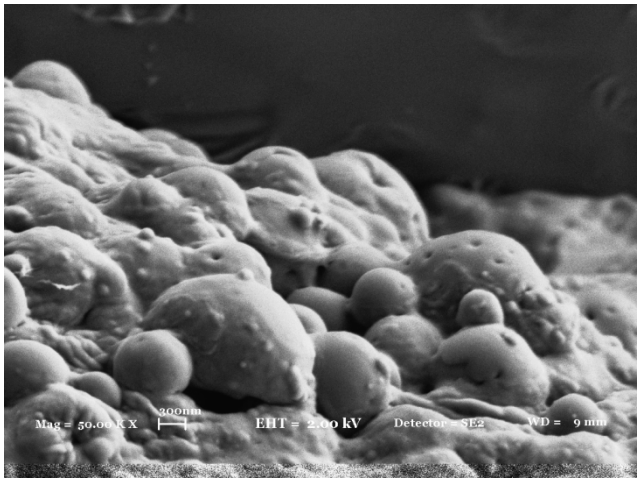


A **B**

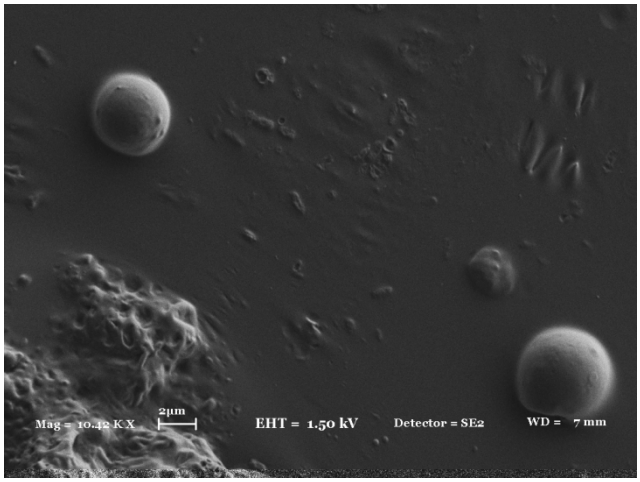


C

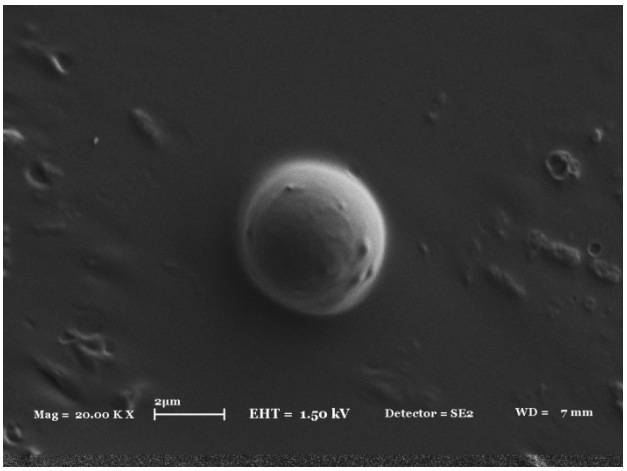
Figure 53. SEM images of L 44 (A, B, C).



A

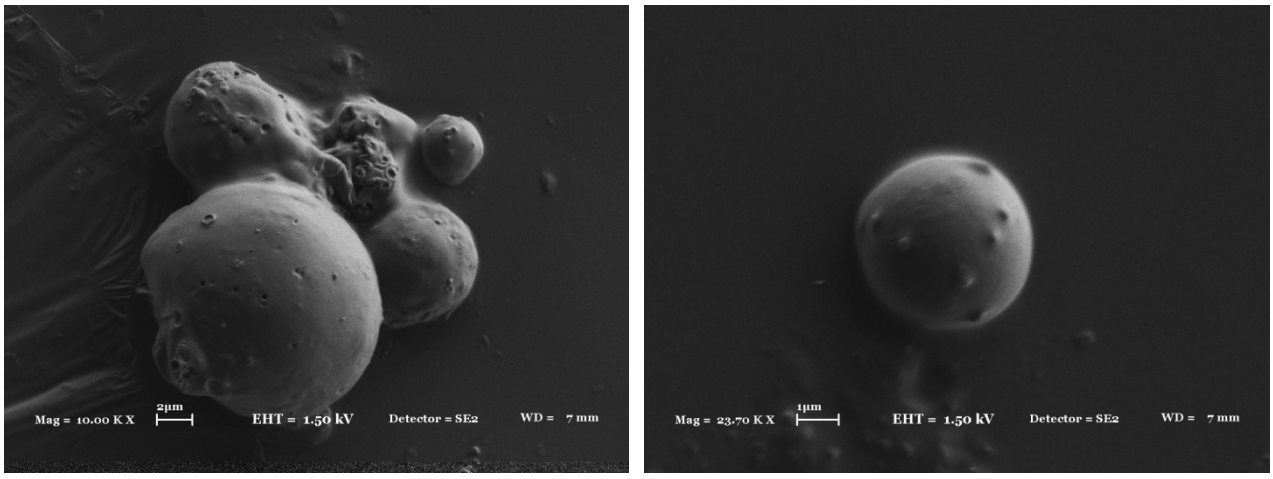


B

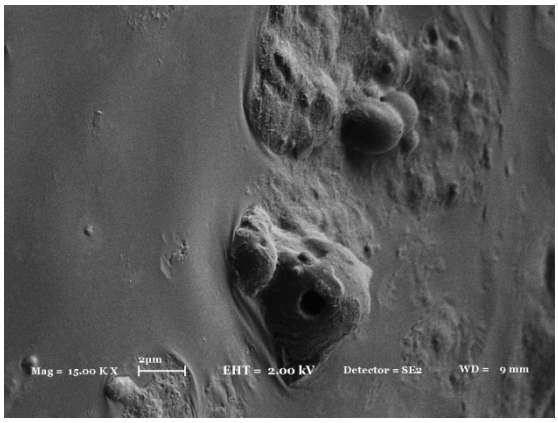


C

Figure 54. SEM images of L 45 (A, B, C).

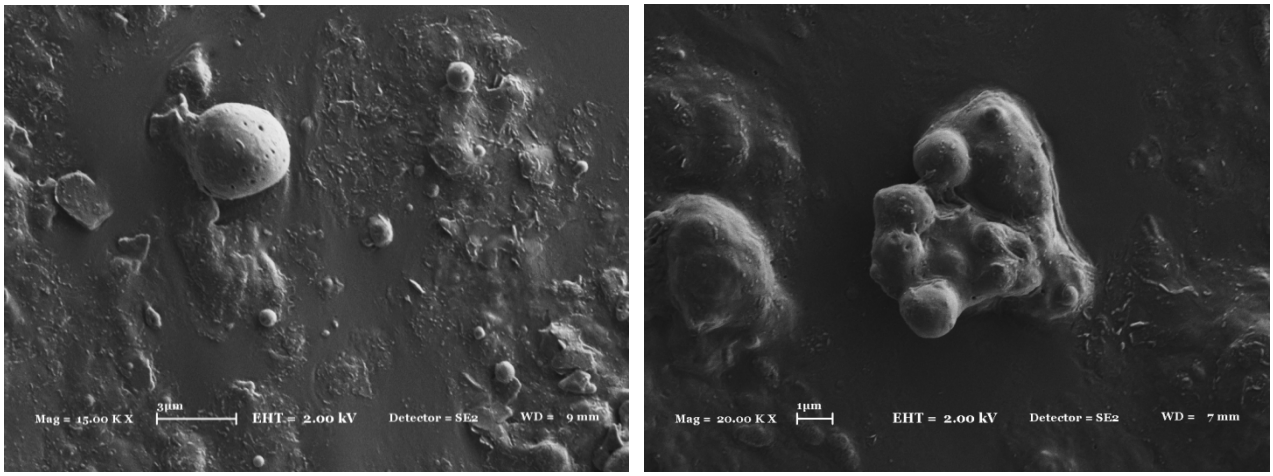


A **B**

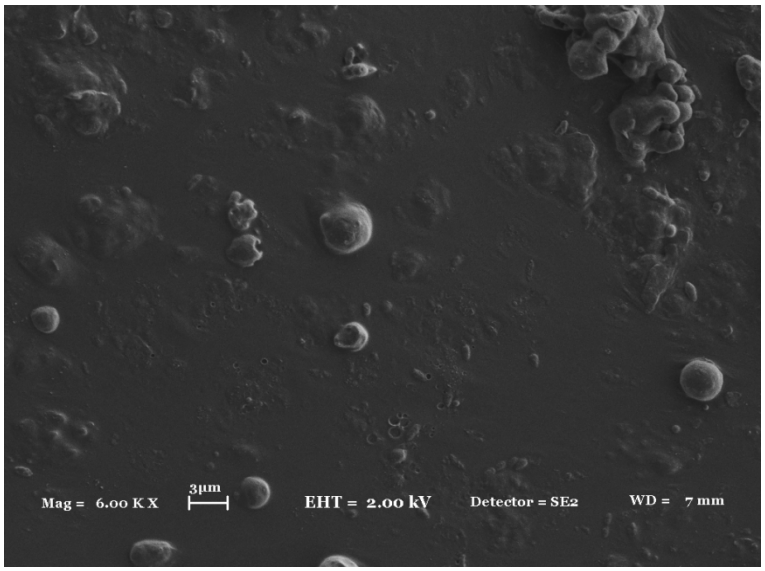


C

Figure 55. SEM images of L 46 (A, B, C).



A **B**



C

Figure 56. SEM images of L 47 (A, B, C).

3.3.6. AFM IMAGES of LIPOSOMES

AFM of L 37 was illustrated in Figure 57.

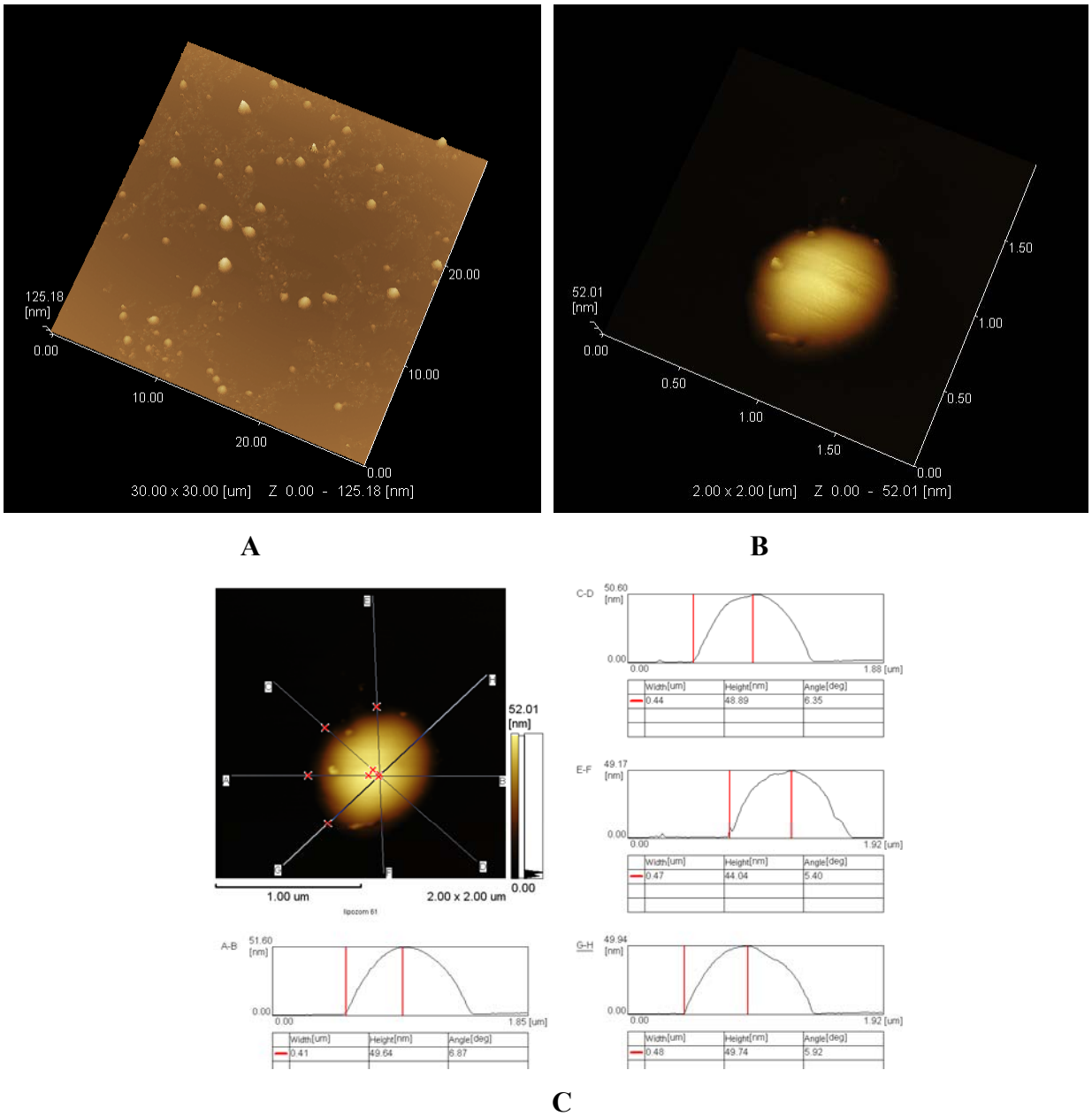


Figure 57. AFM images of L 37 (A, B, C).

3.3.7. RESULTS of PHYSICAL STABILITY STUDIES on LIPOSOME FORMULATIONS

The results of the stability studies of L 37 liposomes carried out under normal (25 ^{0}C and 60 % relative humidity) and refrigerator (4 ^{0}C) conditions covering 3 months were shown in Table 14.

Table 14. Results of stability studies on L 37 for three months (n=3).

Time (day)	Average diameter (nm) at 4^{0}C	Average diameter (nm) at 25^{0}C	Zeta potential at 4^{0}C (mV)	Zeta potential at 25^{0}C (mV)	Encapsulation capacity at 4^{0}C (%)	Encapsulation capacity at 25^{0}C (%)
1	1338 ± 66.6	1405 ± 33.6	-10.4 ± 0.21	-17.6 ± 0.20	14.95	14.95
3	1341 ± 68.5	1494 ± 27.2	-16.7 ± 0.45	-18.4 ± 0.17	14.80	14.57
7	1459 ± 38.4	1527 ± 20.2	-20.6 ± 0.80	-23.6 ± 1.02	13.93	14.17
14	1468 ± 52.3	1727 ± 28.2	-19.6 ± 0.30	-25.4 ± 0.06	13.72	13.38
21	1487 ± 54.9	1818 ± 23.7	-21.9 ± 0.52	-37.8 ± 0.09	13.17	12.06
30	2056 ± 28.7	2850 ± 28.2	-25.2 ± 0.55	-34.7 ± 0.55	12.70	11.55
60	2515 ± 21.7	4187 ± 22.21	-29.1 ± 0.60	-38.8 ± 0.61	11.91	10.75
90	3442 ± 45.2	Not determined	-26.8 ± 0.81	-39.7 ± 1.44	11.06	9.82

Table 14 a. Student's t test results of encapsulation capacity (^{0}4 C and ^{0}25 C) of L 37 formulation.

FORMULATION	L 37 (^{0}4 C and ^{0}25 C)
Student's t test	insignificant p>0.05

3.4. RESULTS of GEL and LIPOGELOSOME FORMULATIONS

Rheological, FT-IR, DSC results of gel and lipogelosome formulations were given in this Chapter.

3.4.1. RESULTS of POLYMER IDENTIFICATION STUDIES with FT-IR

FT-IR results of polymer were presented in Figure 58-59.

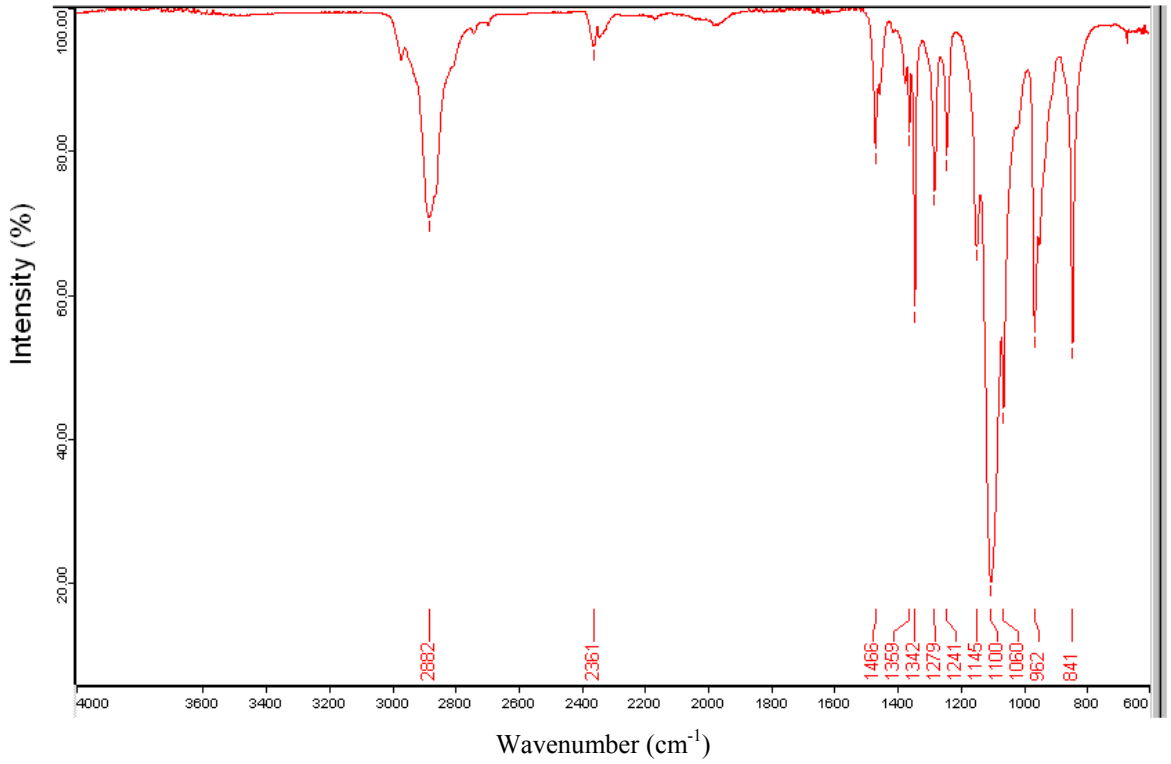


Figure 58. FT-IR spectrum of PXM 407.

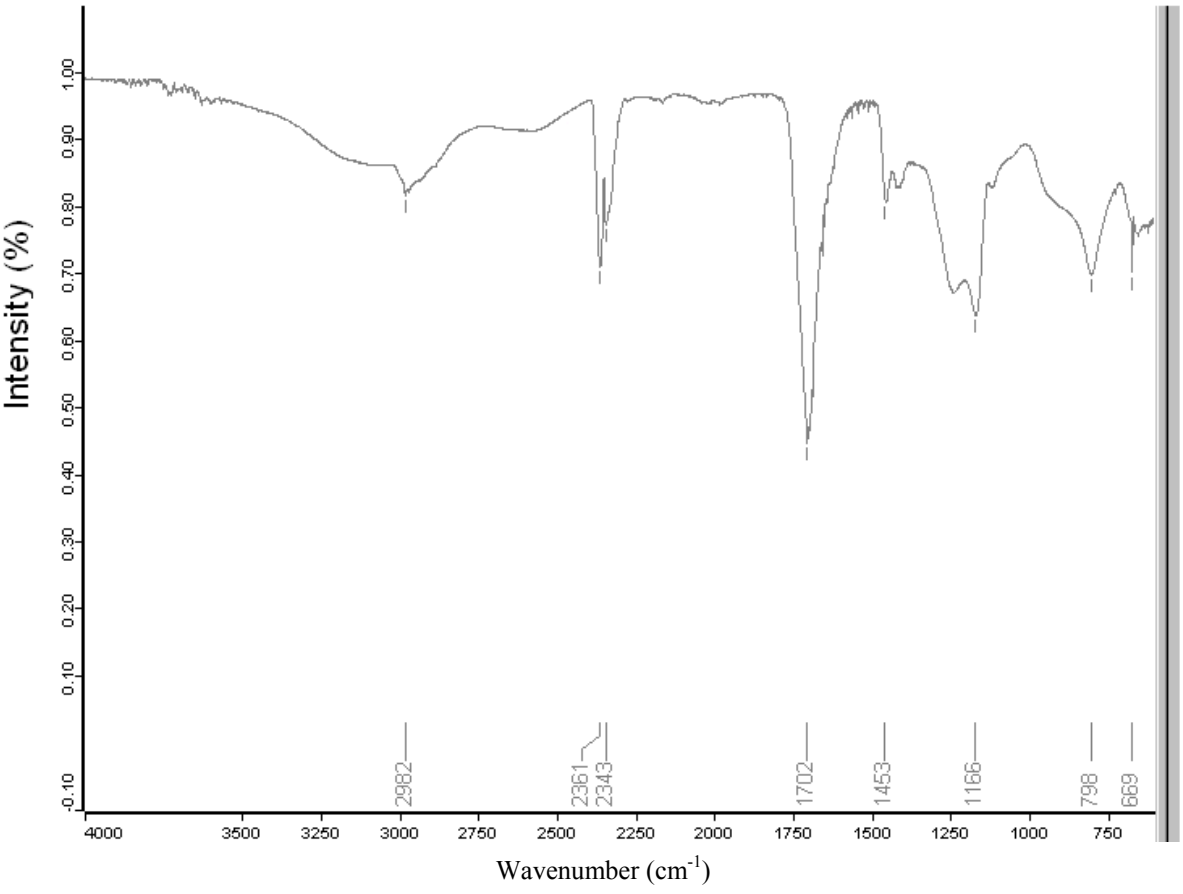


Figure 59. FT-IR spectrum of U 21.

3.4.2. RESULTS of POLYMER IDENTIFICATION STUDIES with DSC

DSC results of polymers were shown in Figure 60-61. Phase transition temperatures were found as 61.2 ^{0}C and 98 ^{0}C for PXM 407 and U 21, respectively.

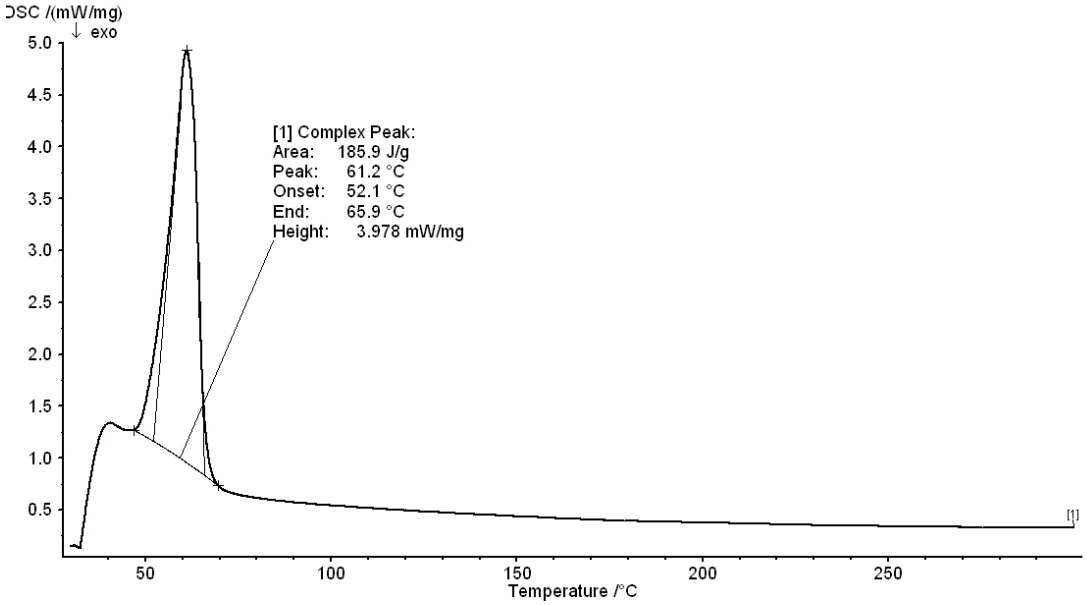


Figure 60. DSC thermogram of PXM 407.

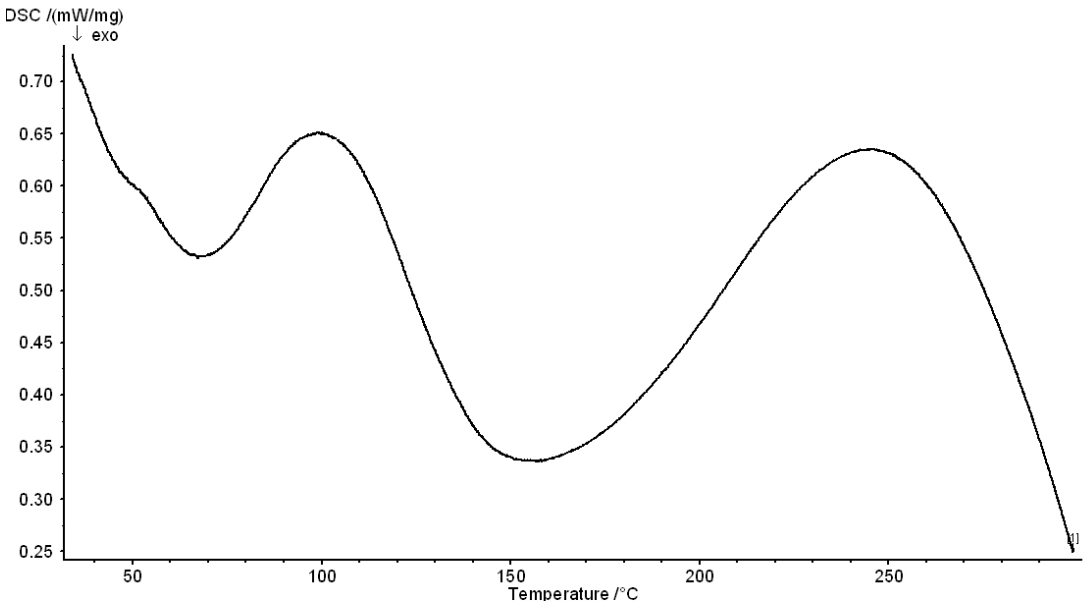


Figure 61. DSC thermogram of U 21.

3.4.3. RESULTS of RHEOLOGICAL STUDIES on GEL and LIPOGELOSOMES

Rheological diagrams of gel and lipogelosome formulations were in Figure 62-65.

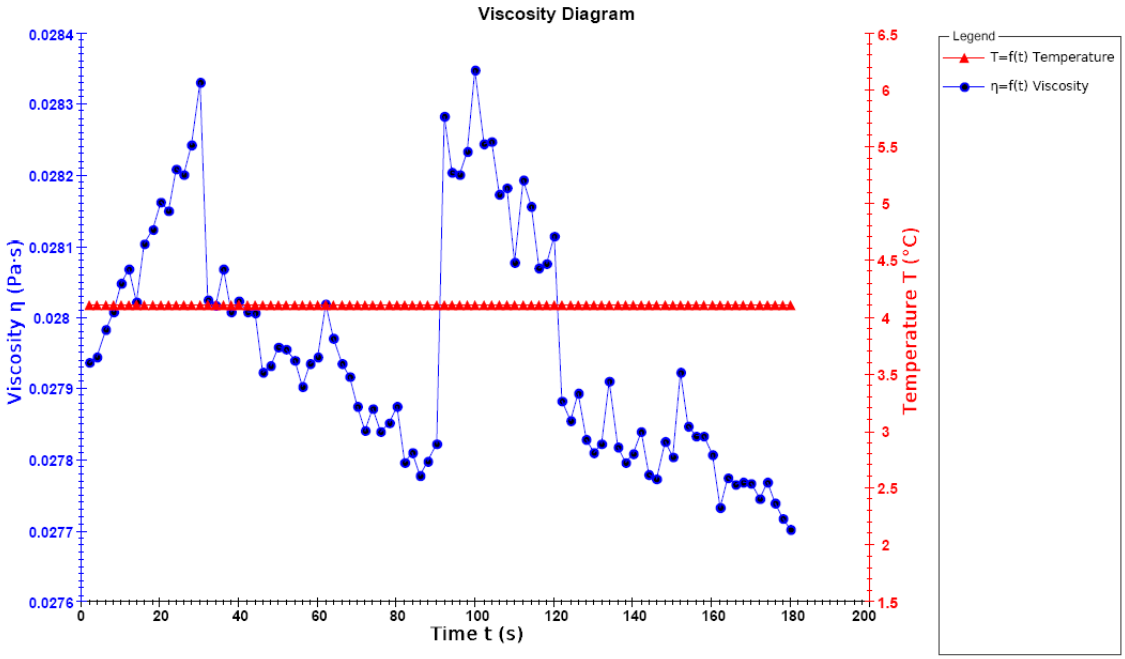


Figure 62. Viscosity diagram of LG 16 at 4^0C.

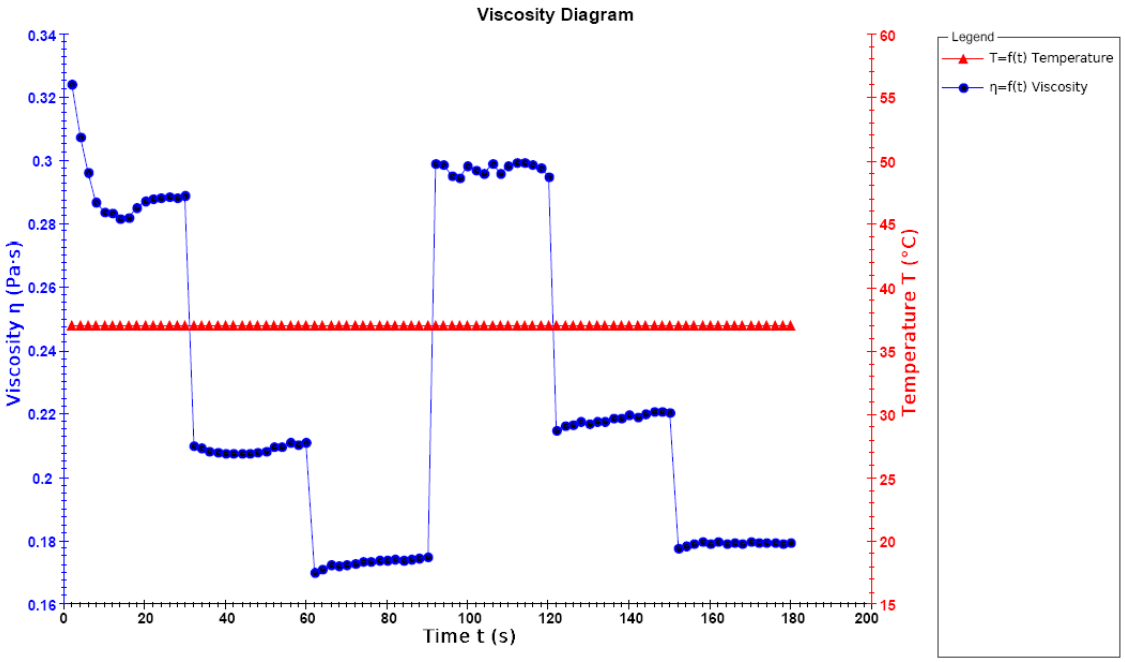


Figure 63. Viscosity diagram of LG 16 at 37^0C.

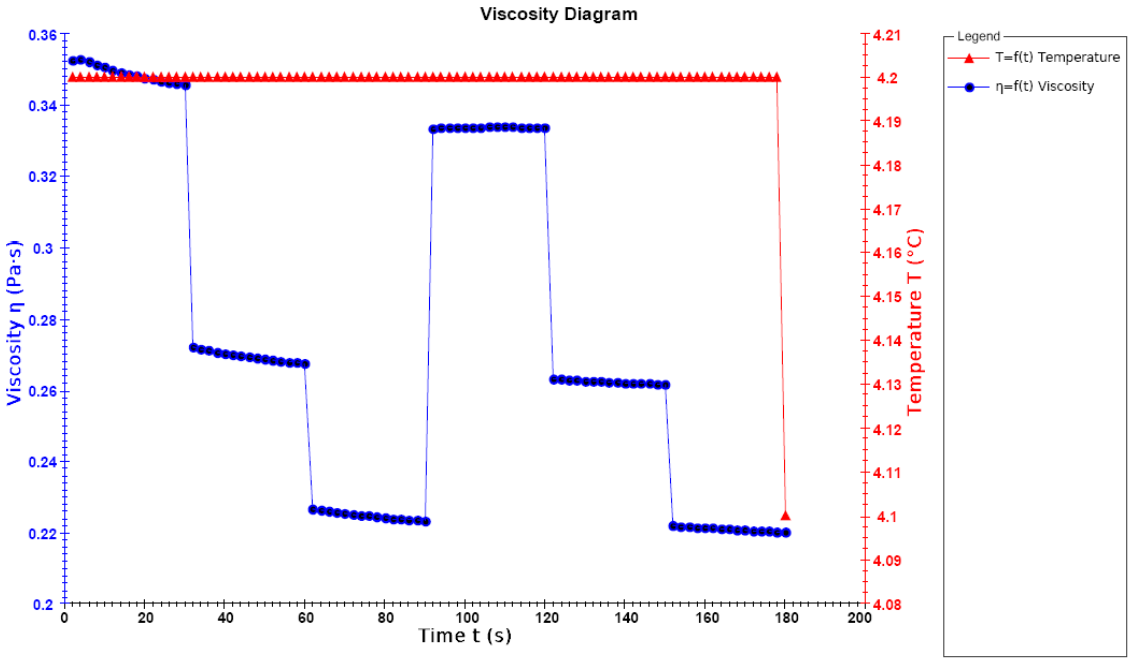


Figure 64. Viscosity diagram of L 8 at 4 ^{0}C.

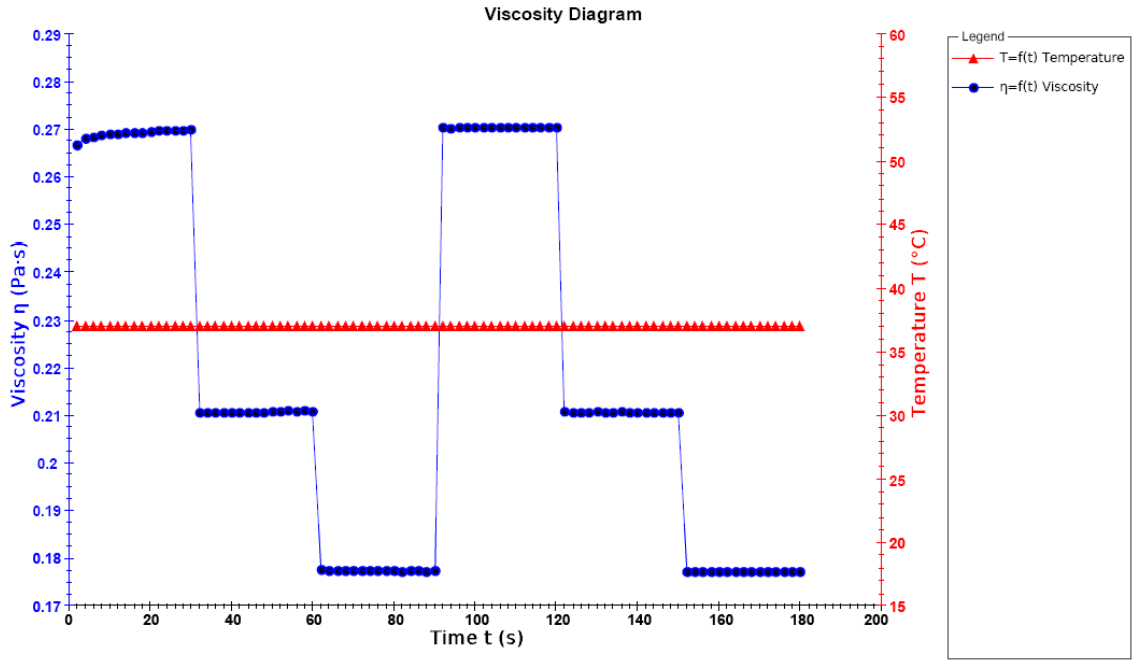


Figure 65. Viscosity diagram of LG 8 at 37^{0}C.

3.5. RESULTS of *IN VITRO* RELEASE STUDIES of Na-HA from LIPOSOMES, LIPOGELOSOMES and COMMERCIAL FORMULATION

Results of in vitro release studies of Na-HA from liposomes, liposogelosomes and the commercial formulation were shown in Figure 66. In addition, fitting to kinetic models of L 37, LG 8 and marketed Formulation of Na-HA CP (Commercial Product) were shown in Table 15.

In Table 15, the fitting to kinetic models of Na-HA from LG 8 and L 37 formulations were investigated. For this purpose; zero-order, first-order, Higuchi and Hixson-Crowell kinetic models were employed for fitting. The best fitting model was zero-order for all formulations including L 37, LG 8 and commercial product of Na-HA.

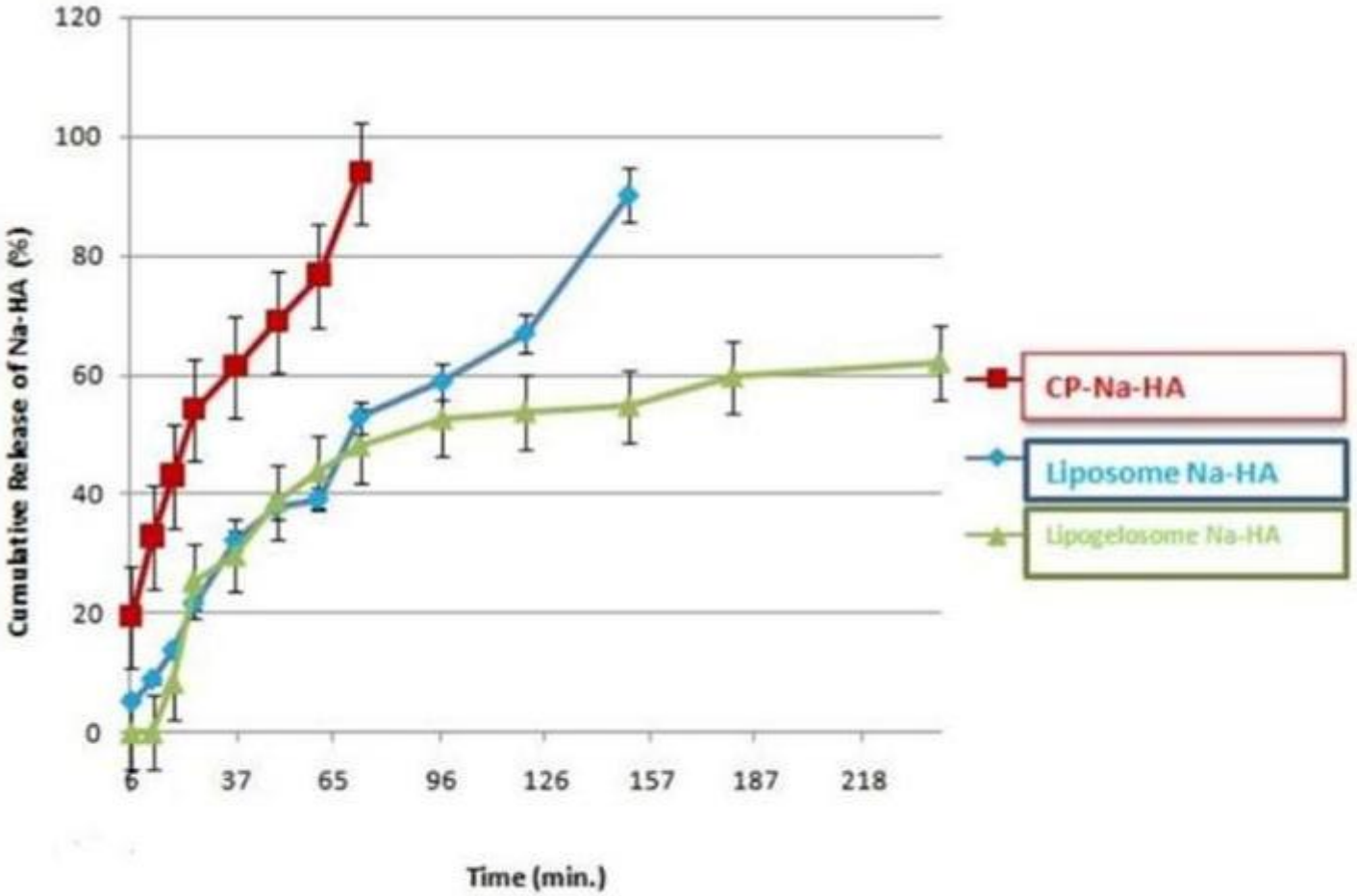


Figure 66. Comparative release of Na-HA from liposome dispersion (L 37), lipogelosome (LG 8) and CP of Na-HA (n=6).

Table 15. Fit to kinetic models of L 37, LG 8 and commercial formulation of Na-HA.

Kinetic Model		**L 37**	**LG 8**	**Commercial Formulation of Na-HA**
Zero Order	**Slope**	0.56	0.17	0.99
	Intercept	6.31	21.22	21.89
	R	0.99	0.96	0.98
	r^2	0.98	0.92	0.96
First Order	**Slope**	0.01	0.01	0.01
	Intercept	1.03	0.29	1.41
	R	0.89	0.76	0.92
	r^2	0.78	0.58	0.84
Higuchi	**Slope**	0.05	0.04	0.07
	Intercept	3.16	2.74	4.96
	R	0.96	0.90	0.97
	r^2	0.91	0.80	0.92
Hixson-Crowell	**Slope**	0.01	0.01	0.01
	Intercept	8.86	8.65	9.21
	R	0.94	0.86	0.94
	r^2	0.88	0.73	0.88

3.6. PRELIMINARY RESULTS of SKIN HYDRATION STUDIES in HUMAN VOLUNTEERS

Changes of moisture, pH and sebum content of volunteers were given in Table 16. In addition, three dimensional images of volunteers were presented in Figure 67-71.

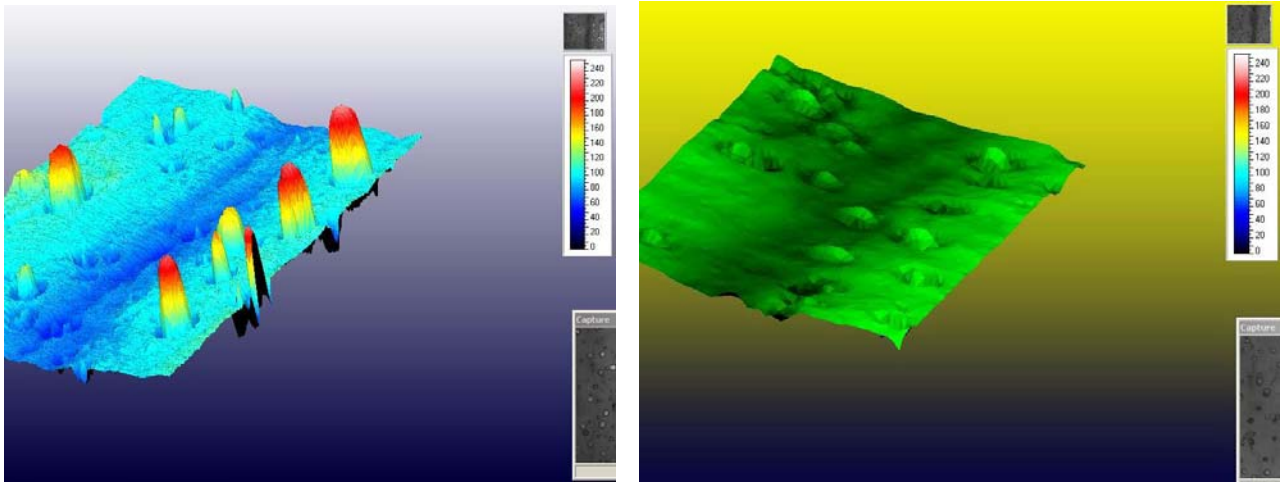
Table 16. Changes of moisture, pH and sebum content of human skin in volunteers.

Group Name	Formulation	Increment of Sebum Content (%)*	Increment of Moisture Level (%)*	pH value (mean)*
A	LG 8	14.83 ± 0.73	30.36 ± 0.12	5.30 ± 0.20
B	LG 7	8.07 ± 0.14	7.5 ± 0.08	4.93 ± 0.03
C	LG 6	25.62 ± 1.25	11.56 ± 0.15	5.21 ± 0.06
D	LG 5	1.17 ± 0.02	6.68 ± 0.52	5.10 ± 0.25
E	CP	13.25 ± 0.35	14.15 ± 0.39	5.14 ± 0.23

(* n=3)

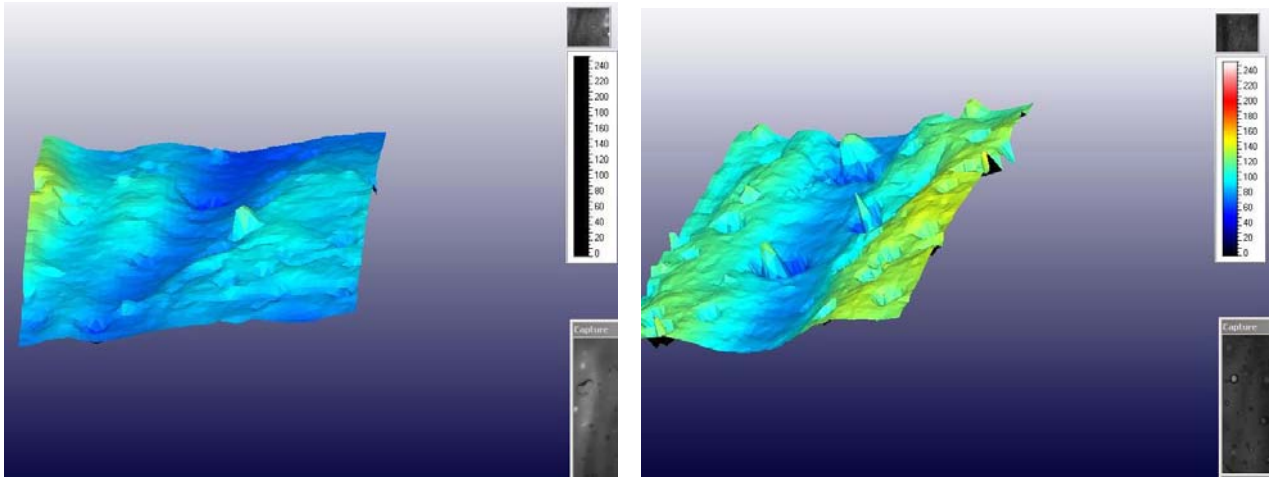
Table 16 a. Formulation components.

LG 8	U 21 (0.5 %) Gel + L37 liposomes
LG 7	U 21 (0.5 %) Gel + L36 liposomes
LG 6	U 21 (0.5 %) Gel + 1% Na-HA
LG 5	U 21 (0.5 %) Gel
CP	Commercial product of Na-HA



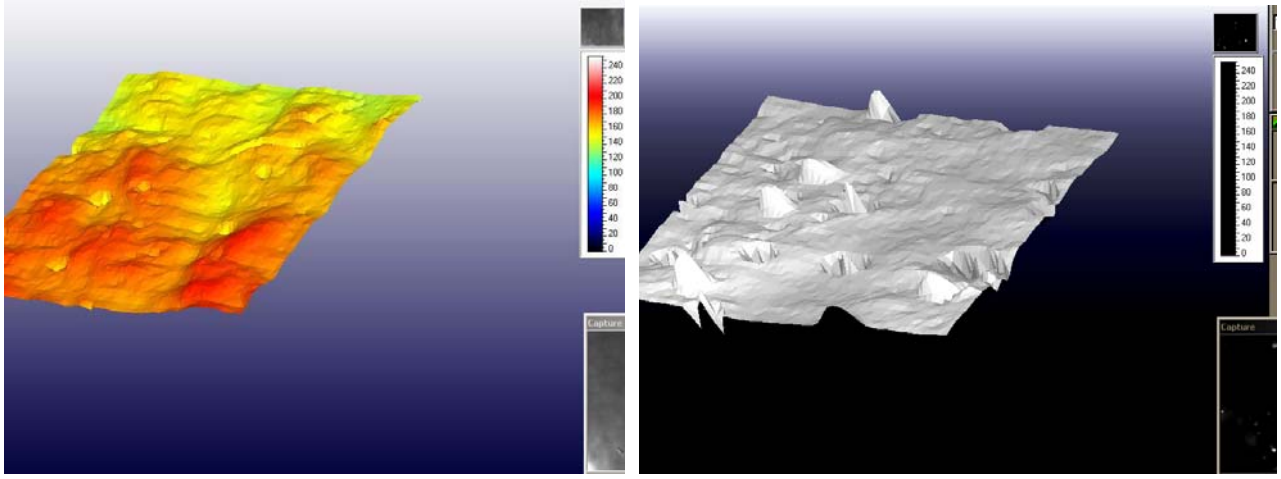
Before After

Figure 67. 3D image of skin of A group (LG 8), before-after two weeks.



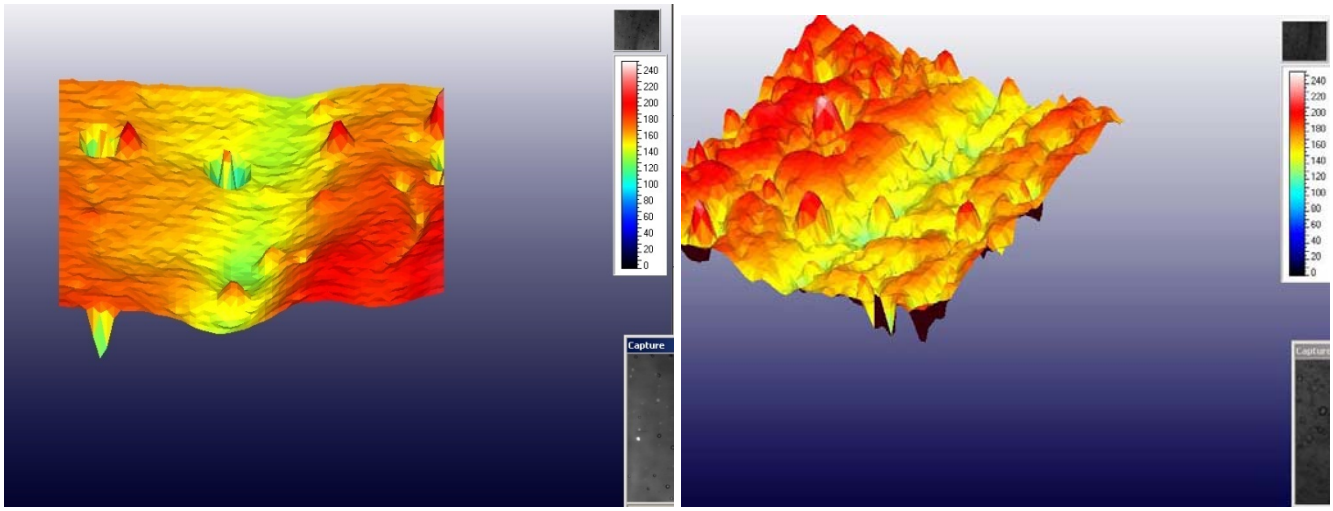
Before After

Figure 68. 3D image of skin of B group (LG 7), before-after two weeks.



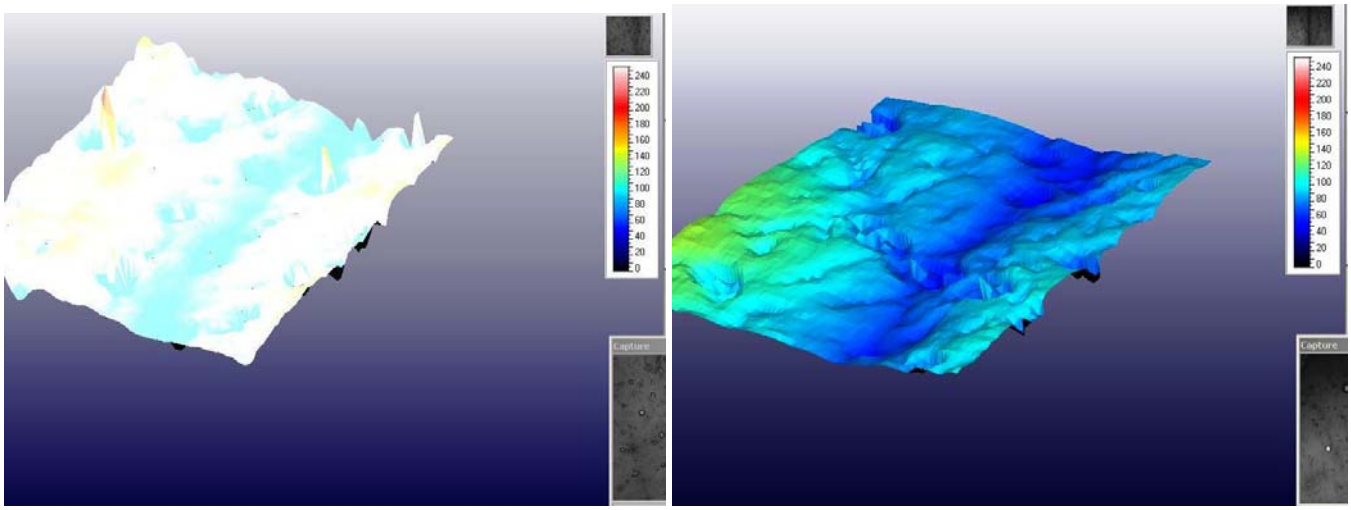
Before After

Figure 69. 3D image of skin of C group (LG 6), before-after two weeks.



Before After

Figure 70. 3D image of skin of C group (LG 5), before-after two weeks.



Before After

Figure 71. 3D image of skin of CP group before-after two weeks.

4. DISCUSSION

In this Chapter of thesis, the all results were discussed respectively the obtained from data about Na- HA liposomal formulation and lipogelosome formulation.

4.1. THE CHOICE of Na-HA

Na-HA is an essential part of the extracellular matrix. Very high concentrations are found in tissues such as the umbilical cord (4 $mg.g^{-1}$), synovial fluid (3-4 $mg.mL^{-1}$), and vitreous humor (0.1-0.4 $mg.g^{-1}$). The mean concentration of Na-HA is 0.02 % (200 $mg.kg^{-1}$). Although a higher Na-HA concentration is found in connective tissues, the greater part of it (56.7 %) is found in the skin (38). In its normal state in tissues, the Na-HA is present as a free polymer, although in some tissues such as cartilage, it is linked to different structural glycoproteins (proteoglycans) or in other tissues to specific cell receptors (37).

Since its discovery in human tissue, Na-HA and its derivatives has been largely studied and applied in the biomedical area. The appeal of this polymer has been accentuated by its high level of biocompatility. It has been used in viscosurgery to allow surgeons to safely create space between tissues. As a microcapsule it can be used for targeted drug delivery. Viscosurgical implants are constructed from Na-HA (51).

Na-HA is reported to have a radical scavenger capacity. Therefore, it was incorporated into semisolid formulations in order to give UV-protection. Hence, determination of the release profile of Na-HA from semisolid formulations is important to predict its topical availability. In the literature, there are no publications up to date regarding the biopharmaceutical characterization of semisolid formulations containing drugs or cosmetics with higher molecular mass such as Na-HA (45).

Na-HA is also a common skin care ingredient. In skin tissue, HA helps transport of essential nutrients from the bloodstream to living skin cells, hydrates the skin by retaining water, and acts as a cushioning and lubricating agent against mechanical and chemical damage. Since 2003, the FDA has approved hyaluronan injections for filling soft tissue defects such as facial wrinkles. Na -HA products have the advantages of longer lasting effects and decreased risk of allergic reaction (51, 52).

The skin's dermis layer is made up of about 70 % water and contains nearly 50 % of human body's total HA allotment; there it helps to support and hydrate the skin, resulting in a healthy and attractive appearance (72).

Na-HA has a smaller molecular size as HA (making it especially penetrative), and is able to hold more water than any other natural substance up to 1000 times its weight in water (73).

In this thesis, it was decided to work extra small size of Na-HA (45 kDa) so, when applied topically to the skin it can reach deep down into the dermis. Its hydrating properties will result in a smoother and softer skin with decreased wrinkles and an all-around fuller appearance. It helps the skin bring and absorb more water more effectively and it reduces any sort of trans-epidermal water loss (TEWL). Although HA and its various derivatives have been used in skin-care products and cosmetics for some time, there have been no published clinical studies on its topical application. Noga Yerushalmi at al. studied bioadhesive liposomes, in which Na-HA is the surface-anchored bioadhesive ligand, are being tested *in vitro* in order to evaluate their bioadhesivity (73).

The method for determination of Na- HA was performed by using UV spectrophotometer. Validation of the proposed method was performed with the evaluation of linearity, precision, accuracy and stability. Data are presented Chapters 3.1.2 and 3.1.3 which demonstrate that the spectrophotometer method was accurate, precise and linear for the determination of Na-HA. For the Na-HA in 10 mM Tris buffer (pH: 5.5), the RSD and CV values were found as 6.1902 and 0.062, respectively (Figure 22). In addition, for the 2.5 % Triton X-100 containing Na-HA in 10 mM Tris buffer (pH: 5.5), the RSD and CV values were found as 10.4372 and 0.104, respectively (Figure 23).

4.1.1. STABILITY of Na-HA

Stability of Na-HA in different conditions was determined by FT-IR method. The spectra of different conditions of Na-HA were very similar, indicating that vortex, heating, ultra-sonication process and freeze drying did not affect the physical structure of Na-HA (Chapter 3.1.1.5).

4.2. LIPOSOME FORMULATIONS

Table 5 displays the formulation of liposomal Na-HA the factors related to find optimum liposomal Na-HA formulation. The percentage of Na-HA entrapment in the

liposomes, appearance, mean of particle size and size distribution, formulation stability, and zeta potential values were considered to select the optimum liposome formulation. So, the optimum formulation was selected depending on the formulations from L 32 to L 47 (Chapter 2.1.2.2.2).

In this thesis, two types of liposome formulations were designed (gel and liquid) (74). While gel state liposomes were containing PL 100H and DPPC, liquid state liposomes were containing DMPC. Phase transition temperature of gel state liposomes was higher than liquid state liposomes. The hydrophilic substance (like Na- HA) has higher encapsulation efficiency in gel state liposomes than liquid state ones. It was found encapsulation efficiency of gel state liposomes were around 14.95 % (L 37) (Table 13). Milky dispersion was observed L 37 liposome formulation in (7:1:2) molar ratio. As shown in Table 11 a, there is significant difference between L 36 and L 37 statistically ($p>0.05$). So the optimum liposome formulation was found as L 37. Multilamellar vesicles with and without the Na-HA were prepared by using thin film technique and the composition of formulation L 37 was (PL 100 H: SA: CHOL), (7:1:2) (31).

4.3. CHOICE of BUFFER SOLUTION

In the formulations, Tris (pH: 5.5) buffer was used. Tris buffer was found as the most effective and has the highest stability than other buffers for liposomes, so 10 mM Tris (pH: 5.5) was preferred for formulations (73). When Na-HA formulations were applied to the skin, the buffer solution was Tris at pH: 5.5 due to the pH of normal skin.

4.4. INCORPORATION of CHARGE INDUCERS

SA was employed as positive charge inducer. The charge inducer was incorporated into the liposome bilayer in order to stabilize liposome dispersions physically. This effect depends on electrostatic interaction between vesicles and it provides electrostatic charge to the vesicle surface (74).

It has been reported that, the positively and negatively charged liposomes exhibit greater absorption of Na-HA than the neutral liposomes with Na-HA (73). It was also reported that the encapsulation efficiency is increased with the incorporation of charge inducer for vesicles (73).

4.5. CHOLESTEROL INCORPORATION

Some sterols such as cholesterol were employed for incorporation into the liposome. CHOL is known to serve as a fluidity buffer; it has a small fluidizing effect when vesicles

are in the gel state. The other observation was increasing cholesterol content (up to 33 %) in the formulation decreased Na-HA loading (73). In vitro drug leakage from the liposomes increased with increase in Na- Ha to lipid ratio in the formulation. The results obtained in this study were in parallel with the previous findings (17, 32, 73, 76).

4.6. PREPARATION of LIPOSOME, GEL and LIPOGELOSOME FORMULATIONS

Due to the type of liposomes (MLV) and the high lipid content, which leads to a considerably increased ratio of aqueous volume inside the vesicles in comparison with the surrounding aqueous volume, prepared liposome formulations were suitable for entrapping water–soluble molecules with high encapsulation efficiency. In pre-formulation studies, different ratios of phospholipid: charge inducer: stability agent were employed in molar ratios of (7:1:2), (7:2:1), (10:1:4), (9:1:10), (9:1:25) and (5:2:3), respectively. These molar compositions were taken into consideration in experiments depending on the triangle phase diagrams and they led the formulations to liposome formation. Among these, (7:1:2) ratio was chosen as the best composition for the entrapment of Na-HA (hydrophilic drug) which is parallel to literature (17, 73). The optimum formulation of liposome was prepared using (PL 100H: SA: CHOL) in (7:1:2) molar ratio (L 37).

The percentage of entrapped Na-HA in the liposome formulations was in between 6-15 % and the amount of free Na-HA increased for other eliminated formulations from L 32 to 47.

Efficient liposomal therapeutics requires high drug loading and low drug leakage. Na-HA was hydrophilic so, the percentage of entrapped Na-HA amount was about 14.95 % for L 37 (Chapter 3.3.3).

Microscobic observations of the liposomes were imaged with different imaging techniques (Chapter 3.3.4, 3.3.5, and 3.3.6). According to SEM images, liquid state liposomes (L 34) were clearly observed not intact and having gaps on the surface of bilayer structure (Figure 43). But, one of the gel state liposomes (L 37) was obviously observed intact in bilayer structure (Figure 46). In addition, SEM images were in good agreement with mean particle size distribution results (Chapter 3.3.5).

Type of liposomes was determined by PLM. According to PLM results, MLV type liposomes were mainly observed in all formulations (Chapter 3.3.4).

Optimum formulation (L 37) was imaged by AFM as well. Finally, the data showed homogeneous and intact bilayer structure and this was confirmed by AFM

(Chapter 3.3.6) images. As shown in Table 13 a, there is no significant difference between L 37 and L 43 ($p<0.05$) statistically. Although, the encapsulation capacity of L 43 was found higher than L 37, the final decision of formulation was L 37 for the economic reason, as the molar ratio of L 37 was (7:1:2) lower than L 47 (10:1:4). In other words, there will be higher phospholipids consumption for (10:1:4) formulation than (7:1:4) one.

The best formulation was decided according to a couple of important characterization parameters (like mean particle size and entrapment percentage and stability). For that reason, the (7:2:1) molar ratio might be the best liposome formulation due to its high stability and the optimum liposome formulation was L 37. The L 37 formulation was containing PL 100H: SA: CHOL in (7:1:2) molar ratio. Then L 37 formulation was incorporated into the U 21 gel with (1:1) w/w and lipogelosome formulation was obtained (LG 8) (30).

The prepared topical liposomal gel formulations (lipogelosome) were clear, homogenous and convenient for the administration to the humans. Viscosity of liposome preparations has to be satisfactory for topical administration. This can be achieved by their incorporation in a vehicle suitable for topical application like gels. One of the limitations of conventional topical dosage forms on the skin is relatively short residence time of the drug at the site of application. Because a controlled release and prolonged retention on the skin are often required for the desired moisture effect, research efforts have been directed to using hydrophilic polymers with bioadhesive characteristics to improve Na-HA delivery.

It has already been proven that liposomes are compatible with polymers derived from cross-linking (poly acrylic acid) polymers (Carbopol derivatives) (28). Therefore, it seems logical to choose a gel made of C 940 as a vehicle for liposomal Na-HA incorporation. For topical application, usually 0.5 – 2 % (w/w) C 940 gels are used. In this study, the chosen concentration was 1 % (w/w) having in mind the desired application, topically as a local depot for controlled release of incorporated Na-HA which will remain for a prolonged time period on the surface of the skin.

4.6.1. MEAN PARTICLE SIZE and SIZE DISTRIBUTION, POLY DISPERSITY INDEX and ZETA POTENTIAL

The mean particle size of all liposome formulations, with and without the entrapped Na-HA was in the range of 750–3000 nm (Table 11). The best formulation (L 37) was concluded to be the positively charged liposomes, which exhibited high physical stability, and relatively high percentage of drug entrappment. According to the results, mean particle

size distribution of empty liposomes was smaller than Na-HA liposomes because of encapsulation capacity of them. So, the best formulation (L 37) was selected according to the mean particle size distribution analysis, encapsulation percentage, PDI values (<0.5) and zeta potential parameters.

The type of liposomes was determined by preparation method, mean particle size and polarized microscope technique and was decided as MLV (73). Yellow and blue crosses shown in Figure 33-40 were typical properties for liquid crystals and can be seen by PLM. Number of bilayers seen in Figure 33-40 was more than two so, the vesicles were MLV type.

Also, **the zeta potential** values of liposomes were evaluated. The zeta potential is a function of the overall charge of a particle, and changes in size reflect aggregation or fusion (76). The most stable liposomes are around 25 mV (76). In this study, zeta potential was found as -20.9 mV ± 0.46 for the optimum liposome formulation (L 37) (Table 11). The maximum stability and the minimum aggregation occur at that zeta potential value (25 mV) so that expected from DLVO theory. When the zeta potential goes below the critical value (25 mV), the attractive forces supersede the repulsive forces and flocculation occurs. These loosely packed particles or flocs settle faster than the deflocculated particles because of their larger sizes (76, 77).

When the results are evaluated from **PDI** viewpoint, Na-HA loaded liposomes have larger particle size than empty liposomes (Table 11). It was determined as 1899 nm ± 13.01 for L 37. In addition, it was reported that homogenous and stable liposome dispersions have < 0.5 PDI value (55). In this study, PDI value was found as 0.356 ± 0.01 for L 37.

4.7. EVALUATION of STABILITY STUDIES of LIPOSOMES

Physical stability is very important for the integrity of liposomes. In order to provide a stable formulation for topical application in which liposomes were distributed uniformly and their structure was preserved, liposomes were kept for 3 months at 4^0C and 25^oC (Relative humidity: 60 %). During those accelerated stability experiments, two parameters (particle size and entrapment capacity) were monitored and no change occurred in the integrity of liposomes. But, the significant changes were observed in the particle size of liposomes, $p>0.05$ (Table 10). According to this table, refrigeration condition was better than room temperature (25 ^{0}C) for liposomes. This observation was in agreement with literature (73).

When compared with the previous studies, the drug leakage of less than 5 % of the initial load in a period of 4 weeks at refrigeration condition was found within the limits (77).

According to the stability results, the Na-HA leakage was less than 5 % of the initial load in a period of 4 weeks at refrigeration condition and room temperature of 25 ^{o}C. So, our vesicles were found stable enough for topical applications. As it can be seen in Table 13, the percentage amount of liposome-associated Na-HA in liposome remained in the same amount. It was observed the changes of particle size of liposomes during storage (Chapter 3.3.7). Liposome became larger than their initial sizes because of aggregation occurring during the sedimentation of the vesicles due to the gravity (13).

4.8. EVALUATION of *IN VITRO* RELEASE STUDIES from LIPOSOME, LIPOGELOSOME and COMMERCIAL PRODUCT

The objective of this study is to develop a delivery system which is containing Na-HA for skin hydration, with high loading capacity and stable Na-HA encapsulation. The optimum Liposome formulation (L 37) composed of (PL 100H: SA: CHOL) was prepared by the method of thin film hydration (54). Na-HA release from the liposomes (L 37), lipogelosome (LG 8) and commercial product (CP) containing Na-HA was studied. As a result LG 8 lipogelosome formulation containing Na-HA formulation has longer release than L 37 and CP of Na-HA (Figure 66).

Almost similar Na-HA release patterns have been observed for liposome and lipogelosome formulations, which indicate that the concentration of the gelling agent in a range of 1 % affects the release rate slightly.

As Na-HA does not associate with the lipid bilayers, the encapsulated aqueous volume is of major importance to achieve a high loading capacity. Therefore, the encapsulation efficiency is related only to the quantity of aqueous phase that is immobilized between the phospholipid bilayers and in the centre of liposomes (aqueous core).

In order to evaluate the effect of incorporated Na-HA in liposomes on the release rate, comparatively, drug release rate from basic liposome dispersion (L 37), liposome gels or lipogelosome (LG 8) and marketed product of Na-HA have also been estimated. Release of Na- HA from liposomes embedded into the gel base (U 21) was significantly slower and

(53 %) released within 120 min than the release from Na-HA liposomal formulation (67 %). Incorporation of liposomal Na -HA into gel resulted in a prolonged drug release rate (Figure 66). Lower release rate from liposomal gel systems compared to basic liposome dispersions could be a result of the influence of the viscosity of the gel matrix followed by slower drug penetration. Gelling agent forms a structured vehicle and due to this network the gelling agent functions as if a retarding steps of the release of Na-HA. This result was found in parallel to the previous studies (32).

The mathematical models were used to evaluate the kinetics of Na-HA release from liposome, lipogelosome and marketed product. The model that best fits to the release data was selected depending on the correlation (r) and determination (r^2) coefficients in various models (Zero order, First-order, Higuchi, and Hixson-Crowell kinetic models). The model giving the highest 'r' value was considered as the best fitting model to the release data.

Highest correlation and also determination coefficients were obtained for the zero-order for Na-HA release kinetics from the liposome (L 37) and liposomal gel (LG 8), suggesting that liposomes could act as reservoir systems for continuous delivery of encapsulated Na-HA. According to zero-order release kinetics, r^2 value 0,974 for L 37 and r^2 value 0,953 for LG 8 were found (Table 15), respectively.

4.9. PRELIMINARY EVALUATION of SKIN HYDRATION STUDIES of HUMAN VOLUNTEERS

Abovementioned results lead us to prepare liposomes in the formulation of (L 37) and lipogelosome in the formulation of (LG 8) for Na-HA. *In vitro* release studies of Na-HA in above mentioned formulations and marketed product of Na-HA were also performed. Finally, gel alone (LG 5), gel with Na-HA (LG 6), lipogelosome without Na-HA (LG 7), lipogelosome with Na-HA (LG 8) and commercial product (CP) of Na-HA were applied to the human volunteers throughout two weeks twice a day. Then, the increase in sebum content and moisture level and mean of pH values were determined for these formulations:

These parameters (sebum content, moisture level, mean of pH values) were evaluated for formulations of LG 5 (gel), LG 6 (gel Na-HA), LG 7 (liposomal gel without Na-HA), LG 8 (lipogelosome with Na-HA) and commercial product containing of Na-HA and were applied to the skin of 15 female volunteers.

As can be seen in Table 16, results showed that the moisture level increase of LG 8 formulation was found higher than the others (30, 36 % ± 0.12). The sebum content increase of LG 6 formulation was found higher than the others (25.62 % ± 1.25). pH values were in between 4.90-5.30 for all formulations. Decreases of wrinkle deepness were illustrated in Chapter 3.6.

The preliminary evaluation of skin hydration studies showed that there are two mechanisms governing for derma-cosmetics obtained with administered liposomes and lipogelosomes; first the vesicles are adsorbed to the outer layers of the stratum corneum and deposit of Na-HA into the bilayers was occur. Secondly, ultra structure changes occur in the deep of stratum corneum and bilayer lipids mix with intracellular lipids (8).

Finally, the percentage of Na-HA encapsulated into liposomes was over 14%.
It was reported by several authors that factors such as lipid composition, charge on the liposome surface, total lipid content and lamellarity could influence the drug deposition into the different skin layers (73). Furthermore, the membranes of large MLVs are more flexible, and due to their heterogeneity some budding of the liposome bilayer can occur, which could result in better adaptation to the surface of the skin and enable some infiltration of bilayer into the pores in stratum corneum lamellar .Integration of phospholipid molecules with the skin lipids might have served further, to help retain the drug molecules within the skin, thus leading to prolonged presence of drug molecules at the receptor site and localized drug action in the skin.

These conclusions suggested the skin hydration use of the prepared lipogelosome formulations as local depots for sustained release of incorporated Na-HA over a prolonged period of time (32).

Thus, liposomes are lipid vesicles and are considered to be safe and effective drug carriers for topical application. So, liposomal gel formulation (lipogelosome) of high molecular weight substance as NA-HA was relevant formulation as being effective for skin hydration. Interaction of Na-HA in the skin with phospholipids, a component of the lipogelosome and skin, may well be a factor in the diffusion of the drug in the skin.

All parameters like changes in humidity levels, sebum content and the pH level on the skin surface were determined and evaluated by using the Multiprobe Adapter Systems (Corneometer, sebumeter, pH-meter, respectively).

The changes of deepness of wrinkles on the skin surface were measured by Skin-Visiometer. The increment of moisture level was found (30.36 ± 0.12) % for LG 8 formulation. As a result, LG 8 formulation has improved skin hydration and better compliance than the conventional formulations.

In order to obtain general results, it was planned to carry out the same in vivo studies on the more crowded volunteer groups.

SUMMARY

In this research, the effectiveness of Na-HA on skin was investigated in lipogelosome formulations. For this purpose, 16 different liposome formulations were prepared by thin film technique with using Na-HA (45 kilo dalton) and without Na-HA in various type of phospholipids; dimyristoylphosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoracglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC) and phospholipon 100H (PL 100H).

The liposomes were characterized by various methods e.g., determination of zeta-potential, mean particle size, size distribution and antrappment capacity. Imaging of vesicles by scanning electron microscopy (SEM), atomic force microscopy (AFM) and polarized light microscopy (PLM) studies for the characterization of liposome, gel and lipogelosome formulations and the entrapment capacity of Na–HA were examined.

Poloxamer derivatives (Poloxamer 188 and Poloxamer 407) and Carbopol derivatives (C 940 and U 21) were used as gelling agents to prepa re liposome loaded gel formulations. *In-vitro* release of Na-HA liposome, Na-HA lipogelosome and marketed formulation of Na-HA were studied by USP Apparatus 4 method. The release kinetic of Na-HA was investigated by using zero-order, first-order, Higuchi and Hixson-Crowell kinetics.

The optimum liposome formulation was PL 100H: SA: CHOL (7:1:2) containing Na-HA (L 37). The entrapment capacity was found 14.95 %. Then L 37 formulation was incorporated into the U 21 gel in (1:1) (w/w) ratio (LG 8). The preliminary *in vivo* studies were performed on 15 female volunteers. Na-HA or without Na-HA liposome, lipogelosome and marketed formulation were applied to volunteers for two weeks twice a day.

All parameters like changes in humidity levels, sebum content and the pH level on the skin surface were determined and evaluated by using the Multiprobe Adapter Systems (Corneometer, sebumeter, pH-meter, respectively). The changes of deepness of wrinkles on the skin surface were measured by Skin-Visiometer. The best results were obtained with LG 8 of lipogelosome formulation.

REFERENCES

1) Meisnera D., Mezeib M. Liposome Ocular Delivery Systems. Advanced Drug Delivery, 16, 75-93, (1995).
2) Ozer A.Y. Application of Light and Electron Microscobic Techniques in Liposome Research. In: Mozafari M.R. (ed). Nanomaterials and Nanosystems for Biomedical Applications, Dordrecht, The Netherlands, 145-153, (2007).
3) Lasic D.D. Applications of Liposomes. Handbook of Biological Physics, Amsterdam, The Netherlands, 1, 491-519 (1995).
4) Keller B.C. Liposomes in Nutrition. Trends in Food Science and Technology, 12, 25-31, (2001).
5) Talegaonkar S., Azeem A., Ahmad F., Khar R, Pathan S., Khan Z. Microemulsions : A Novel Approach to Enhanced Drug Delivery. Recent Patents on Drug Delivery & Formulation, 2, 238-257, (2008).
6) Vitas A., Diaz R., Gamazo C. Effect of composition and Method of Preparation of Liposomes on Their Stability and Interaction with Murine Monocytes Infected with Brucella abortus. Antimicrobial Agents and Chemotherapy, 40, 146–151, (1996).
7) Laura M., Dosio F., Cattel L. Stealth Liposomes: Review of the Basic Science, Rationale, and Clinical Applications, Existing and Potential. International Journal of Nanomedicine 1, 297–315, (2006).
8) Remington. The Science and Practice of Pharmacy, Philadelphia, USA, 21, 766-780, (2005).
9) Lasic D.D. The Spontaneous Formation of Unilamellar Vesicles. Journal of Colloid and Interface Science, 124, 428-435, (1988).
10) Szoka F., Papahadjopoulos D. Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evoparation. Proc. Natl. Acad. Sci., 75, 4194-4198, (1978).
11) Szoka F., Olsona F., Heath T., Vail W., Mayhew E., Papahadjopoulos D. Preparation of Unilamellar Liposomes of Intermediate Size (0.1–0.2 μm) by a Combination of Reverse Phase Evaporation and Extrusion Through Polycarbonate Membranes. Biochimica et Biophysica Acta (BBA) - Biomembranes Volume, 601, 559-571, (1980).

12) Lasic D. D. The Mechanism of Vesicle Formation. Biochem. J., 256, 1-11, (1988).
13) Torchillin V. P., Weissig V. Liposomes Second Edition, Oxford University Press, New York, USA, 2, 5-40, (2003).
14) Liu R.R., Cannon J.B., Paspal S.Y.L. History of Liposomes. Water-Insoluble Drug Formulation, New York, USA, 2, 375-409, (2008).
15) Mazda F., Özer A.Y., Ercan M.T., Hıncal A.A. Preparation and Characaterization of Urea Niosomes *In Vitro* and *In Vivo* Studies. S.T.P. Pharma Sciences, 7, 205-214, (1997).
16) Barenholz Y., Liposome Application: Problems and Prospects. Current Opinion in Colloid & Interface Science, 6, 66-77, (2001).
17) Magdassi S., Delivery Systems in Cosmetics. Colloids and Surfaces A: Physicochemical and Engineering Aspects 123, 671-679, (1997).
18) Reinberg A., Koulbanis C., Soudant E., Nicolai A., Mechkouri M., Smolensky M. Day-Night Differences in Effects of Cosmetic Treatments on Facial Skin. Effects on Facil Skin Appearance. Chronobiology International, 7, 69-79, (1990).
19) Raskovic D., Piazza P. Liposomes: A Promising Future for Dermatocosmetology and Clinical Dermatology. Journal of Liposome Research, 1, 737-751, (1993).
20) Physicians' Desk Reference, 57th ed. Montvale (NJ). Thomson PDR, 1423-1761, (2003).
21) Physicians' Desk Reference, 57th ed. Montvale (NJ). Thomson PDR, 2419-2423, (2003).
22) Duman G., Yurdakul D., Aslan İ., Gümrü. S., İnanç İ., Yıldız B., Taralp A., Yeşilada E., Şahin F., Erol D.D. Oral Hygiene with Pluronic F127 and Antibacterial Activity. 14th International Symposium on Recent Advances in Drug Delivery Systems, Utah, USA, February 15-18, (2009).
23) United States Patent Application 20070048364 A1 Cl 424/451. Free Flowing Granules Containing Carbomer, (2007).
24) Kumar R, Katare O. P. Lecithin Organogels as a Potential Phospholipid-Structured System for Topical Drug Delivery: A Review. AAPS PharmSciTech, 6, 298-310, (2005).
25) Morlan Z.U., Rios R.C, Montes A.C., Contreras L.M.M, Segundo E.P., Quintanar A.G., Guerrero D.Q. Determination of Poloxamer 188 and Poloxamer 407 Using

High-Performance Thin-Layer Chromatography in Pharmaceutical Formulations. J of Pharm and Biomed Analysis, 46,799-803, (2008).

26) Viegas T.X., Henry R.L. Osmotic Behavior of Poloxamer 407 and Other Non-ionic Surfactants in Aqueus Solutions. Int J Pharm, 160, 157-162, (1998).

27) Kweon H.Y., Yoo M. K., Lee J.H, Wee W.R., Han Y.G., Lee K.G, Cho C.S. Preparation of a Novel Poloxamer Hydrogel. J Appl Poly Sci, 88, 2670-2676, (2003).

28) United States Patent Application 20040258648 A1 Cl 424/70.16. Polymer Compositions and Process For Preparing Polymers, (2004).

29) European Patent Application EP0220797 A2 Cl A61K 9/50. Process for The Preparation of Liposome, (1987).

30) F. Farshi, A.Y. Özer, S. Tavassoli, A. Sungur and A.A. Hıncal, A Clinical Trial: *In Vivo* Studies on Dexamethasne Sodium Phosphate Liposomes in the Treatment of Human Aphthous Stomatitis, J. Liposome Res, 6, 699–712, (1996).

31) Türker S., Erdoğan S., Özer A.Y., Ergün E.L., Tuncel M., Bilgili H., Deveci S. Scintigraphic Imaging of Radiolabelled Drug Delivery Systems in Rabbits with Arthiritis. Int J of Pharm, 296, 34-43, (2005).

32) Türker S., Erdoğan S., Özer A.Y., Bilgili H., Deveci S. Enhanced Efficacy of Diclofenac Sodium-Loaded Lipogelosome Formulation in Intra-Articular Treatment of Rheumatoid Arthritis. Journal of Drug Targeting, 16, 51-57, (2008).

33) United States Patent 7553664 B2 U.S. Cl. 435/378. Three-Dimensional Skin Model, (2009).

34) United States Patent 6273367 B1 U.S. Cl. 244/123. Cover-Skin Structure, (2001).

35) Yin D., Yang W., Ge Z., Yuan Y. A Fluorescence Study of Sodium Hyaluronate/Surfactant Interactions, Carbohydrate Research, 340,1201-1206, (2005).

36) Saari, H., Konttinen Y.T., Friman, C., Sorsa, T. Differential Effects of Reactive Oxygen Species on Native Synovial Fluid and Purified Human Umbilical Cord Hyaluronate, Inflammation, 17, 403-415, (1993).

37) Frasher, J.R.E., Laurent, T. C., Laurent, U. B. G. Hyaluronan: Its Nature, Distribution, Functions and Turnover, Journal of Internal Medicine, 242, 27–33, (1997).

38) Romagnoli D., Belmontesi M. Sodium Hyaluronate–Based Fillers: Theory and Practice. Clinics in Dermatology, 26, 123-159, (2008).

39) O'Regan, M., Martini, I., Crescenzi, F., De Luca, C., Lansing, M. Molecular Mechanisms and Genetics of Hyaluronan Biosynthesis, International Journal of Biological Macromolecules, 16, 283-286, (1994).
40) Balazs E.A., Freeman M.I., Klo'ti R. Sodium Hyaluronate and Replacement of Vitrous and Aqueous Humor. Mod Prob Opthalmol, 10, 3-21, (1972).
41) K. Meyer, J.W. Palmer. The Polysaccharide of the Vitreous Humor. J Biol Chem, 107, 629, (1934).
42) Simoni R.D., Hill R. L., Vaughan M., Hascall V. The Discovery of Hyaluronan by Karl Meyer. J Biol Chem, 277, 27, (2002).
43) Murthy KR. History, Developments and Future Thoughts of Intraocular Lens. Indian J Ophthalmol, 37, 58, (1989).
44) United States Patent 5017229 A1 U.S. Cl. 106/162. Water in Soluble Derivatives of Hyaluronic Acid, (1991).
45) Alkrad J.A., Mrestani Y., Neubert R.H.H. : The Release Profiles of Intact and Enzymatically Digested Sodium Hyaluronate From Semisolid Formulations Using Multi-Layer Membrane System. Eur J Pharm and Biopharm, 56, 37-71, (2003).
46) Chou C.L., Li H.W., Lee S.H., Tsai K.L., Ling H.Y. Effect of Intra-articular Injection of Sodium Hyaluronate in Rheumatoid Arthritis Patients with Knee Osteoarthritis, J Chin Med Assoc, 71, 411–415, (2008).
47) Das S. Sodium Hyaluronate in Rheumatoid Arthritis: Some Facts. Journal of the Chinese Medical Association, 71, 601, (2008).
48) Chevalier X. Mechanisms of Action of Sodium Hyaluronate. Osteoarthritis and Cartilage, 9, 300-307, (2001).
49) Patrick J Bowler. Impact on Facial Rejuvenation with Dermatological Preparations. Clin Interv Aging, 4, 81–89, (2009).
50) Prestwich G.D., Vercruysse K.P. Profiles Therapeutic Applications of Sodium Hyaluronate and Hyaluronan Derivatives. Pharmaceutical Science & Technology Today, 1, 42-43, (1998).
51) F. Manna, M. Dentini, P. Desideri, O. De Pita, E. Mortilla and B. Maras. Comparative Chemical Evaluation of Two Commercially Available Derivatives of Hyaluronic Acid (Hylaform from Rooster Combs and Restylane from Streptococcus) Used for Soft Tissue Augmentation. J Eur Acad Dermatol Venereol, 13, 183–192, (1999).

52) Baumann L. Replacing Dermal Constituents Lost Through Aging with Dermal Fillers. Seminars in Cutaneous Medicine and Surgery, 23, 160-166, (2004).

53) United States Patent Application 20090209456. Compositions And Methods For Improving Facial And Body Aesthetics, (2009).

54) Bangham, D.A., Standish, M.M., Wathins, C.J : Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids, J. Mol. Biol., 13, 238-252, (1965).

55) Rouser G, Fleischer S, Yamamoto A. Two Dimensional Thin Layer Chromatographic Separation of Polar Lipids And Determination of Phospholipids by Phosphorous Analysis of Spots. Lipids, 5, 494–496, (1970).

56) Colas J.C., Shi W., Rao V.S.N.M., Omri A., Mozafari M.R., Singh H. Microscobic Investigations of Nisin-Loaded Nanoliposomes Prepared by Mozafari Method and Their Bacterial Targeting. Micron, 38: 841-847, (2007).

57) Schmolka, I. R. Artificial skin. I. Preparation and Properties of Pluronic F-127 Gels for Treatment of Burns. J. Biomed. Mater. Res., 6, 571-582, (1972).

58) Riccia, E. J. , Bentleya, M. V. L. B. , Farahb, M. , Bretasb , R. E. S. Marchetti , J. M. Rheological Characterization of Poloxamer 407 Lidocaine Hydrochloride Gels. Eur J Pharm Sci, 17, 161-167, (2002).

59) Bhardwaj, U., Burgess, D. J. A novel USP Apparatus 4 Based Release Testing Method for Dispersed Systems. Int J of Pharm, 388, 287-294, (2010).

60) http://www.courage-khazaka.de/download/pdf/scientific_mpa_low.pdf (Dikstein S. Instrumental Analysis in Individual Cosmetic Consultation. Cosmetics & Toiletries, 98, 1, (1983).)

61) http://www.courage-khazaka.de/download/pdf/brochure_scientific_lo.pdf (Paepe K. D., Lagarde J. M., Gall Y., Roseeuw D., Rogiers V., Study of the Human Skin Microrelief by a Standardised Light Transmission Method. Skin Research and Technology, 6, 3, (2000).)

62) Kayaalp S.O. Turkish Drug Guidelines, Golden Media, Istanbul, Turkey, 463-464, (1999).

63) Song H. Systematic Studies of the Interaction Between Amyloid ß-Protein and Lipids, Ph.D Thesis, Mainz, Germany, 28-30, (2005).

64) Barani H., Montazer M. Review on Applications of Liposomes in Textile Processing. Journal of Liposome Research, 18, 249-262, (2008).

65) O'Regan, M., Martini, I., Crescenzi, F., De Luca, C., Lansing, M. Molecular Mechanisms and Genetics of Hyaluronan Biosynthesis, International Journal of Biological Macromolecules 16, 283-286, (1994).

66) Zhen W., You X., Liu B., Zheng Z., Pu Y., Siong L., Li Q. Formation of Atractylone Liposomes by Rapid Expansion from Supercritical to Surfactant Solution. Asia-Pac. J. Chem Eng., 10, 462, (2010).

67) Lasic D.D. Application of Liposomes, Handbook of Biological Physics ed. by Lipowsky R., Sackman E. Elsevier Science, 1, 10, (1995).

68) Lash J. and Schubert, R.: In Liposome Technology, 2nd edn,Vol.II, (ed. Gregoriadis G.), CRC Press, Boca Raton, 233, (1993).

69) Rainberg A., Koulnabis C., Soudant E., Nicolai A., Mechkouri M., Smolensky. Day-Night Differences in Effects on Facial Skin Appearance. Chronobiology International, 7, 69-79, (1990).

70) Desai K. G., Park H.J. Study of Gamma-Irridation Effects of Chitosan Microparticles. Drug Delivery, 13, 39-50, (2006).

71) Yerushalmi N., Margalit R. Hyaluronic Acid-Modified Bioadhesive Liposomes as Local Drug Depots: Effects of Cellular and Fluid Dynamics on Liposome Retention at Target Sites. Archives of Biochemistry and Biophysics, 349, 21–26, (1998).

72) Parka S.N., Leeb H. J., Leeb K.H., Suh H. Biological Characterization of EDC-Crosslinked Collagen–Hyaluronic Acid Matrix in Dermal Tissue Restoration. Biomaterials, 29, 1631-1641, (2003).

73) Mazda, F. Studies on Liposome and Non-Ionic Surfactant Vesicle Formulations Containing Urea. Master Thesis, Hacettepe University, Ankara, (1993).

74) Sinico C., Manconi M., Peppi M., Lai F., Valenti D., Fadda A. M. Liposomes as Carriers for Dermal Delivery of Trretinoin: *In Vitro* Evaluation of Drug Permeation and Vesicle–Skin Interaction. Journal of Controlled Release, 103, 123-136, (2005).

75) Sułkowski W.W., Pentak D., Nowak K., Sułkowska A. The Influence of Temperature, Cholesterol Content ond pH on Liposome Stability. Journal of Molecular Structure, 744, 737-747, (2005).

76) Awada D., Taboda I., Lutza S., Wessolowskib H., Gabel D. Interaction of $Na_2B_{12}H_{11}SH$ with Liposomes: Influence on zeta potential and particle size. Journal of Organometallic Chemistry, 690, 2732-2735, (2005).

77) Kumar R, Singh B,xs Bakshi G, Katare O. P. : Development of Liposomal Systems of Finasteride for Topical Applications: Design, Characterization, and *In Vitro* Evaluation. Pharm Dev Tech, 12, 591–601, (2007).

RESUME

İsmail ASLAN was born in Bakırköy, on 15 March 1982. He attended to primary, elementary and high school in İstanbul, and later went on to complete a Bachelor of Science degree in Pharmacy at the Marmara University, İstanbul, completed in 2005. İsmail completed military service as lieutenant at Ankara in December 2006. After military service, he started at Yeditepe University in İstanbul as a Research and Teaching Assistant. His graduate program, Cosmetology, was carried out at the Yeditepe University Institute of Health Sciences for a Master of Science Degree, completed 2010.