

LIPOSOMES ENCAPSULATING COSMETIC RAW MATERIALS

by

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ABSTRACT

LIPOSOMES ENCAPSULATING COSMETIC RAW MATERIALS

Vesicles are highly ordered self-assemblies consisting of concentric bilayers formed by self-aggregation of amphiphilic building blocks in the presence of water. Their enclosed structures makes them perfect vehicles for studies in many areas from targeted drug delivery to cosmetic raw material encapsulation.

For a substance to operate onto the skin effectively it is necessary to penetrate through stratum corneum, outermost layer of the skin. As vesicles have structures very similar to skin membrane, they have the ability to fuse with skin outer layer. Due to this unique characteristic, usage of vesicles is promoted lately in cosmeceuticals industry.

In this study, a novel liposome composition has been introduced by using skin lipids such as ceramides, cholesterol and cholesteryl sulfates. Effect of different downsizing methods on liposome size and stability is compared. To increase the vesicle stability and keep away possible bacterial infections, a new dispersion media consisting of ethanol has been introduced. Encapsulation efficiencies of the obtained liposomes are investigated with some cosmetic raw materials like filaggrin, madecassoside and heterosides. These molecules are chosen specifically for their common therapeutic effects on skin diseases such as eczema and atopic dermatitis. Filaggrin has been used for the first time in an encapsulation study with this research.

ÖZET

KOZMETİK HAM MADDELERİ KAPSÜLLEYEN LİPOZOMLAR

Vesiküller, su varlığında amfifilik yapısal blokların kendiliğinden kümelenip ortak merkezli çift katmanlı bir yapı oluşturması ile meydana gelen yüksek düzenlilikte yapılardır. Vesiküller, içe dönük kapalı yapıları sayesinde hedefe yönelik ilaç taşımacılığından kozmetik hammadde kapsüllenmesine kadar çok geniş bir alanda kullanılmak üzere mükemmel araçlardır.

Bir maddenin cilt üzerinde etkili bir şekilde çalışabilmesi için cildin en üst tabakası olan stratum corneum tabakasının içinden geçebilmesi gereklidir. Vesiküller cilt yapılarına benzerliği dolayısıyla cildin bu dış tabakasını rahatlıkla geçebilirler. Bu eşsiz özellikleri, vesiküllerin son yıllarda kozmetik endüstrisinde kullanımını gittikçe arttırmaktadır.

Bu çalışmada, seramit, kolesterol ve kolestril sülfat gibi cilt lipitleri kullanılarak yeni bir lipozom bileşimi hazırlanmıştır. Çeşitli lipozom boyutu küçültme tekniklerinin lipozom boyutu ve dayanıklılığına etkileri karşılaştırılmıştır. Lipozom dayanıklılığını arttırmak ve olası bakteriyel enfeksiyonları önlemek amacıyla etanol içeren yeni bir yayılma ortamı geliştirilmiştir. Filaggrin, madecososide ve heteroside gibi çeşitli kozmetik hammaddelerin elde edilen lipozomlardaki kapsülleme verimlilikleri ölçülmüştür. Bu maddeler egzema ve atopik dermatit gibi bazı cilt hastalıklarına karşı ortak iyileştirici etkileri bulunması dolayısıyla özellikle seçilmişlerdir. Bu araştırma ile filaggrin ilk kez bir kapsülleme çalışmasında kullanılmaya başlanmıştır.

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LIST OF SYMBOLS

a_0	Polar head surface are of the lipid
l	Chain length of the hydrocarbon tail
P	Molecular packing parameter
T_c	Phase transition temperature
V	Volume of the liposome
ζ	Zeta potential

LIST OF ACRONYMS/ABBREVIATIONS

CMC	Critical micelle concentration
Cryo-EM	Cryogenic electron microscopy
DLS	Dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG	1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FEM	Freeze Fracture Electron Microscopy
KH ₂ PO ₄	Potassium dihydrogen phosphate
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NaOH	Sodium Hydroxide
PBS	Phosphate Buffer Solution
PC	Phosphatidylcholines
PDI	Polydispersity Index
PG	Phosphatidyl glycerol
SC	Stratum Corneum
SEM	Scanning Electron Microscopy
STEM	Scanning Transmission Electron Microscopy
SUV	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
TAG	Triacylglycerol
ULV	Unilamellar Vesicles
UV	Ultraviolet Light
UV/VIS	Ultraviolet Visible Spectrophotometer

1. INTRODUCTION

1.1. Surfactants

Surfactants are amphiphilic molecules with the ability to self-aggregate in the solvents above a certain concentration where the tails form the aggregate core and the heads are in contact with the surrounding liquid. They have both polar and non polar functional regions in their structures. The polar head groups generally include one of the heteroatoms like sulfur, oxygen, phosphorous and nitrogen whereas the nonpolar tail groups consist of hydrocarbon chains of alkyl or alkyl benzenes. Heteroatoms in the polar regions form functional groups such as alcohols, esters, phosphates, thiols and so on that gives the surfactant its chemical characteristics. This functional polar portion can be referred as hydrophilic part which is covalently bonded to the nonpolar chain portion that is named as hydrophobic part.

Due to having polar-nonpolar duality in their structure, surfactants are partly soluble in both nonpolar and polar media and this characteristic leads them showing a special behavior in aqueous media. In order to avoid unfavorable interactions between hydrophobic tail and water, surfactant locates itself at the interface of the air/water with the head group in the water side and the tail group in the air side resulting formation of an adsorbed layer of surfactant on the surface [1].

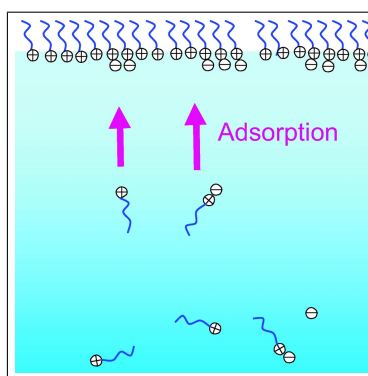


Figure 1.1. Adsorption of surfactants at the interface of the air/water [2]

To avoid further increase of the free energy in their system, when a certain concentration is reached a spontaneous and thermodynamically driven process starts and the surfactants self-organize [3]. This concentration is defined as *critical micelle concentration*, CMC. It is specific for each surfactant and depends on the parameters like temperature, pressure, and presence of other surfactants and electrolytes in the environment [4]. Aggregation process of the surfactants in aqueous medium is demonstrated in Figure 1.2.

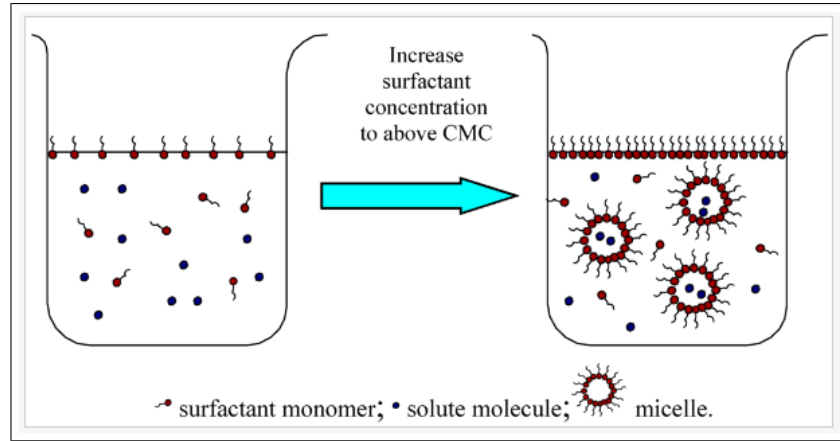


Figure 1.2. Schematic representation of self-aggregation concept in water [5]

The measure of the balance in size between hydrophilic head and hydrophobic tail is one of the factors that determines the shape of the aggregate. In order to understand the properties of surfactant solutions such as surface tension, spreading capacity, viscosity, stability and eventually performances in various applications, it is important to predict the aggregate shape and control the self-assembly. Extensive work has been carried out to be able to get the well-defined structures in a predictable and reproducible way upon experiments [6, 7].

First, Tanford [8] proposed of two opposite forces idea to explain why surfactant aggregates form in aqueous solutions. According to this concept, aggregation results from the attractive forces caused by hydrophobic attraction in the hydrocarbon-water interface by gathering with the hydrophilic, steric and ionic repulsive forces between the

head groups [9]. Later, Israelachvili et al. introduced a numerable way of understanding aggregations, *molecular packing parameter*, to predict how the size and the shape of the aggregate at equilibrium would occur from a combination of molecular packing considerations and general thermodynamic principles [6].

The molecular packing parameter, P , can be calculated from the following equation

$$P = V/a_0.l \quad (1.1)$$

where V is the volume of the hydrocarbon tail, l is the chain length of the hydrocarbon tail and a is the polar head surface area of the surfactant as demonstrated in Figure 1.3. This equation allows us a simple insight on the relationship between molecular structure and the shape of the resulting self aggregates. For example; for spherical micelles $0 < P \leq 1/3$, for cylindrical worm-like aggregates $1/3 < P \leq 1/2$ and for bilayer structures like vesicles $1/2 < P \leq 1$ [10].

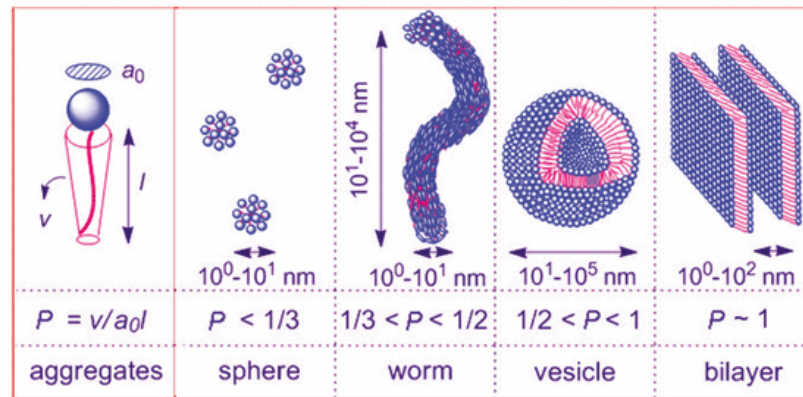


Figure 1.3. The relationship between packing parameters and the morphology of the self-aggregates [11]

Surfactants can be separated into two main groups according to the charge on their polar head group as ionic and nonionic surfactants. Nonionic surfactants carry no specific charge on their head groups and generally formed from polyether chains. They consist of a large organic molecule on one end and a polarity gathering oxygen- rich part at the other hand. Ionic surfactants carry a net charge on their head groups and can be classified as anionics, cationics and amphoteric. Amphoteric surfactants have characteristics of both anionic and cationic ones depending on the pH of the medium. Upon decreasing pH, they pass from anionic to cationic forms and at a certain pH they become a zwitterion meaning that simultaneously possess the both ionic states and renders the molecule overall neutrally-charged. [12,13]

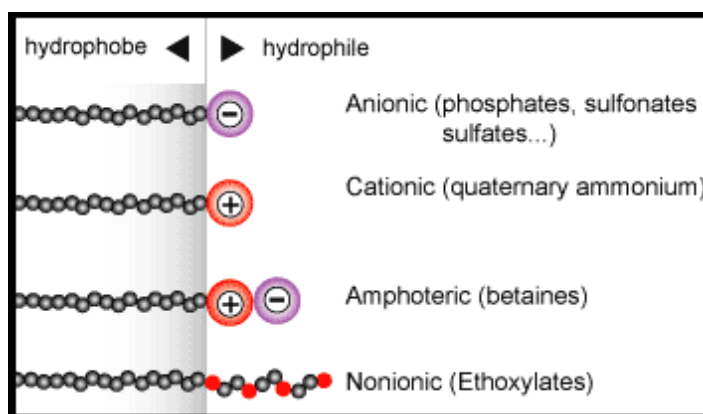


Figure 1.4. The general structures and some examples of anionic, cationic, amphoteric and nonionic surfactants [14]

Surfactants have an important role in a broad range of fields such as displacement of oil and dirt, remediation of soil, water cleaning, wetting of solid surfaces and stabilizing emulsions. They lower the surface tension of the water that makes easier to remove materials from a surface [15]. They can be used as detergents by solubilizing normally insoluble compounds with incorporating them into the aggregate core as seen in Figure 1.5. Surfactants are also used in daily life as hair and body care products, paints, varnishes, textile wetting, coating and printing wares.

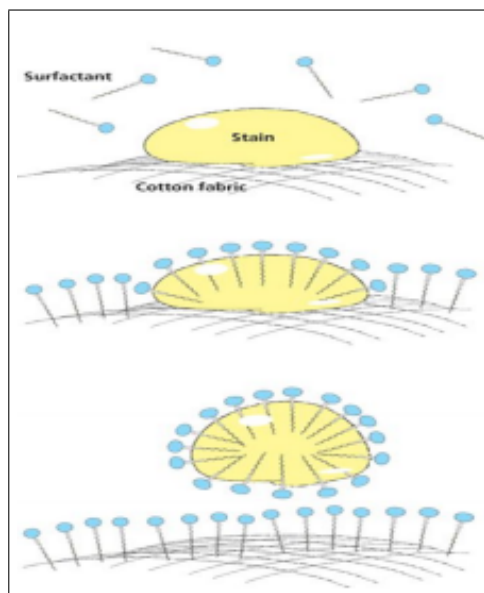


Figure 1.5. Stain removal mechanism of the surfactants from fabric [16]

Carboxylates, sulfates, sulfonates, phosphates, alcohols with long alkyl chains and lipids are the main substances classified under surfactants and among all, lipids constitute a vitally important role as being an amphiphile in biological membranes by establishing the main components of cells and skin barrier in all living organisms.

1.2. Lipids and Their Aggregations

1.2.1. General Information About Lipids

Lipids are naturally occurring amphiphilic compounds composed of mainly carbon, hydrogen and oxygen atoms. They are essential biomolecules for the function and structure of living creatures by occupying a big percentage of the total chemical composition of a cell. Lipids, in general, contribute to cell structure, provide energy storage and take part in biological processes. Because of having an amphiphilic characteristic, they are not fully soluble in water and other polar solvents, instead they are dispersed in water as droplets and form emulsions. Major lipid groups include fats, oils, phospholipids, triglycerides, sphingolipids, waxes and steroids (Figure 1.6).

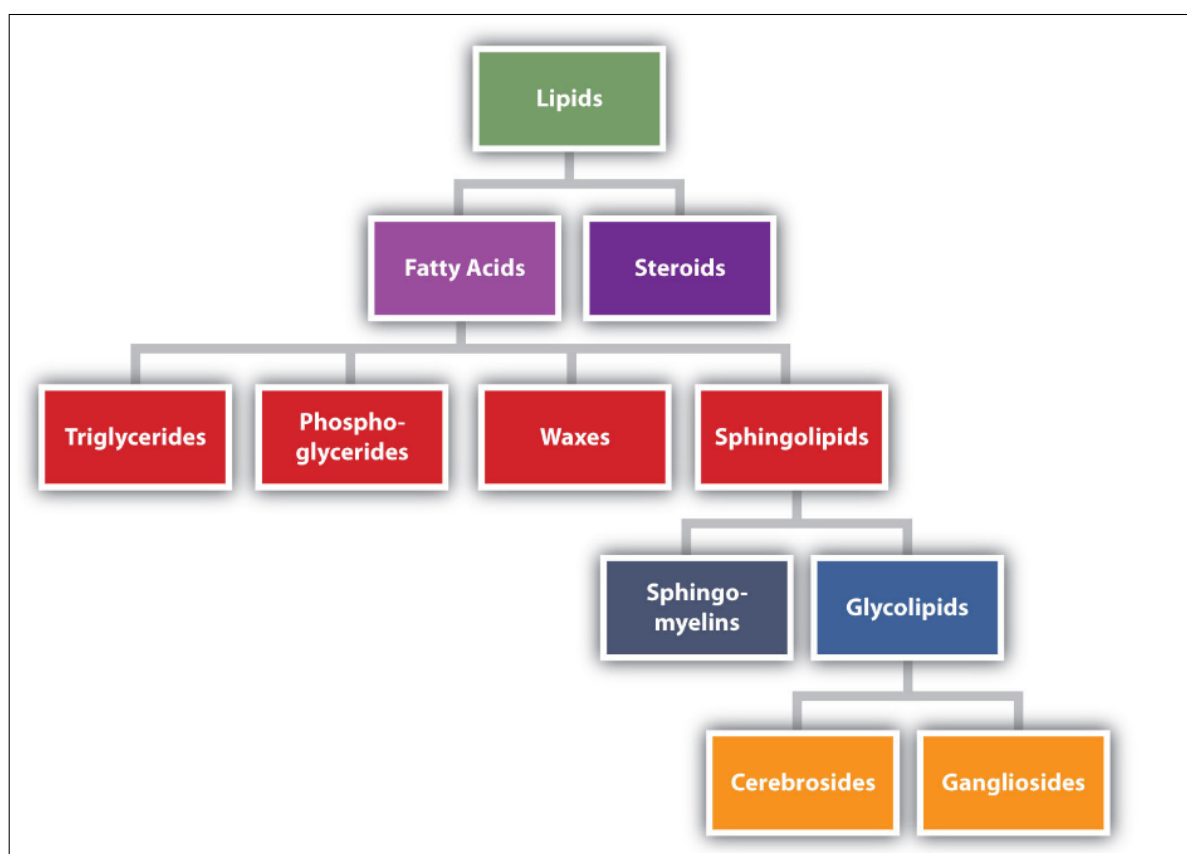


Figure 1.6. Lipid organization based on structural relationships [17]

1.2.1.1. Steroids. A steroid is an organic compound with four fused rings arranged in a specific configuration composed of seventeen carbon atoms as three six-membered and one five-membered rings. They differ with the functional groups attached to this four-ring core and with the oxidation states of these rings. The sex hormones estradiol and testosterone, the dietary lipid cholesterol and the anti-inflammatory drug dexamethasone can be shown as examples of steroids [18]. Steroids plays an important role in providing structure to cell membranes by modifying the membrane fluidity and in cell signaling which activate steroid hormone receptors [19]. Cholesterol is the most fundamental steroid molecule because all the other steroids are made from it.

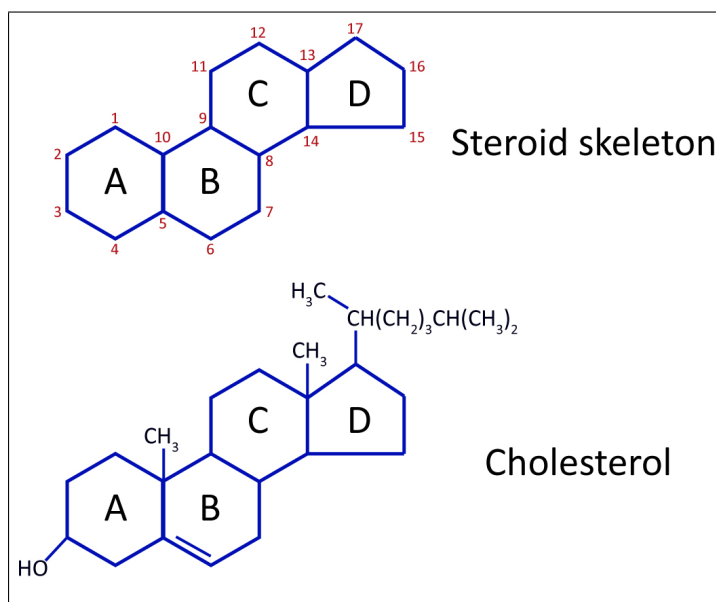


Figure 1.7. The common structure of a steroid molecule and the structure of cholesterol [20]

1.2.1.2. Fatty Acids. Fatty acids are the building blocks of many important complex lipids such as triglycerides, phospholipids, sphingolipids and waxes. A fatty acid is mainly a long hydrophobic chain, either saturated or unsaturated, with a hydrophilic carboxylic acid part on it (Figure 1.8). Thus, these amphiphilic molecules have dual solubility characters. Most naturally occurring fatty acids have an unbranched chain with even number of carbon atoms between 14 and 22 carbons in chain length [21,22].

The most abundant storage form of fat in animals and plants is triglyceride which is also known as triacylglycerol, TAG in short. The term “acyl” in the name is the adjective form of acid of fatty acids because they are formed from three fatty acids joined together by a single glycerol molecule. Glycerol is a small carbohydrate molecule with three carbons. The formation pattern of TAGs is shown in (Figure 1.8). TAGs function primarily in energy storage, thermal insulation and cell and organ protection [23].

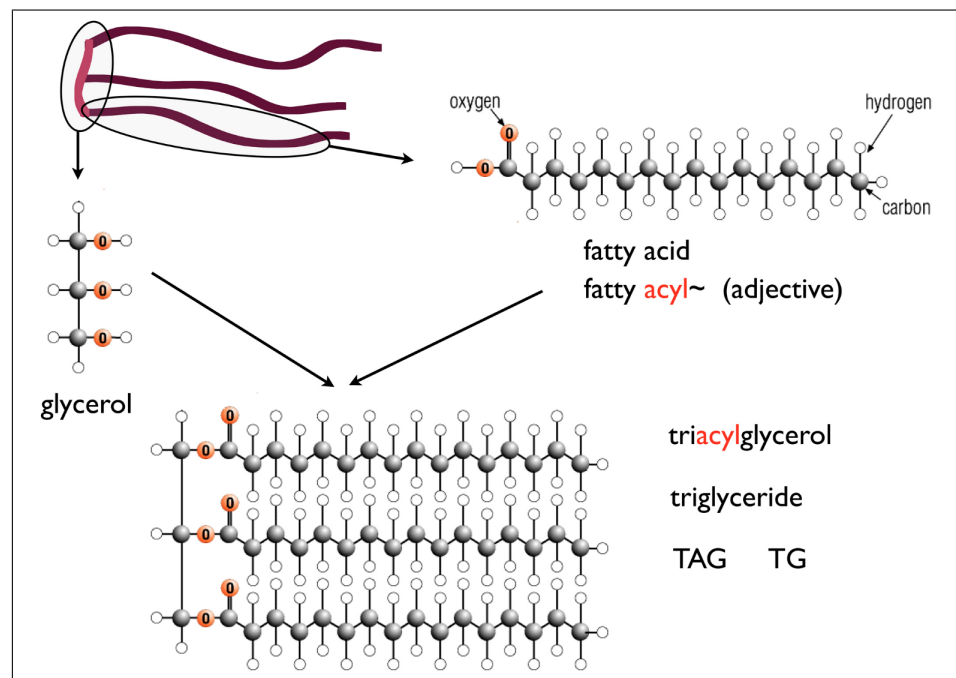


Figure 1.8. Schematic representation of formation of triglycerides from fatty acids and glycerol [24]

Another group of fatty acids which is very similar to TAGs in structure is phospholipids. Like TAGs, phospholipids also have glycerol in their architecture but they differ from fatty acids in two pattern ; they have double fatty acid chains instead of three on their hydrophobic parts and have distinctive phosphate head groups which supplies them unique properties (Figure 1.9).

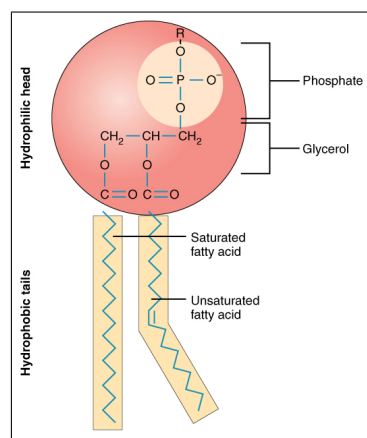


Figure 1.9. Phospholipid structure [25]

These special molecules have amphiphilic character like surfactants and they execute spontaneous formation of bilayers when placed into an aqueous medium. They self-arrange into spherical structures named as liposomes with a double layer of phospholipids. The hydrophilic head groups are oriented towards the aqueous surrounding solution, while the tail portions are repelled by hydrophobic interactions and attracted to each other inside the bilayer. The reason why they form bilayers, instead of unilayered micelles like other surfactants is the double chain on their hydrophobic tails. Phosphatidylcholines, PC, which has an extra choline structure in the head groups, and phosphatidylglycerols, PG, are the two most common types of phospholipids used in liposome formation. Phospholipids have important functions in biological mechanisms, like forming the cell membrane and intracellular organelles, supplying the supporting matrix and surface for many catalytic processes, actively participating in signal transduction and providing precursors for signaling processes and macro molecular synthesis [26–28]. Phospholipids have a variety of usages in industry too. Being fully natural, they can be used in drug delivery systems as carrier of drugs and personal care products as skin restoratives and moisturizers.

Sphingolipids are a class of fatty acids carrying a backbone of sphingosine which contains a set of aliphatic amino alcohols (Figure 1.10). These compounds play important roles in cell membrane structures, signal transmission and cell recognition. They also perform regulatory actions by interacting with specific proteins. Ceramides are notable classes of sphingolipids that are responsible for cellular signaling, differentiation, proliferation and programmed cell death [29].

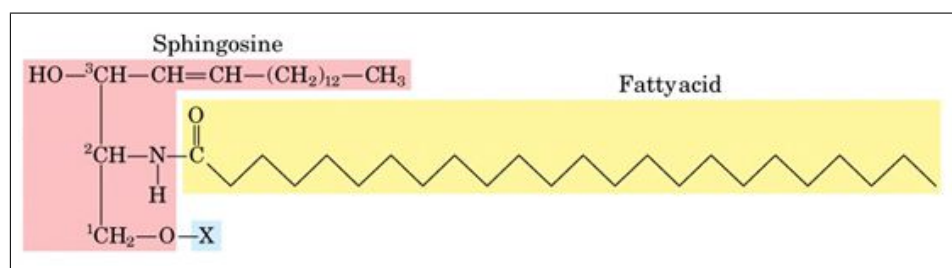


Figure 1.10. General structure of sphingolipids [30]

Fats that consist of very long alkyl chains are called waxes. Synthetic waxes are long-chain hydrocarbons (i.e. alkanes or paraffins) without any functional groups whereas natural waxes may include various types of substituted long chain compounds, such as fatty acids, primary and secondary long chain alcohols, ketones and aldehydes. They are widely used in coating and packaging applications, cosmetics, foods, adhesives, inks, castings, crayons, chewing gum, polishes and candles [31].

1.2.2. Vesicles as Lipid Aggregates

Self-aggregates of lipids are called vesicles. They are enclosed structures in which at least one flexible amphiphilic lipid bilayer separates an inner compartment filled with the solvent from the bulk external solvent medium. Vesicles can be formed spontaneously without external help or with external help such as sonication or hand-shaking [32, 33]. The accumulation of the bilayer sheets produces vesicles and these vesicles give rise to growth of the bigger giant vesicles [34].

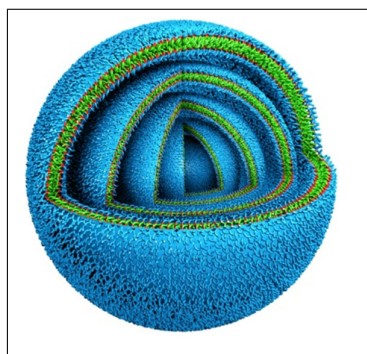


Figure 1.11. 3-D representation of multilamellar lipid vesicles [35]

Vesicles can differ in size from nanometers to micrometers and vary in membrane layers as being unilamellar or multilamellar vesicles (Figure 1.12). Unilamellar vesicles, ULVs, have one single bilayer around the inner aqueous parts. These vesicles can either be small ULVs, SUVs, or large unilamellar vesicles, LUVs. SUVs have a diameter between 20 and 100 nm whereas LUVs can go as big as 1000 nm. Recently, there has been also formed even bigger vesicles named as giant unilamellar vesicles, GUVs, which has diameters up to 50 μm . Vesicles can also exist as multilamellar vesicles,

MLVs, consisting of multiple lipid bilayers. MLVs are found generally in more concentrated surfactant systems, while ULVs are tend to be formed in dilute systems. Of all the types, MLVs are the simplest vesicles to prepare and obtain more homogeneous size distributed vesicles but the major disadvantage of them is the comparatively low encapsulation capacity which is several fold less than LUVs of comparable size. Fundamentally, size is prescribed by the thermodynamic properties of the lipid used in the system [36], [37].

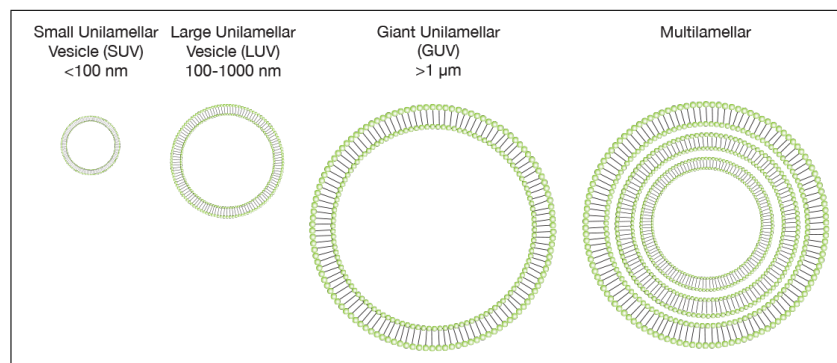


Figure 1.12. Schematic representation of vesicle types [38]

Stability of vesicles is an important concept to determine the end product application. Formation of stable vesicles is determined by some unique elastic properties of the lipid films. For example, having a low bending stiffness and a high intrinsic lateral pressure affects vesicle stability in a positive manner. The phase transition temperature, T_c , of the lipid bilayers is also one of the most important properties that helps to understand the link between temperature changes and the stability of the vesicles. Commonly, at a given temperature a vesicle can exist in either a liquid or a solid phase which is also referred to a *gel* phase. Upon increasing the temperature up to T_c , a phase transition occurs from gel phase to liquid one which allows free diffusion through vesicles.

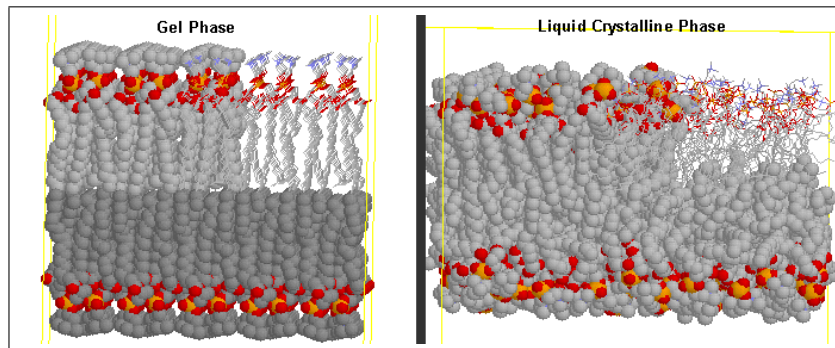


Figure 1.13. Differences in gel and liquid phases of the vesicles [39]

T_c is measured numerically with differential scanning calorimeter, *DSC*, and has a direct influence on mobility, permeability and the fluidity of the vesicles. Lipids with longer alkyl chain lengths will have lower mobility and consequently higher phase transition temperatures. Head groups and the degree of unsaturation are also the other determining factors of phase transition temperature. Vesicles are found to be more stable if the bilayer undergoes fluid to-solid phase transition [40].

Vesicles are very popular due to their ability to mimic biological membranes and are used in various applications such as drug delivery, nanotechnology and two-dimensional-crystallization of proteins. Vesicles take a great advantage in encapsulation studies and are used as targeted delivery agents. Upon having an aqueous core and a lipid bilayer in its structure, they are capable of encapsulating both lyophilic and lyophobic materials. They also protect the encapsulated materials from degradation and reduce drug-related toxicities in drug delivery applications. They are used in topical studies due to being biodegradable, biocompatible and stable in colloidal solutions. Also, one of the newest search areas of the vesicles is making models for artificial cells to understand the evolution of living species [37, 41]. Vesicles are named as liposomes when they are formed from phospholipids as explained detailed in the next section.

1.3. Liposomes

Liposomes are special kind of vesicles that are formed from self-assembled phospholipids in aqueous media. In 1964, Bangham [42] and his co-workers for the first time observed that phospholipids in watery medium construct closed bilayer structures. Later, these closed bilayer structures were named as liposomes by Sessa and Weissmann [43]. They are spherical microscopic lipid bilayers with an inner aqueous cavity that enables them ready for encapsulation of hydrophilic substances. They can carry hydrophobic substances in their bilayer regions as well. They can be filled with drugs, cosmetic raw materials, proteins, genes and other macromolecules to be used in targeted topical and drug delivery. Being biocompatible with cell membrane enables them to be used in-vivo applications.

In nature, phospholipids are found in cell membranes which are composed of stable bilayers. In a cell, two polar portions of these layers faces through outer and inner aqueous medium while the nonpolar portions exist between these polar regions hiding from the water and compose the cell membrane as demonstrated in Figure 1.14. When membrane of phospholipids are disrupted, they reassemble spontaneously into tiny spheres, smaller than a normal cell in bilayers and referred as liposomes [44].

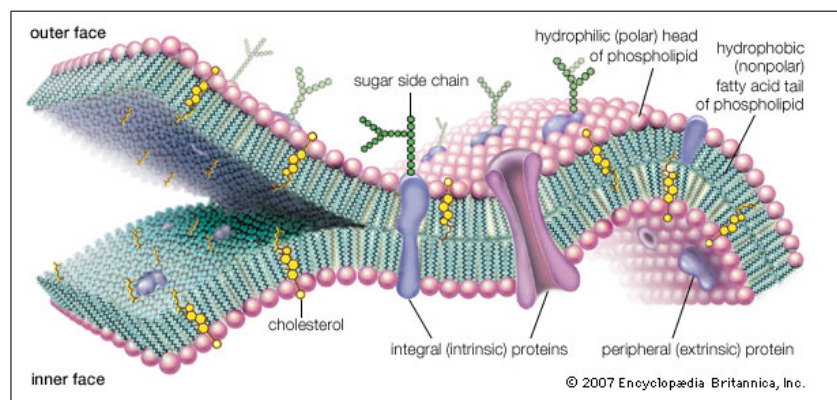


Figure 1.14. Phospholipid bilayer structure of cell membrane [45]

Liposomes are observed almost in all living organisms naturally and can be prepared artificially from lipid mixtures. When choosing the lipids and the exact lipid ratios to construct a liposome, it is important to calculate P of the lipids used. In lipid mixtures, P of each lipid is multiplied by its mole fraction and the additive result determines the lipid aggregate shape. The sum has to be in the range of 0.74- 1.0 to obtain liposomes as aggregates [46].

1.3.1. Preparation of Liposomes

Phospholipids spontaneously form liposomes but main matter of the fact is getting the aggregate with the right structure, size, high entrapping efficiency and low leakage of encapsulated material in a reproducible way. There are lots of ways for manufacturing liposomes which can be examined under several common stages such as drying, hydration, down-sizing, purification and lastly characterization of the final product (Figure 1.15). These stages are classified under three main methods as mechanical dispersion, solvent dispersion and detergent removal.

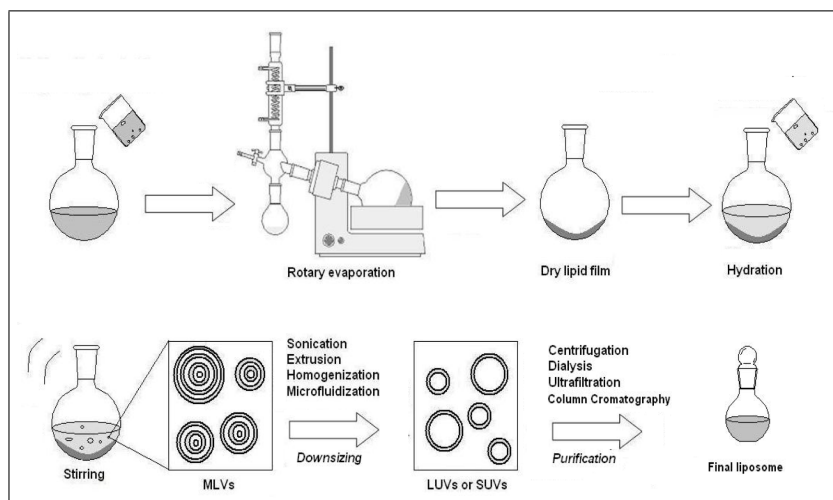


Figure 1.15. Representation of liposome production [47]

Mechanical dispersion method starts with mixing all the lipids and hydrophobic materials to be entrapped in an organic solvent homogeneously, and drying down the solution onto the side of the container glassware with rotary evaporator under reduced

pressure. Lyophilizer can also be used to obtain complete dryness. Then, dispersion of this solid residue is achieved by addition of the aqueous medium. If the substances desired to be entrapped are hydrophilic, they are also solubilized and added with this aqueous medium. Upon hydration, the residue peels off the glass vessel by swelling and forms MLVs to be processed in further steps. This method is extremely useful for encapsulating of hydrophobic substances whereas the yield for hydrophilic materials is quite low.

Solvent dispersion method includes injection of the dissolved organic lipid solution into the watery medium that contains the materials entrapped. MLVs are immediately formed at the interface between organic and aqueous regions. The main drawbacks of this method are getting heterogeneous size distribution of liposomes and difficulties in removal of possible azeotropes of water with organic solvents.

In detergent removal method, the detergents at their CMCs are used to solubilize lipids. As the detergent is removed, the micelles become increasingly richer in phospholipid and aggregate to form LUVs. The removal is achieved either by dialysis or column chromatography and the resultant vesicles are perfectly homogeneous in size. Although this method is the best for obtaining reproducible liposomes, extra awareness and further processing is needed to remove the remaining traces of detergents within the liposomes.

By means of reproducibility and applicability, mechanical dispersion method is the simplest and easiest way to produce homogeneous sized liposomes among all the three methods. After preparation of MLVs, liposomes are processed in a series of additional steps to modify their characteristics like size, shape, stability and encapsulation efficiency.

Following the formation of MLVs, various down sizing methods are applied to reduce vesicle sizes because they are too large to work with and highly heterogeneous in population. Ultra-sonication and extrusion are the most preferred techniques for size reduction.

Sonication includes disruption of MLVs suspensions by using sonic radiation energy and produces SUVs with diameters in the range of 20-100nm. Most common instrumentations for this process are bath and probe tip sonicators. Bath sonicators are applied for large volumes of dilute MLVs whereas probe tips are more suitable for smaller volume of suspensions that require high energy input to break down the vesicles. As seen in Figure 1.16, probe sonication is an open system that includes contamination risk both from environment and metal tip of the probe. On the contrary, it is not needed to open the suspension vessel in bath sonicators and so they provide a safer alternative for down sizing step. They also enable regulation of the suspension temperature which is highly important for performing the process above T_c of the lipids to avoid phase transitions. Because, sonication below the T_c of the lipids results structures that contain defects within the bilayer. Also, possibility of lipid degradation is fewer in bath sonication due to applying milder energy to system and it supplies a more reproducible way of adjusting vesicle size. Mean size and distribution of the vesicle in this down sizing method is influenced by the factors like composition, concentration, temperature, sonication time and power, volume and sonicator tuning.

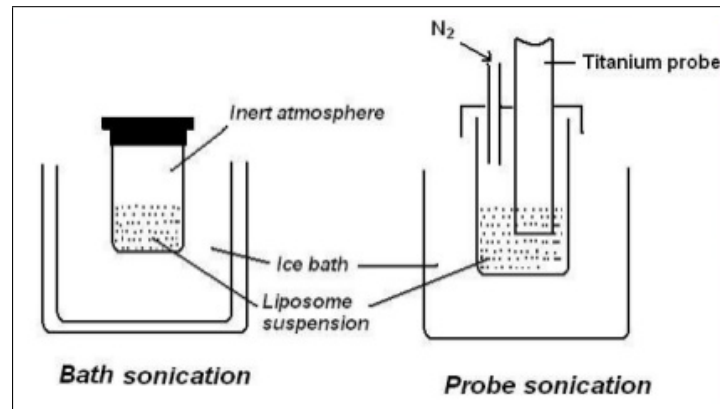


Figure 1.16. A demonstrative picture of bath and probe sonicators [48]

Membrane extrusion is another technique for down sizing vesicles which includes an extruder unit with a membrane filter of defined pore size. Upon multiple repeated extrusions through polycarbonate membranes, homogeneous size distribution of SUVs that converted from MLVs is achieved at around the membrane pore sizes. This method is more advantageous over sonication in many respects. It is very simple, reproducible,

nondestructive and applicable for suspensions with high vesicle concentrations. Also, sustaining the suspension temperature is no longer a problem in membrane extrusion due to not having warming-up troubles. Only drawback of this technique is working volumes are so small that extruder syringes fit in just a small amount of solution changes from 0.25 to 1 ml and the process has to be repeated several times to obtain the desired volumes for future steps [49–51].

Industrial production of liposomes requires additional attention because sterility and stability issues gain more importance for the end products. Removal of carcinogenic raw material and solvent residuals is the main handicap to overcome in big scale productions. To prevent this, usage of carcinogenic raw materials is eliminated from the process and ethanol injection is applied as a dispersion method. This method is easy to scale up and ethanol is a harmless solvent that accepted by the authorities at a maximum rate of 0.1%.

1.3.2. Enhancing The Liposome Stability

Liposome stability can be investigated in physical, biological and chemical aspects that are also relevant with each others. Liposomes tend to show coalescence or flocculation behaviors by fusing within the dispersion. This is a thermodynamically driven process and causes a decrease in vesicle shelf lives. Also possible degradation of phospholipids will result in leakages of the entrapped substances and broken down lipids will interact with the leaking materials. Lipids can degrade either by hydrolysis of the ester linkages between glycerol backbones and fatty acids or by oxidation of the unsaturated acyl chains on their hydrophobic regions. Sustaining the size distribution in the desired range is another issue that is important for liposome quality. Furthermore, environmental factors such as temperature, light, oxygen, and heavy metal ions may influence the liposome stability and initiate chemical or physical changes [50].

There are several preventive steps to overcome problems related to degradations. Protecting lipids from light exposure, using nitrogen or argon gases and adding antioxidants while preparing liposomes avoid oxidative degradations and increase the shelf

life. EDTA, Ethylenediaminetetraacetic acid, like preservatives can also be used to remove heavy metal traces from the product. To prevent degradations upon hydrolysis it is important to control T_c of the lipids and the environment of the aqueous medium. Lipids with convenient T_c s, should be chosen not to have unconformity troubles. Buffers with low salt concentration should be used protect the vesicle structure and maintain the vesicle solution pH [52].

Addition of cholesterol in lipid composition stabilizes the bilayers by providing membrane fluidity, elasticity and permeability as well. A rigid and more saturated membrane with higher ratio of cholesterol is increases the stability and reduces the leakage problems.

Liposome stability can be enhanced upon adjusted storage conditions. In one study, it is reported that vesicles are stable up to 6 months when they are stored at 4°C instead of 25°C [53]. Freeze-drying of liposome suspensions with lyophilizer, an instrument for removal of water traces in the frozen state at extremely low pressures, shows an increase in the shelf life. Suspensions can be stored under lyophilizer by adding cryoprotectants like sugars or polyols as stabilizing agents. Freezing without stabilizers can cause damages on the vesicle structures [54].

Charge on the liposomes is also a determinant factor on both in-vivo and in-vitro stability. In a study done by Caselles et al, liposomes with neutral charge containing phosphatidylcholine showed the highest stability and the ones with positive charge exhibited similarly to those with neutral charge. On the other hand, the stability of negatively charged liposomes was dependent on their composition and those containing only one class of negatively charged phospholipids were very unstable while liposomes containing also PC were more stable [55].

1.3.3. Characterization of Liposomes

Characterization of liposomes is required to be done immediately after the preparation because vesicle quality can change very fast upon storage. A reproducible, pre-

cise and rapid methodology should be established to obtain a predictable end product. Some of the main characteristics of the liposomal formulations are vesicle mean size and shape, homogeneity, surface charge, encapsulation efficiency, stability, in- vivo drug release, T_c and lipid composition in the aggregate [56].

1.3.3.1. Size and Size Distribution Determination. For delivery studies, vesicle size is the key factor that determines the encapsulation amount within the aggregate and permeability through cell membrane. Size distribution and average size of the particles is mostly measured by Dynamic Light Scattering, DLS. Working principle of this technique is based on radiating a monochromatic light beam onto a solution including spherical particles and measuring the change in wavelength of the incoming light. This change results from the fluctuations of the light scattered from the particles that are in Brownian Motion. Motion is created by the collisions between suspended particles and solvent molecules. DLS is a time effective and practical technique for vesicle size distribution measurements due to being fully automatized and it is also suitable for routine measurements with its modest cost [57,58].

1.3.3.2. Shape Visualization of Nanoparticles. Shape morphology assessment for nanoparticles is performed with various electron microscopy techniques such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Scanning Transmission Electron Microscopy (STEM) which is a combination of SEM and TEM, Cryo Electron Microscopy (Cryo-EM) and Freeze Fracture Electron Microscopy (FEM). Each of them has a specific purpose of usage with little differences and applicable for various types of samples [59]. Electron microscopes can also be used for size determination of nanoparticles but it is not very time and cost efficient method for that specific purpose.

SEM is used for analyzing electrically conductive materials and when the sample is non-conductive, it is coated either with low-vacuum sputter coating or high-vacuum evaporation methods by an ultra thin coating of electrically conducting material. Environmental SEM mode is also available for the non-conductive specimens if the material

is not suitable for coating treatments [60]. SEM is only appropriate for completely dried samples whereas Cryo-EM allows observation of frozen specimens in their native states at cryogenic temperatures [61]. Both techniques are disadvantageous in a manner that processing samples (i.e. coating, freezing) may cause conformational or physical changes and this can alter the resultant images.

FEM is based on freezing a specimen rapidly and cracking it on a plane through the tissue. This method is particularly useful for lipid membrane examinations and protein binding studies [62].

STEM is the most suitable technique for vesicle characterization due to allowing sample imaging without any additional treatment except air drying of the specimen. STEM is performed similarly to SEM by scanning a focused beam of electrons over a thin sample while collecting some desired signal to produce image and differentiates in a way that it supplies higher resolution by creating backscattered electrons and X-rays [63].

1.3.3.3. Surface Charge Determination. Zeta potential (ζ) analysis is an effective tool for determining surface charge properties of the nanoparticles in an electrolyte solution. Dispersed particles are surrounded by an electrical double layer region which is formed as a result of the attractions between surface charges and oppositely charged thin layer of ions (Figure 1.17).

First layer, *Stern Layer*, is directly bounded to the particle and consists of oppositely charged ions. Second layer is called “*Diffused Layer*” and formed from ions that are less firmly associated with the core. There is a characteristic boundary location within this double layer associated with particle movement named as “*Slipping Plane*” and liquid underneath of the slipping plane remains attached to the surface. Zeta potential represents the potential at this boundary and quantifies the charge stability of colloidal nanoparticles. A high zeta potential indicates higher repulsions and promotes greater stability within the liposomal formulation. Flocculations may occur in

dispersions with low zeta potentials due to attractive forces between similarly charged particles.

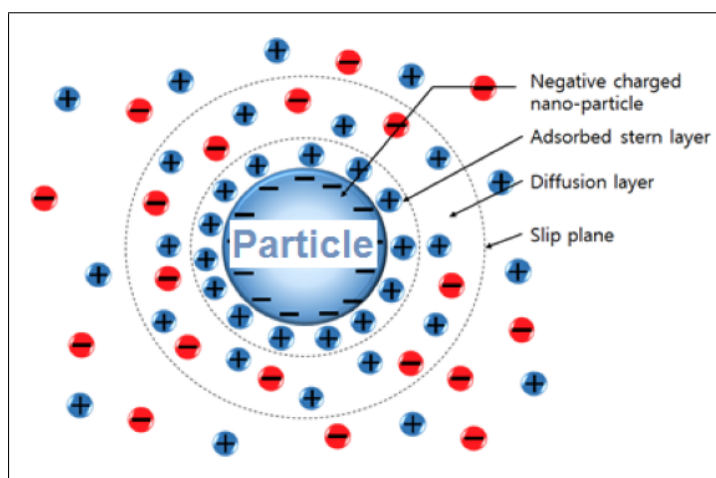


Figure 1.17. Electric double layer around a charged nanoparticle suspended in a dispersed medium [64]

Unlike size and shape determinations, particle environment e.g., pH, ionic strength, and concentration have a great effect on zeta potential measurements. Therefore, successive evaluations is needed to obtain an accurate result [65].

1.3.3.4. Entrapment Efficiency. Yield of encapsulation can be estimated by column chromatography techniques. High Performance Liquid Chromatography (HPLC), Size Exclusion Chromatography (SEC) and High Performance Thin Layer Chromatography (HPTLC) is the major methods to indicate entrapped material percentages. If the material entrapped is absorbed in the Ultraviolet - Visible region, Ultraviolet-Visible (UV/Vis) spectrophotometer can also be used to follow up the encapsulated drug amount.

Important point to take into consideration in all these methods is that liposomal formulations consist of both un-encapsulated and encapsulated materials around vesicles. Hence the free, un-encapsulated fractions should be separated with a refinement technique like ultracentrifuge.

1.3.3.5. In Vitro Drug Release Studies. Drug release studies are usually performed with dialysis method. A portion of liposomal formulation is placed in a dialysis membrane at 37°C under continuous magnetic stirring and receptor medium is sealed to avoid evaporations. Samples are taken at several time intervals and drug releasing properties are monitored by UV spectrophotometer for about 8 hours [56].

1.3.3.6. T_c Determination. DSC is used to determine critical temperatures of the liposomal formulations. Each sample is generally scanned between 20°C to 200°C with a scanning rate of 10°C/min or 20°C/min [66].

1.3.4. Liposome Applications

Recently, liposomal formulations have gained a great attraction due to their controllable chemical and physical characteristics. They are used in different branches of science like mathematics (i.e. topology of two-dimensional surfaces in three-dimensional space by bilayer elasticity), physics (i.e. aggregation behavior, fractals, soft and high-strength materials), biophysics (i.e. permeability, phase transitions in two-dimensions, photo physics) and pharmaceuticals (i.e. drug action studies). Amphiphilic characters of the vesicles enable them to be used as powerful solubilizing systems for a wide range of compounds as well [67].

It has been shown that liposomal formulations increases therapeutic activity and decreases toxicity. So, the most common implementations of liposomes are in clinical studies as targeted and controlled drug delivery, gene therapy and drug encapsulation. They are non-toxic, biocompatible and biodegradable delivery vehicles. Liposome technology enables drug carriage to a tissue or cell area that is normally inaccessible to the free drugs by also giving the chance to control of drug releasing rate [68].

Vesicular systems can encapsulate and deliver both hydrophobic and hydrophilic materials effectively into the cell membrane and so they are appropriate substances for

topical applications which include skin delivery investigations. In cosmetic industry, liposomal formulations are used as penetration enhancers for cosmeceutical products.

Lipid composition, surface characteristics, size, bilayer phase behaviors are the important factors directly related to the end usage of liposomal formulations. Due to the ease on controllability of all these parameters, liposomes are very encouraging materials to be engaged in a variety of commercial applications.

1.3.5. Liposomes in Cosmetic Industry

Cosmeceuticals are the fastest growing branch of the personal care industry. According to a study done by "Research and Markets", an online market research organization, entire global cosmetic industry sales were about 460 billion USD in 2014 and is considered to be 675 billion USD by 2020. Almost 36% of this share is occupied by skin care market which includes moisturizers, cleansers, skin masks, anti-acne and anti-aging products [69]. Although beauty industry is originated in very ancient times and developed a lot, still a plenty of new trends come out within the market that urge progress and revenue. Liposomes are one of the most novel vehicles that are extensively used as raw material carriers in cosmetic industry with their encapsulation and skin mimicking abilities.

Liposomes are biodegradable, biocompatible, flexible and nontoxic vesicles that can encapsulate both hydrophilic and hydrophobic active ingredients easily. Incorporation of liposomes in cosmetic products is remarkably advantageous because lipids are well hydrated and can decrease the dryness level of the skin which is the main reason for the major skin problems. Due to the similarities to biological membranes, liposomes can easily penetrate into the epidermal barrier and provide required raw materials to replenish the skin. They can also increase the chemical and physical stability of the sensitive cosmetic ingredients by acting as a protective vehicle against oxygen and light. Adjustable lipid composition of liposomes makes targeted and controlled dermal release possible [70].

PC is one of the most common lipids for the liposomes used in cosmeceutical products due to its softening and conditioning properties. Vitamins, antioxidants, ceramides and cholesterol are the main ingredients encapsulated into liposomes to be used in topical creams so far. Liposomes can also be used in the treatment of hair loss and dry skin and incorporated in a large fraction of cosmetic market like sunscreens, moisturizers, solid perfumes, lipsticks and hair conditioners. “Capture” was the first liposomal anti-aging cream produced by Dior in 1986 [71].

1.4. Liposomes and Skin

1.4.1. Structure and Function of the Human Skin

The main function of the skin is to defend the body from undesirable exogenic influences such as bacteria, ultra-violet rays of the sun and unpleasant temperature conditions and also to avoid the loss of endogenic substances like water and blood. This indicates that the skin acts as a barrier against transition of materials through the underlying tissue. Skin is composed of three main layers as the epidermis, the dermis and the hypodermis (Figure 1.18).

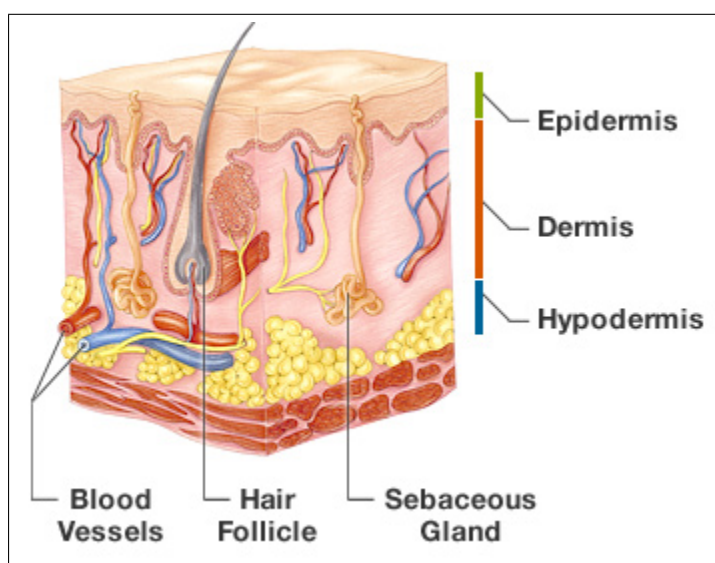


Figure 1.18. Structural layers of human skin [72]

The primary barrier of the skin is located in the epidermis, outermost part of the skin which is called *stratum corneum*, SC. The principal constituent of this vitally important region is lipids and the barrier function depends on the presence of a unique accumulation of lipids in the inter-cellular spaces of the SC. All the substances applied onto the skin always have to pass this protective barrier at first. For this purpose, understanding the lipid organization in this domain is considered to be very crucial for the skin barrier function [73, 74].

Under the SC, there is the viable epidermis layer which generates the SC. The reason for epidermis is called as a “viable” layer is that it is a dynamic, self-renewing tissue which balances the SC cell growths right after any loss of surface skin cells. The epidermis contains no blood vessels and is nourished by diffusion from the dermis. It helps regulating the body temperature.

The dermis, intermediate layer of the skin, is directly adjacent to the epidermis and responsible for the mechanical support of the skin. It contains blood vessels that provide nourishment and waste removal from the skin and nerve endings that provide the sense of touch and heat.

The lowermost and thickest layer of the skin is hypodermis which attaches the skin to underlying bone and muscle system. It stores fat as an energy reserve and contains blood vessels and nerves that are larger than those found in the dermis [75–77].

To better understand the working principles of the skin barrier, it is necessary to get a deeper knowledge on lipid composition of the skin.

1.4.2. Lipid Composition of Human Skin Barrier

Squier et al. in 1991, proved that the main determinant of the skin function is lipid content of the epidermal SC rather than the thickness or number of layers present. Lipids represent a major role in the water permeability of the skin barrier by comparing permeabilities of skin samples before and after lipid extraction [78]. Skin fatty acid,

phospholipid and cholesterol content may alter excessively in many different kinds of mammals but here only human skin composition will be taken into consideration. It is important to know the exact composition of the human skin barrier to develop cures against recent skin problems such as acne, eczema, rosacea and so on.

The major lipids found in human SC are ceramides, cholesterol and free fatty acids which make up approximately 50, 25 and 10 percent of the SC lipid mass, respectively. In Table 1.1, lipid composition of human SC can be seen in detail [79].

Table 1.1. Composition of the human SC lipids

Lipids	Mass percentages
Triglycerides	30.1
Free fatty acids	19.3
Ceramides	18.1
Cholesterol	14.0
Hydrocarbons	10.9
Cholesterol esters	6.9

There have been found eight subclasses of ceramides which differ from each other by the head group structures linked to a fatty acid or a-hydroxy fatty acid of varying hydrocarbon chain length and ceramide1 has been found to play an important role in the formation of the lipid barrier [80,81]. Fatty acids in SC generally has chain lengths between C22 and C24 and they hardly affect the lamellar lipid organization. They are necessary for formation of a lamellar phase since this is the principal ionizable lipid class in the SC. An important constitution of the outermost skin layer is cholesterol sulfate. Although it is found in small amounts, typically around 5 percent w/w, it has a big role in the suppression of proteases in SC, enzymes that are important for the degradation of the desmosomes, cell structures specialized for cell-to-cell adhesion. In the external SC layers cholesterol sulfate is metabolized to cholesterol by cholesterol sulfatase, which increases the activity of proteases [82]. Upon researches done on

isolated skin mixtures, no protein molecules encountered in SC and it is concluded that proteins have no influence on skin barrier [74].

1.4.3. Liposomes as Transdermal Drug Delivery Agents

SC is composed of dead cells and corneocytes planted in the lipid regions. Corneocytes are also made of dead cells that are filled with water and keratin filaments. Principal lipid constituents of SC are saturated fatty acids, ceramides, cholesterol and some cholesterol esters and they are somehow systematized in a multiply bilayered organization [83]. Transdermal drug delivery is the topical application of drugs to the skin in the treatment of skin diseases and it uses the skin as an alternative route for the delivery of active drugs. SC is mostly in the scope of transdermal delivery subject because it acts as a primary barrier to active material transportation.

Liposomes offer a potential value as topical delivery agents in encapsulation studies due to their similarities in lipid bilayer organizations with skin and ease to mimic the SC nature. By altering the lipid composition used, liposomal structures with tailored properties can easily be designed for delivery and encapsulation studies of drugs.

Alternative mechanisms have been proposed to explain working of liposomes as skin drug delivery vehicles so far and in Figure 1.19, one of the models that illustrates how cells accept liposomes is shown as an example. According to this model, liposome lipids adhere onto the skin surface and after releasing the drug, mix with the current lipid matrix [84].

Deformable liposomes and ethosomes are the new liposome classes of intensive research on transdermal delivery because of their elasticity and flexibility features. They are capable of crossing the skin just by squeezing through SC channels even though their sizes are ten times bigger than those channels. Difference in water content between the relatively dehydrated skin surface and the aqueous viable epidermis results this penetration behavior. Ethosomes are different from traditional liposomes in a manner that they contain up to 10% ethanol in their composition which helps them to

disturb skin bilayer organization. Skin penetration enhancing behavior of these special vesicles are mentioned detailed in a separate section as they are used in the scope of this research.

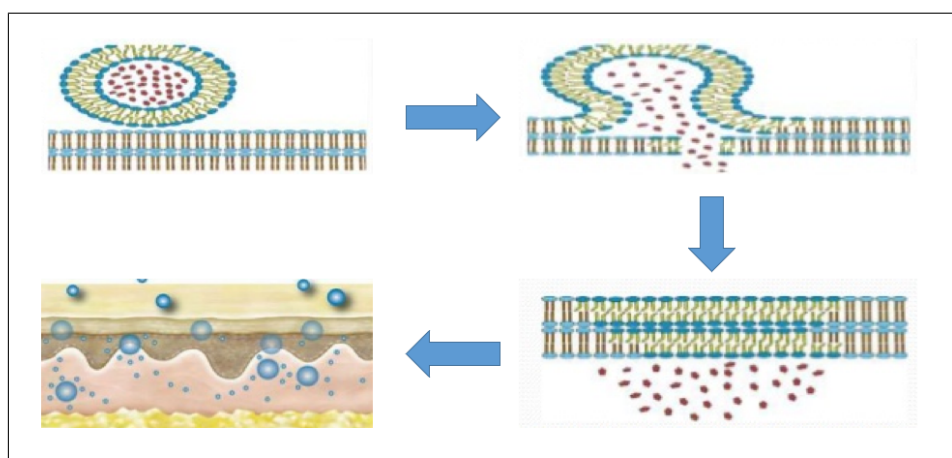


Figure 1.19. Drug release mechanism of liposomes into the skin

1.4.4. Ethosomes as Skin Penetration Enhancers

Ethosomes are vesicles that contain high concentration of ethanol which makes them unique about the ease of penetration through SC. By interacting with the polar head groups in the lipid bilayer of the skin, ethanol reduces the rigidity and so increases the fluidity of the SC which then leads a raise in membrane permeability. Enhanced permeability helps encapsulated drugs penetrate through the deep skin layers. Furthermore, ethanol provides better solubility to drugs and this increases the encapsulation efficiency. It is also reported that higher zeta potential carrying vesicles are more successful to attach inside the skin. In cosmetics, ethosomes are used for increasing stability of the end products and avoiding bacterial infections as well [85].

There are many different ways to obtain ethosomes but the most applicable and simple method is mechanical dispersion. It is the addition of hydro alcoholic solution that contains ethanol with different concentrations to the lipid films in dispersion step.

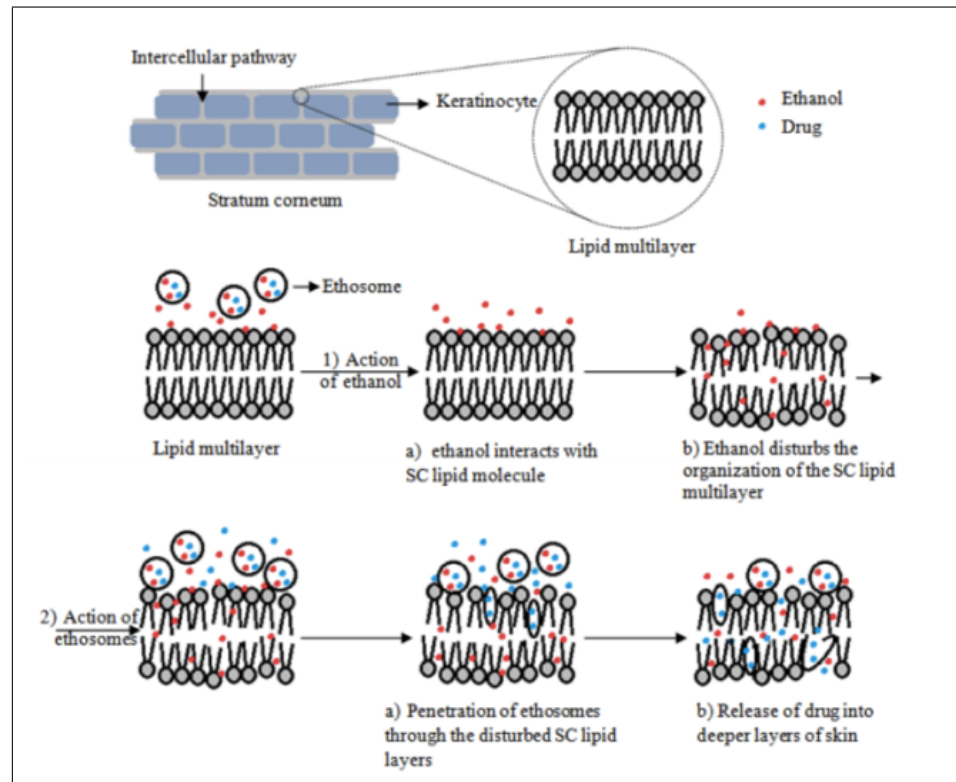


Figure 1.20. Drug release mechanism of ethosomes into the skin [86]

1.5. Eczema and Filaggrin Deficiency

Eczema is the most significant and common skin disease affecting more than 20% of the children in the western nations. It causes a dry, red skin and itchiness. Eczema generally occurs among children in the first six months to five years old. It usually appears on the face but can be observed anywhere of the skin like knees, elbows, hands and back of ears. Frequently symptoms disappear as the patients grow older but some of them will keep on experiencing the complaints during adulthood. It can develop among adults also, even if they have never had it in childhood. There are several types of eczema with different symptoms on the victims and depends on the history of affected person. Eczema is called atopic dermatitis when it is chronic, severe and inflammatory and it is the most common type of eczema.

The exact cause of atopic eczema is still unknown. According to the research results done by Di Nardo and P. Wertz, the ceramide and cholesterol sulphate levels

were significantly lower and the cholesterol values were higher in eczema patients with respect to healthy subjects [87]. Ceramide is responsible for connecting the adjacent bilayers in the skin and maintenance of the necessary ratio of water input and output through the SC. Decreased ceramide levels cause an increase in water loss which then leads to dry skin. Degenerate skin barrier can not protect itself against irritants and allergens and emerges eczema. Later, Irwin McLean's findings on atopic dermatitis showed that some genes control the skin health and filaggrin has the top importance among them. They find out filaggrin loss-of-function mutations and the collapse of skin barrier function are the key reasons behind eczema development. Filaggrin deficiency results in disturbed skin barrier which leads to higher water loss and entrance of allergens through SC [88].

There is currently no permanent cure for atopic eczema, but treatment can help to relief the impacts of symptoms. Avoidance from irritants and eczema triggering factors like soaps, detergents, wool clothings, extreme weather conditions and allergic foods that specific for individuals may help to reduce obtrusive effects of the disease. Moisturizing the skin regularly may assist to get rid of the symptoms resulting from dry skin. Also, there are some topical steroid creams decreasing the cutaneous inflammation by maintaining ceramide levels in the skin barrier. There is not a successful research done on curing filaggrin deficiency among the eczema patients yet and it is lately in the scope of dermatology studies.

1.5.1. Filaggrin Gene and Protein

The term filaggrin is derived from 'filament aggregating protein' and it is a very crucial protein for human skin epidermis. Filaggrin is formed from the breakdown of profilaggrin polyprotein which is a major component of the skin and vital for formation of corneocyte layer that generates the outermost protective skin portion. During epidermal terminal differentiation, profilaggrin dissociates by serine proteases to form monomeric 37 kDa dephosphorylated filaggrin proteins. It has a distinctively high pH value due to its high histidine content in its primary structure. Dryness of the human skin is measured by filaggrin levels because it aggregates with the keratin filament

which is responsible for shaping skin cells in the skin membrane. Without this aggregation outermost barrier layer would be disturbed and water loss would occur. As the corneocyte layer would not form properly, the corneocytes dry out and the lipid layer is easily lost so that the skin becomes dry and cracked.

Filaggrin formation is responsible by FLG gene and faulty work of this gene causes filaggrin deficiency. Around one of tenth of world population inherits this filaggrin loss-of-function mutations which causes 50% reduction in the amount of filaggrin produced in the skin. Filaggrin deficiency has different results on people; some suffer from dry skin, some suffer from vulgaris and some suffer from atopic eczema. Some patients with no filaggrin have more severe results on their skin such as marked ichthyosis vulgaris and cracked skin.

1.5.2. Madecassoside and Heterosides

Madecassoside is a chemical compound derived from centella asiatica plant leaves that is used traditionally as an ancient herbal remedy among eastern nations for many centuries. It has a wide range of biological activities such as anti-inflammatory, antioxidant and wound healing. Even small amounts of it like %0.1 in cosmetic products have the specified benefits when applied to the skin. Due to being a water soluble active reagent within the pH range of 4 and 7, it is very easy to formulate madecassoside with skin care products. Because of anti-inflammatory capability, it has been used to treat psoriasis and chronic lesions of the skin.

Centella asiatica mainly includes two glycosides; madecasoside and asiaticoside. They both increase the collagen synthesis and used as anti-aging and skin firming agents. A mixture of them is called heterosides. This mixture improves the skin barrier function by stimulating ceramide formation, filaggrin synthesis and water recycling through the skin. In cases of atopy prone skins, only madecasoside including creams are recommended. Madecasoside itself supplies protection from inflammatory stress, normalize keratinization process and triggers filaggrin protein formation.

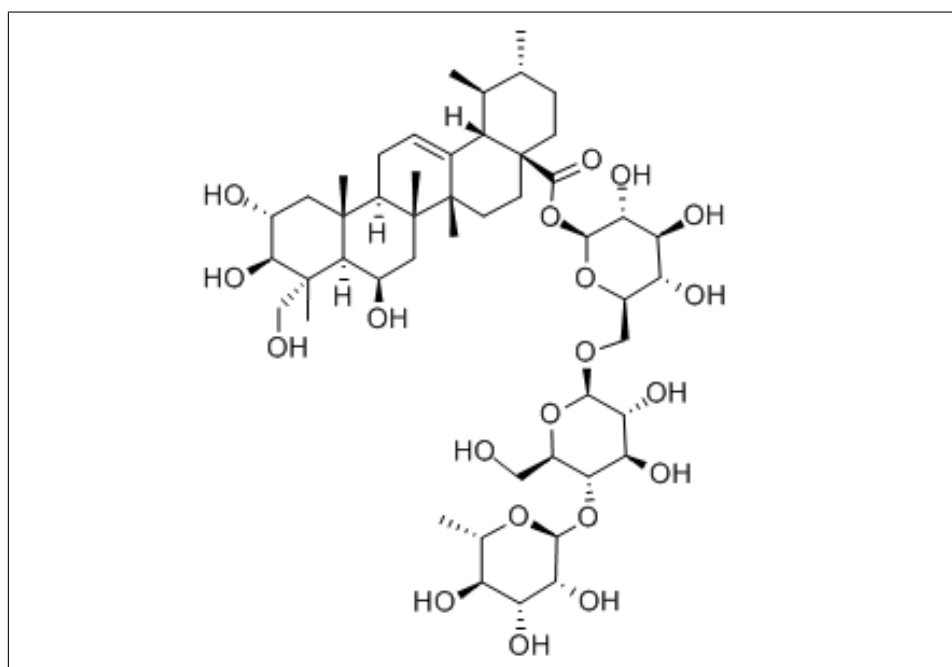


Figure 1.21. Chemical structure of madecassoside [89]

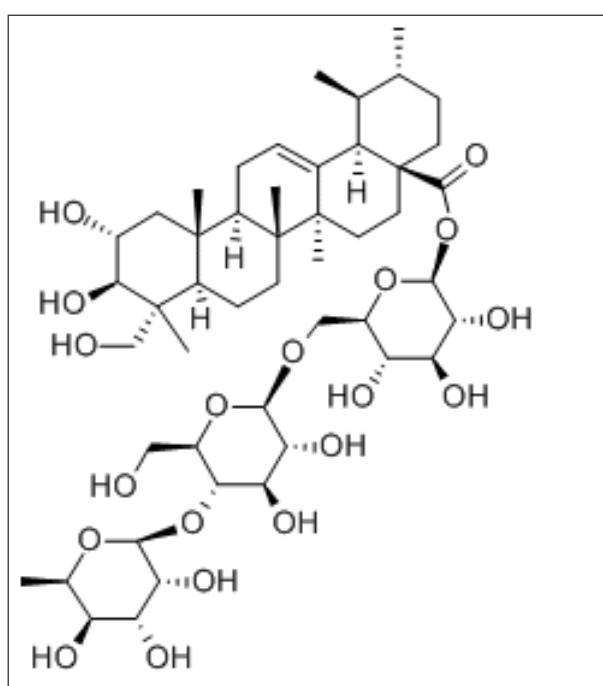


Figure 1.22. Chemical structure of asiaticoside [89]

In this study, madecassoside and heterosides are used in encapsulation studies as alternative cosmetic raw materials to prepare liposomes in purpose of eczema curing due to their filaggrin stimulating properties.

2. AIM OF THE STUDY

Liposomes, have gained a tremendous importance in cosmeceutical industry due to their abilities of enabling the active ingredient penetration through the tough skin sub-layers and enhancing the skin absorption of the applied cosmetic products. Based on this feature, liposomal encapsulations are largely used to lower the costs of skin products by reducing the required amount of active constituent. Studies on liposomes are mostly about maximizing liposomal formulations to skin lipid compositions and increasing the encapsulation yields for skin care ingredients.

Eczema is a kind of inflammatory skin disease that causes red, dry skin with obstructive itchiness. It approximately affects 10 to 20 percent of the world's population and mostly linked to genetic factors, nutrient deficiencies, bacterial infections and dry skin. In 2011, Irwin McLean stated that the lack of a protein named filaggrin in the skin causes dry skin conditions which are strongly related to eczema. So far filaggrin deficiency could not be cured by classical methods or by taking filaggrin supplements.

In this research, by using DMPC, DMPG, ceramide and cholesterol lipids, we have tried to introduce a novel liposomal formulation with a reproducible lipid and suspension medium combination which is very similar to human skin lipid composition to be used in cosmetic raw material encapsulation. The designed liposome has further attempted to encapsulate filaggrin protein. Some centella asiatica leaf extract molecules, madecassoside and heteroside, which are assisting filaggrin protein synthesis in human skin, are also included in the encapsulation studies.

The resultant liposomes are characterized in terms of particle shape and size, size distribution and surface charge properties by making use SEM, DLS and Zeta Potential Analyzer. Encapsulation efficiency is followed and calculated based on UV-Vis spectrophotometry information.

3. EXPERIMENTAL

3.1. Materials

3.1.1. Lipids

1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) is a zwitterionic synthetic phospholipid in white powder form with >%99 purity, purchased from Sigma Aldrich.

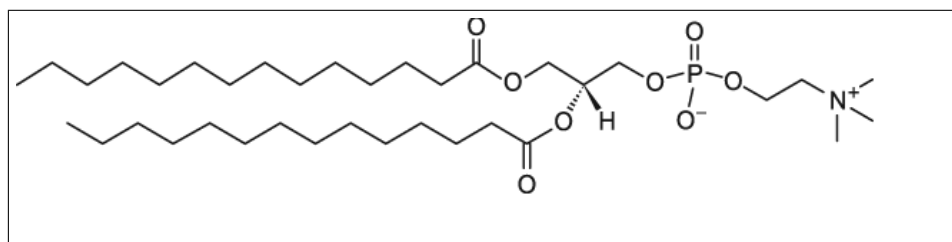


Figure 3.1. Chemical structure of DMPC

1,2-Dimyristoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)]],Sodium salt (DMPG) is an anionic synthetic phospholipid in white powder form with >%99 purity, purchased from Sigma Aldrich.

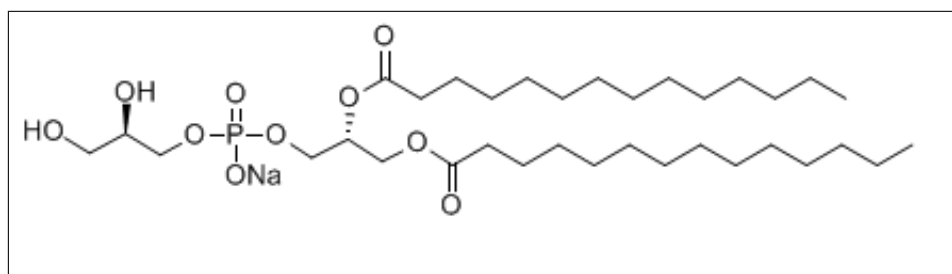


Figure 3.2. Chemical structure of DMPG

Cholesterol is a sphingolipid in charge of controlling cell membrane permeability and fluidity. It is a supportive lipid for liposome preparation and purchased from Sigma Aldrich in white powder form with >%99 purity.

Ceramide is a white powdered lipid with >%98 purity, purchased from Sigma Aldrich. It protects skin from water loss and external infections.

Cholesterol sulfate is a molecule mainly responsible for serine proteases in epidermis which involves in epidermal cell adhesion. It is purchased from Sigma Aldrich in white powder form with >%99 purity.

3.1.2. Encapsulated Materials

Filaggrin is purchased from Thermo Fisher Scientific in a diluted green solution form named R7 format to avoid degradations. It is diluted in 0.05 mol/L Tris-HCl, pH 7.6 containing stabilizing protein and 0.015 mol/L sodium azide. The concentration of filaggrin in R7 is 4 μ g/mL.

Madecassoside and heterosides are given as a gift of Novodist Cosmetic Chemicals company in powder forms with >%99 purities.

3.1.3. Solvents

Chloroform and methanol are purchased from Merck with >%99 purities. They are used for solubilizing lipids prior to the thin film preparation by rotary evaporator.

3.1.4. Suspension Media

Phosphate Buffer Solution (PBS) used for hydration of thin lipid films is prepared in the laboratory. 1.36 gr potassium dihydrogen phosphate (KH_2PO_4 , Merck) is solubilized in 100 mL deionized water. Then 58.2 mL of 0.1M sodium hydroxide solution

(NaOH, Merck) is added onto this mixture and solution is completed to 200 mL with extra deionized water. The final buffer solution is 0.05M and has a pH around 7.01.

Ethanol is purchased from Merck with >%99 purity. It is used in preparation of liposome suspension solution in hydration step.

3.1.5. Breakdown of Liposomes

0.02M Cholic acid, sodium salt solution is prepared by solubilizing 0.43 gr salt in 0.05L deionized water. The salt is purchased from Acros Organics in a white crystalline form with >%99 purity. Sodium cholate is a bile salt that is used to break down the encapsulated liposomes by saturating the bilayers. 1:1 volume ratio of liposome and bile salt solutions are used to achieve full breakage of the liposomes. After the addition of bile salt solution, vigorous mixing applied by vortex and breakdown of liposomes are followed by the disappearance of the turbidity.

3.1.6. Instruments

Rotary Evaporator; BIBBY, Rotary Evaporator, RE100 is used to obtain thin lipid films by evaporating the solvents.

Water Bath; Julabo SW 22 is used for swelling of hydrated thin lipid films. The flasks are left for 12 hours at 55°C which is above T_c of the phospholipids.

Vortex Mixer; Fusion Whirlimixer is used for homogenization of the swelled liposomes by vigorous mixing for 20 min.

Extruder; Avestin Lipofast mini extruder with 100 nm polycarbonate filters and 0.5 mL Hamilton syringes is used for downsizing of liposomes. Each liposome sample is exposed to 11 successive filtrations through polycarbonate filters to ensure the uniformities.

Sonicator; Bandelin Sonorex RK 52 is used as an alternative way of downsizing liposomes. Each sample is sonicated for 15 min by also avoiding temperature increases with the usage of some ice in the sonicator water.

Both downsizing methods are proper for liposome down sizing. To decide which method is more effective for the designed liposomes, either of them are applied and comparisons are done before the encapsulation studies.

Centrifuge and Filtration; Rotafix 32 Hettich Zentrifugen is used for filtration of the encapsulated lipid suspensions to remove the excess and un-encapsulated material from the bulk solution. Vivascience Vivaspin 2 mL concentrator is operated in the process. The ultracentrifugation technique was reported as a simple and fast method for the separation of drug-loaded liposomes from their medium. Each sample is centrifuged at 60000 rpm, +4°C.

UV/VIS Spectrophotometer; Shimadzu PharmaSpec UV-1700 UV/VIS spectrophotometer is used to follow turbidity of the liposomal suspensions. Encapsulation analyses are also followed by the same spectrophotometer. Quartz cuvettes are used to take measurements.

Particle Size and Zeta Potential Analyzer; Brookhaven Instruments 90 Plus Particle Size/Zeta Analyzer is used to apply Dynamic Light Scattering (DLS) method to measure the size, size distributions and zeta potentials of the liposomal solutions. Measurements are done in quartz UV cuvettes after dilution of each sample with the specified hydration medium.

SEM/STEM; XL30 ESEM-FEG/EDAX system is applied to display the shapes, sizes and surface morphologies of the liposomes. Wet samples are dropped onto SEM grids and air dried for 3 hours before examinations.

3.2. Methods

3.2.1. Preparation of Liposomes

Liposomes are prepared using thin film evaporation procedure. First, all the lipids are dissolved fully in the defined ratios of chloroform/methanol solvent medium in 25 mL round bottom flasks. Then evaporation of the solvents is performed by using a rotary evaporator. 5 mL total solvent is used for each preparation. A large round sided flask supplies a larger possible surface area to dry down and form a very thin film. Evaporation is done till all the solvent is gone and a thin film is obtained around the inner surface of the flask. This film is kept waiting overnight or vacuum pumped for further removal of the residual solvent. Different ratios of lipids and solvent mixtures are examined in means of obtaining better lipid film formation. DMPC:DMPG:Cholesterol lipid molar ratio is kept constant as 9:1:3 in each liposomal formulations as indicated in the research done by F.M. Cagdas et al [90] while ceramide and cholesteryl sulfate concentrations are studied to prepare the best liposomal formulation that is compatible with human skin.

Hydration is performed by the addition of an aqueous medium onto the lipid film. A defined ratio of ethanol and phosphate buffer mixture is used as aqueous medium and added to the round bottom flasks containing the lipid films. The final mixtures kept at 55°C in water bath for 12 hours to get a homogeneous colloidal system. Mechanical dispersion is done by vortex mixing for 15 min and MLVs are obtained. Freeze-Thaw is performed on MLV suspensions by freezing the solution within liquid nitrogen and thawing it afterwards in 37°C hot water bath. This process is necessary to increase the homogeneity of vesicle size distribution and avoid any clogging of the lipid membranes. 6 cycles of freeze-thaw is applied onto each vesicle suspension.

MLVs are downsized and converted into LUVs either by extrusion through 100 nm polycarbonate filters or sonication at room temperature (27°C). All extruded samples exposed to 11 passes through filters. An odd number of passages is necessary to avoid

contaminations by larger liposomes that could not pass the filter. If sonication is performed, MLVs are sonicated for 15 min in sonic bath to get LUVs.

Once the liposomes are obtained, they are kept at 27°C water bath which is above the phase transition temperatures of the lipids used.

3.2.2. Encapsulation of Liposomes

Due to having all water soluble raw materials, encapsulation is done in hydration steps. Defined amounts of encapsulated substances are dissolved in PBS/ Ethanol containing aqueous media prior to hydration of the dry lipid films. The presence and the concentrations of the encapsulated materials are followed by using UV/VIS spectrophotometer.

Removal of the excess and un-encapsulated materials from the bulk solutions is performed by ultracentrifugation. 2 mL prepared vesicle samples are put in filtration tubes as shown in Figure 3.3 and ultra centrifuged at 60000 rpm until no un-encapsulated material is left in the upper liposomal solutions. Solutions at the bottom part of the filtration tube are examined periodically with UV/VIS spectrophotometer and elimination of the excess encapsulates is followed. Solutions at the upper part of the tube are completed to 2 mL each time which results in 1:1 dilution.

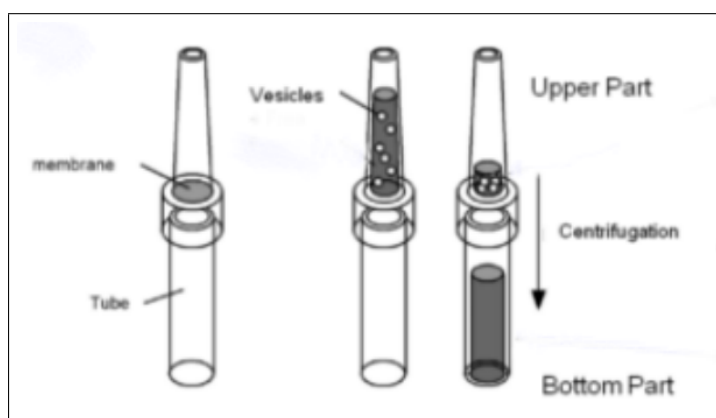


Figure 3.3. Demonstrative picture of filtration procedure [91]

Once the loaded liposomes are all separated from their medium, the lipid bilayer is disrupted with sodium cholate salt solution and the released material is then quantified with UV/VIS spectrophotometer. [92]

4. RESULTS AND DISCUSSION

4.1. Stability and Size Comparison of Down-Sizing Methods

Extrusion and sonication down-sizing methods are both practiced and compared with respect to vesicle size, size distribution and stability. Size analyses are done by Particle Size Analyzer right after the preparation of the liposomal formulations. After 5 days and 15 days, stability of the liposomes are checked.

Homogeneities of the vesicle particle sizes are compared with the polydispersity index (PDI). Size distributions are measured with DLS and the PDI designates how homogeneously the sizes of the vesicles are distributed in the solution. The PDI is dimensionless and scaled such that values smaller than 0.05 indicate highly mono dispersed size distribution whereas values greater than 0.7 indicate that the sample has a very broad size distribution.

Sizes and homogeneities of the extruded, 20 min and 40 min sonicated and neither sonicated or extruded liposomes are compared in Table 4.1

Table 4.1. Comparison of the sizes and homogeneities of sonicated and extruded liposomes

Liposomes	Effective Diameter (nm)			PDI (Homogeneity)		
	0 days	5 days	15 days	0 days	5 days	15 days
Not down-sized	553.7	577.7	508.2	0.266	0.265	0.248
20 min sonicated	217.7	235.3	171.3	0.261	0.293	0.231
40 min sonicated	188.7	212.7	190.7	0.260	0.253	0.095
Extruded	187.9	214.2	192.6	0.191	0.102	0.107

Results show that down sizing is needed for the liposomes otherwise they are too big to be used in skin delivery studies. 200 nm sized and smaller liposomes will sustain a better penetration through the human skin. Although there is not a significant size and homogeneity difference between 40 min sonicated and extruded liposomes, extrusion method is chosen over sonication to avoid filaggrin protein degradation in the continuation of the project. Stability of the extruded liposomes is maintained up to 1 month but after that particle size is started to increase upon possible flocculations. Figure 4.1 indicates an increase in the particle size after a certain storage time.

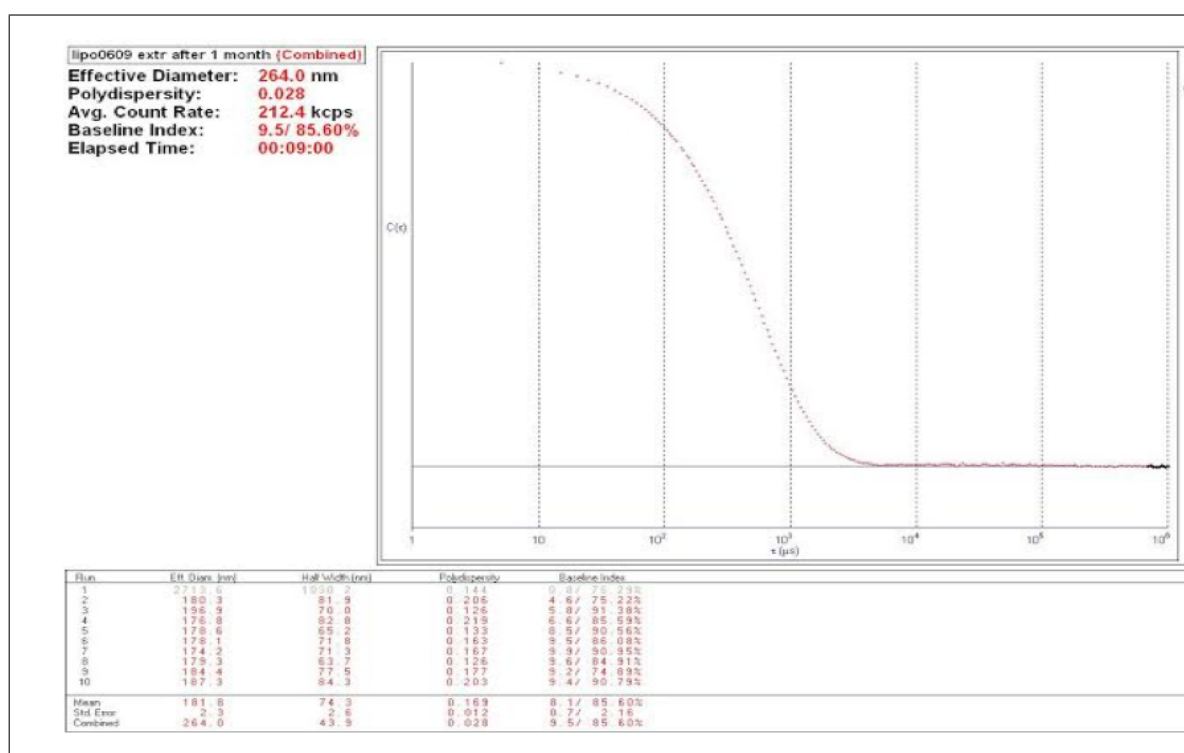


Figure 4.1. The particle analysis of the extruded liposome after 1 month

4.2. Fixing the Liposome Concentration

Due to the limited amount of ceramide amount, the lipid concentrations are decreased in 10 fold and 5 fold to check whether the liposomes are still obtained successfully. Two additional liposomes are prepared with the concentrations of 0.445mg/mL and 0.89mg/mL in 10 mL solutions and compared with the current liposome that is

prepared with 44.5 mg total lipid in 10 mL solvent. As seen in Figures 4.2 and 4.3; decreasing the concentration of the lipids damages vesicular morphology of the vesicles and disables the liposome formation. So the concentration is kept constant but the solvent amount is decreased to 5 mL instead.

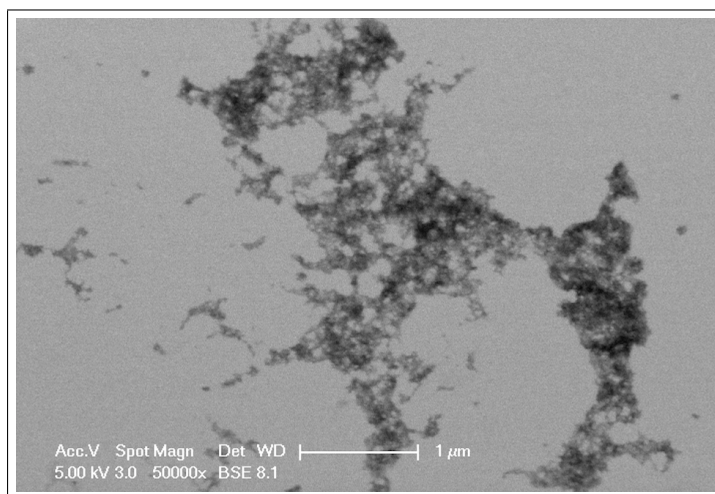


Figure 4.2. STEM image of 1/10 diluted liposome

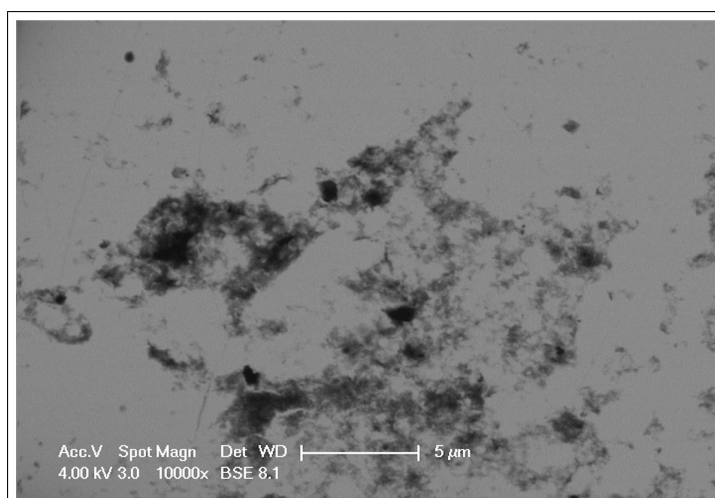


Figure 4.3. STEM image of 1/5 diluted liposome

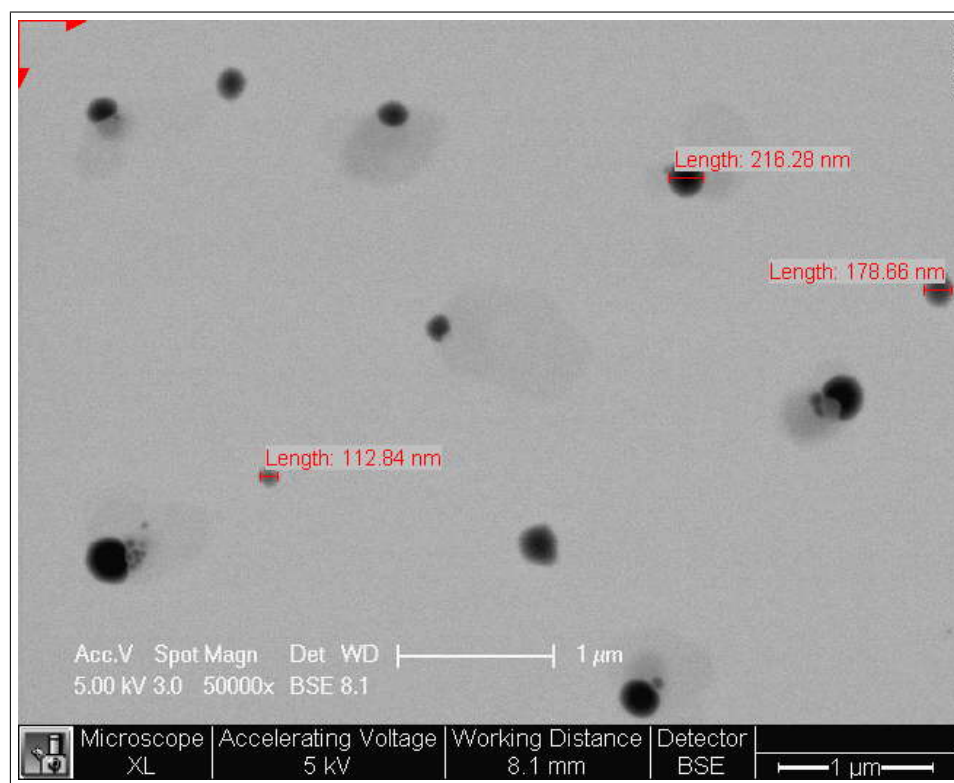


Figure 4.4. STEM image of the liposome prepared with original concentration

4.3. Determination of Optimum Solvent Composition

In the liposomes used for skin delivery purposes, ceramide is an essential compound to complete skin competence. Since it dissolves in methanol faster and better than chloroform, methanol is added in the solvent system. Otherwise a further heating stage is needed to solubilize the ceramide in chloroform and that would harm the other lipids used. For this purpose, solvents with 2:1,1:1 and 1:2 chloroform: methanol volume ratios are used to prepare thin film lipids and analyzed for comparison.

Size and homogeneity measurement results as shown in Table 4.2 indicates that upon increasing the methanol amount, particle size and homogeneity is also increased. For healthier results, STEM analyses of the particles are also carried out.

Table 4.2. Size and homogeneity comparison of the liposomes prepared with the solvents at defined ratios

Chloroform:Methanol (v/v)	Effective diameter (nm)	PDI
2:1	173.3	0.057
1:1	177.1	0.045
1:2	186.8	0.034

In Figures 4.5 and 4.6, perfectly formed vesicle structures of 2:1 and 1:1 chloroform:methanol (v/v) containing liposomes can be seen whereas undesired extra bags around small lipid vesicles are observed in 1:2 liposomes as seen in Figure 4.7. These extra vesicle-like bag formation can be an outcome of using excess methanol which can also act as a surfactant by having both polar and nonpolar regions. This also explains the increased homogeneity in the size distribution of the 1:2 chloroform:methanol (v/v) containing liposomes.

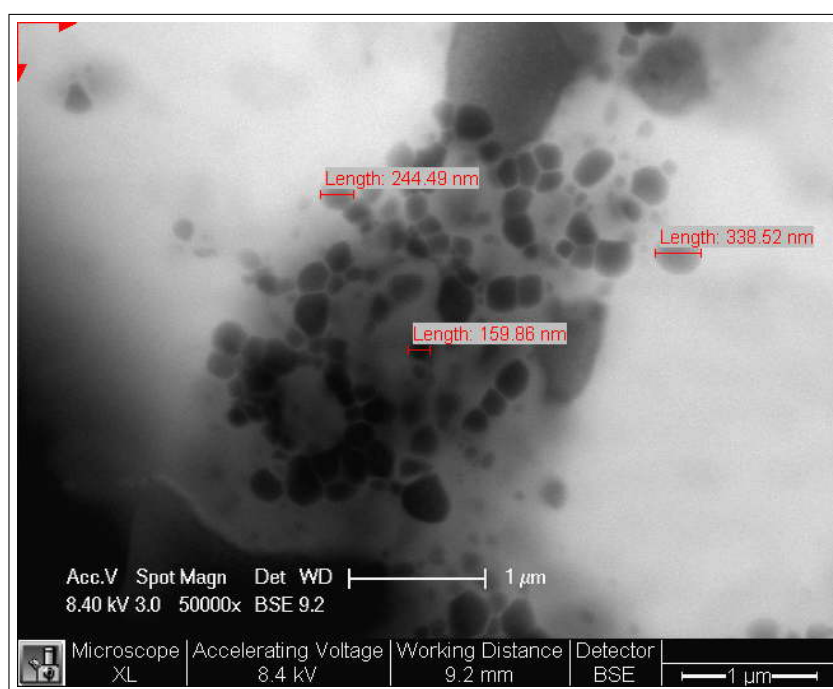


Figure 4.5. STEM image of the liposome prepared with 2:1 chloroform:methanol (v/v)

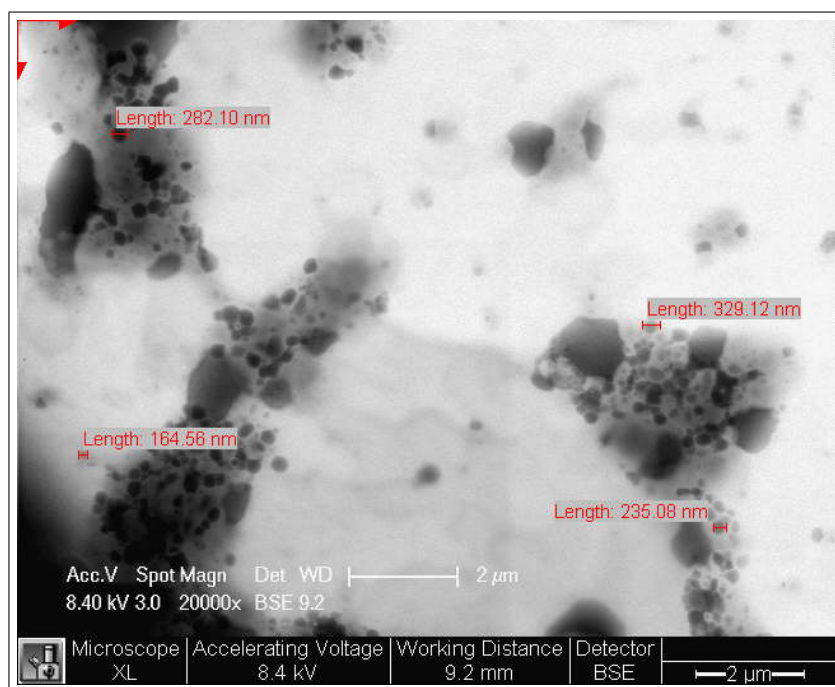


Figure 4.6. STEM image of the liposome prepared with 1:1 chloroform:methanol (v/v)

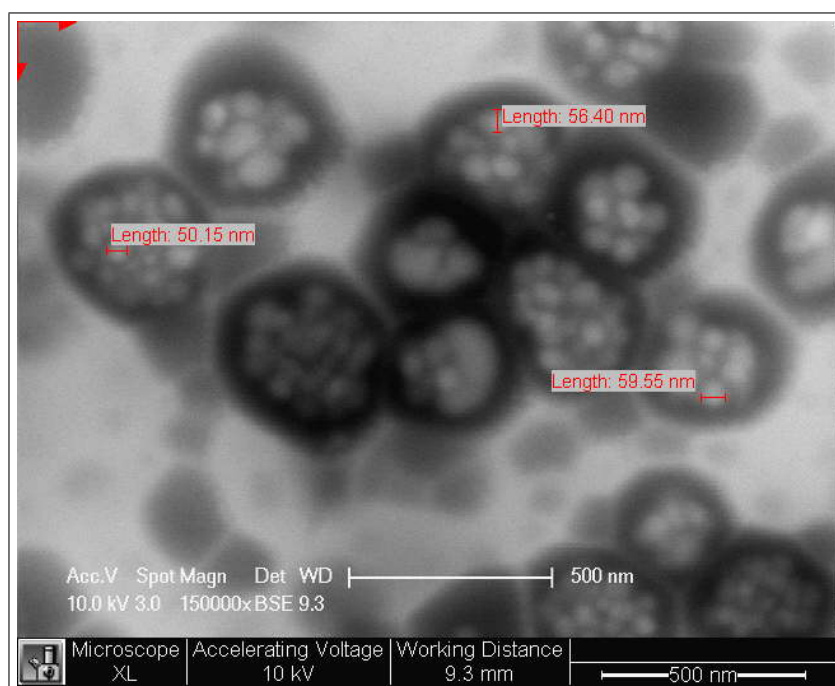


Figure 4.7. STEM image of the liposome prepared with 1:2 chloroform:methanol (v/v)

Liposomes prepared with the solvent of 1:1 chloroform:methanol ratio are pursued for the further experiments in order to solubilize ceramide molecules better in the continuation of the study.

4.4. Increasing Liposome Stability with Ethanol

Ethanol is added to the liposomal formulations by mechanical dispersion method, in the hydration step where the buffer solution is added to disperse the thin lipid film. For this purpose, three hydro-alcoholic solutions are prepared with different PBS: Ethanol volume ratios and results are compared with respect to size, shape and stability.

Liposomes that are dispersed with 1:1 PBS: Ethanol foam excessively (Figure 4.8) after vortexing stage and give non proper STEM images as seen in Figure 4.9. They also have bad size distribution results as marked in Table 4.3. So it is concluded that increasing ethanol ratio up to 1:1 is not an appropriate dispersion approach for hydrating liposomes.



Figure 4.8. Liposome dispersed with 1:1 PBS:Ethanol solution

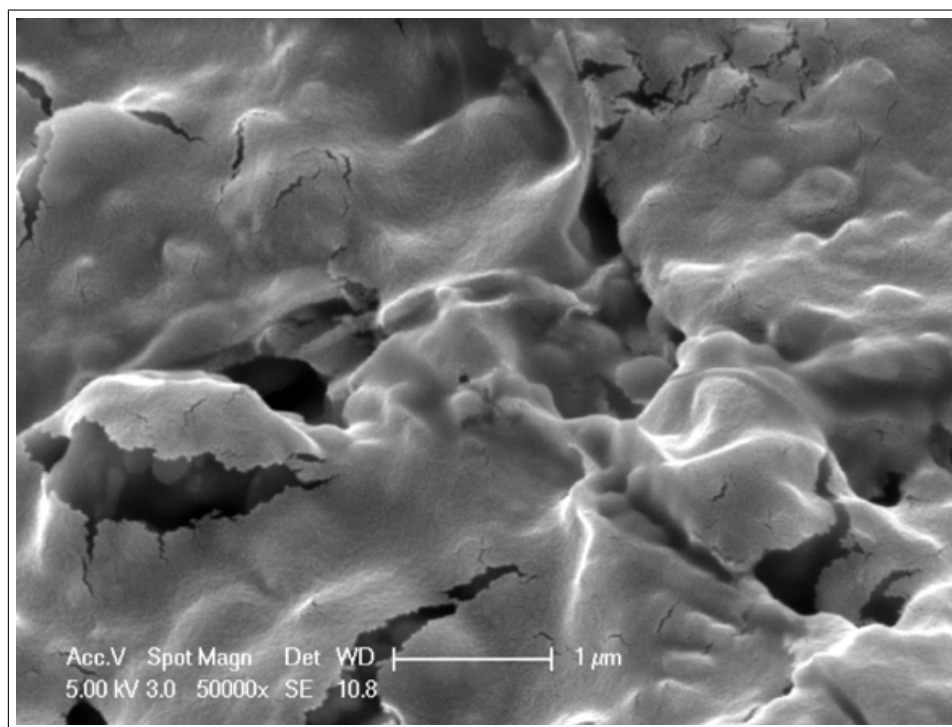


Figure 4.9. SEM image of 1:1 PBS:Ethanol (v/v) dispersed and lyophilized liposomes

According to the particle size analysis as shown in Table 4.3, 2:1 PBS: Ethanol ratio containing liposomes are better for skin delivery purposes due to being smaller in size and better in homogeneity.

Table 4.3. Size and homogeneity comparison of the liposomes dispersed with various PBS:ethanol solutions

PBS:Ethanol (v/v)	Effective diameter (nm)	PDI
4:1	240.6	0.160
2:1	200.0	0.066
1:1	596.5	0.317

Unlike from the previous studies, SEM images are investigated instead of STEM images to see if there is any solvent effect on imaging. SEM samples are prepared with a different method. 2 mL of liposomal solutions are taken into glass vials and dried under

lyophilizer for overnight. Dried liposome samples (Figure 4.10) are put on a carbon SEM grid which are then coated with an electro conducting material, copper. Sample imaging is applied over that coating to be able to get images from a non conducting sample.



Figure 4.10. Lyophilized liposomal formulations

In STEM imaging, liposomal solutions are directly dropped on a copper grid and left for air drying for a few hours. Then, both scanned and transmitted electron beam is used for sample imaging.

According to the images in Figures 4.11 and 4.12 , SEM can also be applied for imaging of liposome samples but due to the convenience of the sample preparation, STEM method is proceeded for further imaging of the liposomal solutions.

Also, SEM images of the samples give combined salient structures instead of independent spheres that are observed in STEM analysis which make the examination of the vesicles more difficult.

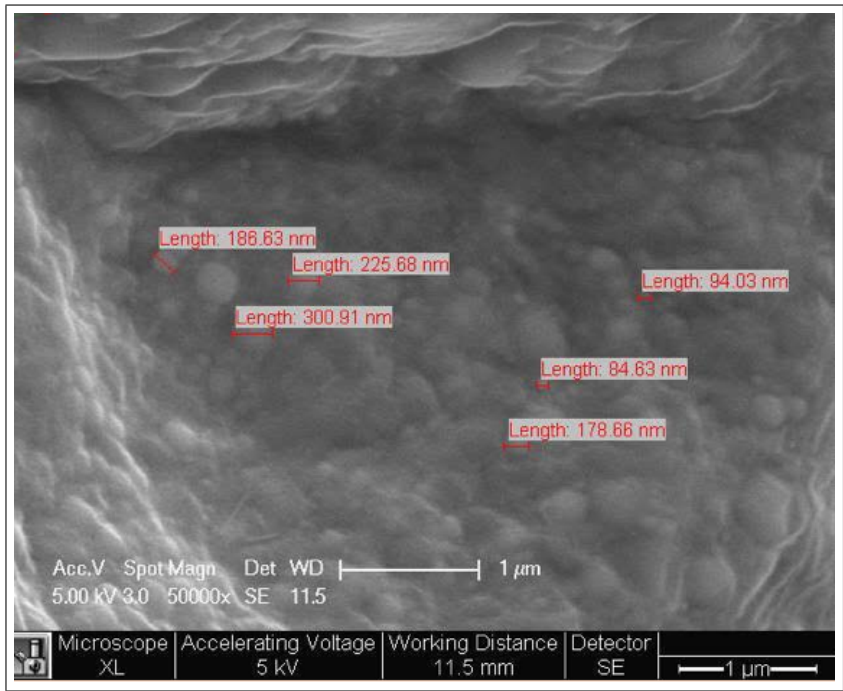


Figure 4.11. SEM image of 4:1 PBS:Ethanol (v/v) dispersed and lyophilized liposomes

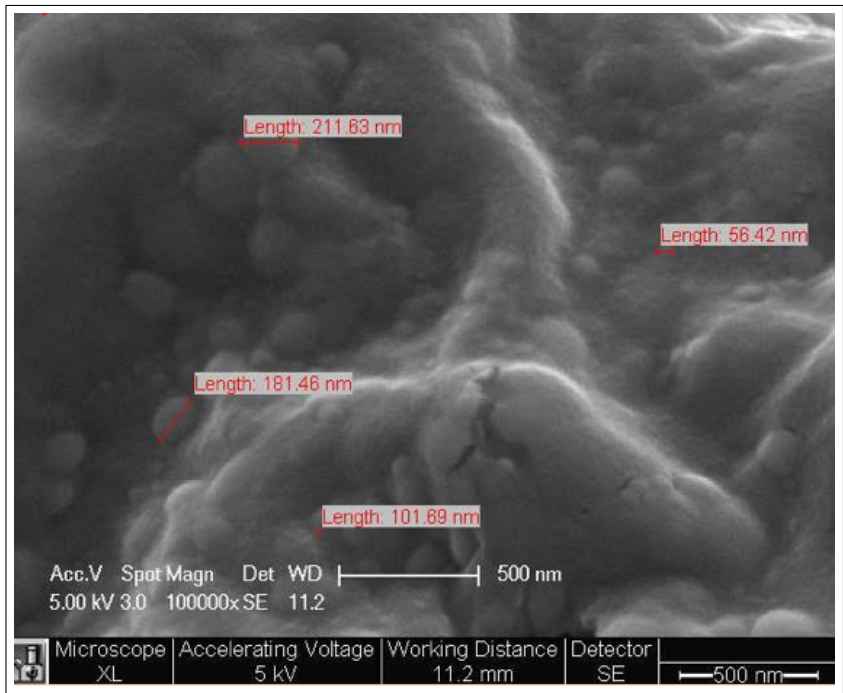


Figure 4.12. SEM image of 2:1 PBS:Ethanol (v/v) dispersed and lyophilized liposomes

4.5. Ceramide Addition to the Lipid Mixture

Skin lipids are composed of around 40% molar ratio of ceramides. Ceramides are the supportive compounds of the intercellular lipids which gives the SC its resistance and protective function. These lipid chains are mostly in a solid crystalline or gel state and supply an ordered, firm structure to the SC.

Liposomal formulations are prepared by addition of ceramide in three different molar ratios and characteristics are compared to the those prepared with DMPC: DMPG: Cholesterol 9:1:3 molar ratios. According to the size and size distribution analyses shown in Table 4.5 and STEM images, all of the prepared formulations generated approximate physical characteristics. To better mimic the natural skin membrane, liposomes with higher ceramide concentrations (i.e. third and fourth samples) are preferred.

Table 4.4. Mole percentages of the lipids used in the liposomal formulations

Sample	DMPC	DMPG	Cholesterol	Ceramide	Total mmole	Solvent (mL)
1	69.3	7.7	23	0	3.82	5
2	60.3	6.7	20	13	3.82	5
3	51.3	5.7	17	26	3.82	5
4	42.3	4.7	14	39	3.82	5

Table 4.5. Size and homogeneity comparison of the liposomes prepared with the defined ceramide amounts

Sample	Effective Diameter (nm)	PDI
1	232.8	0.187
2	201.9	0.041
3	237.2	0.171
4	260.3	0.256

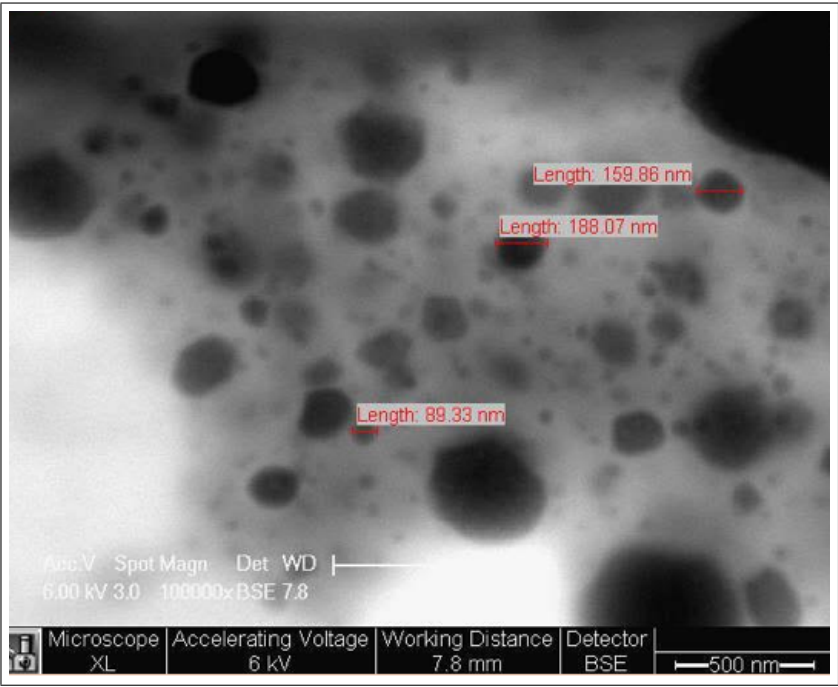


Figure 4.13. STEM image of sample 1

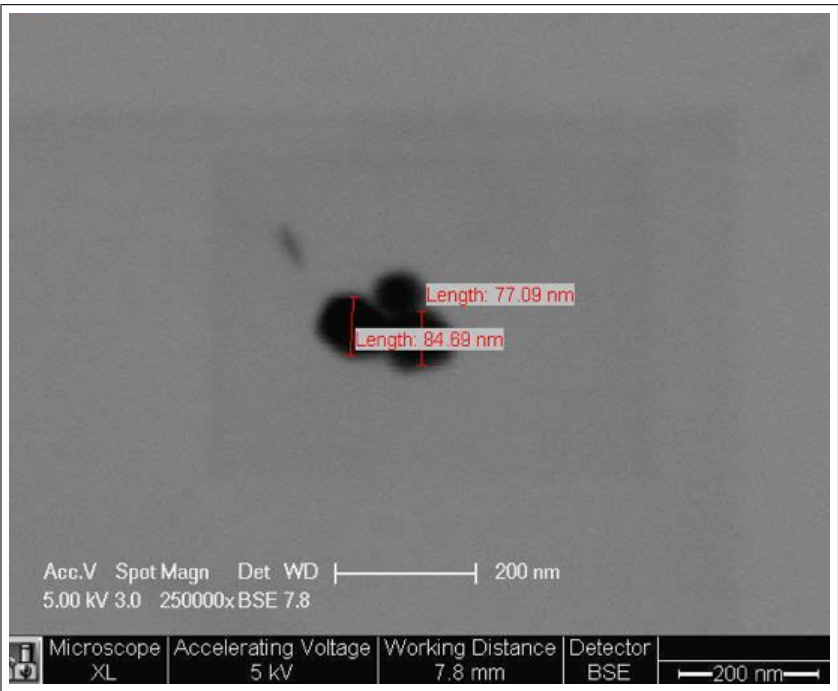


Figure 4.14. STEM image of sample 2

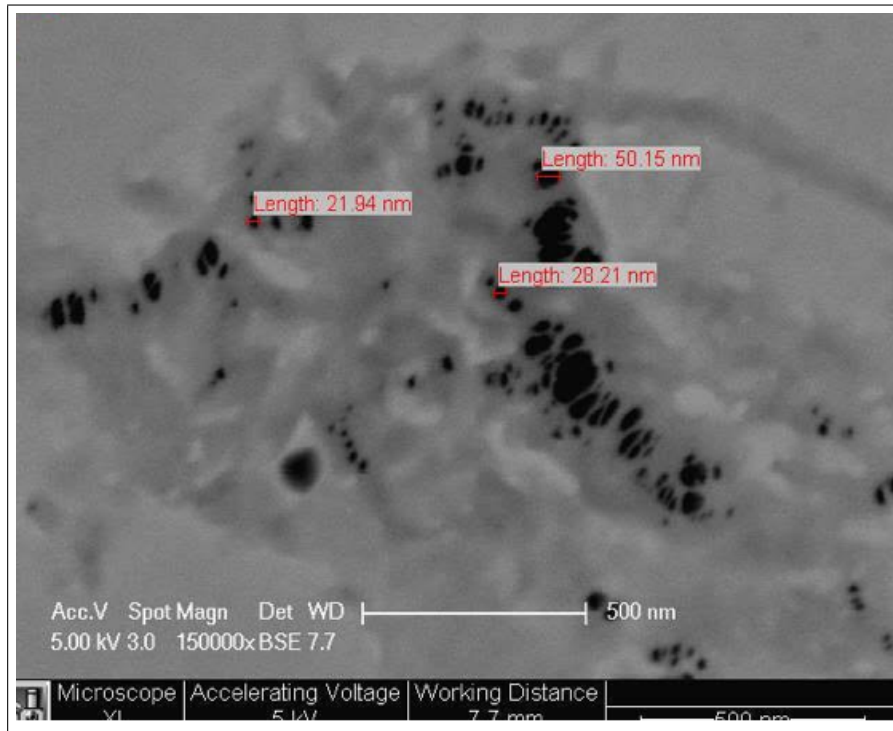


Figure 4.15. STEM image of sample 3

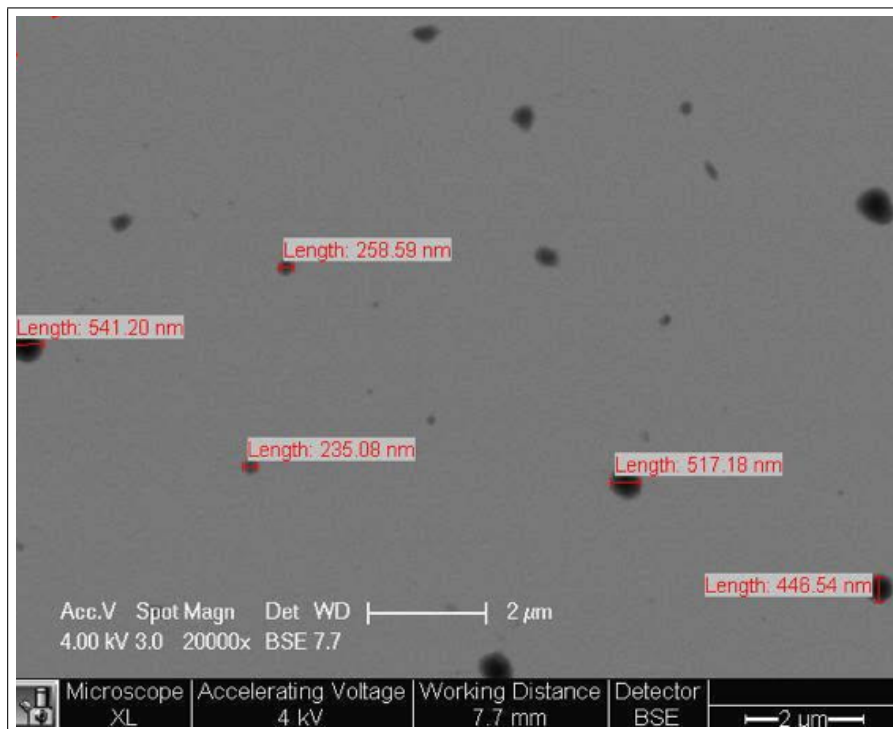


Figure 4.16. STEM image of sample 4

STEM images of the samples show that liposomes with the highest ceramide content (i.e. Figure 4.16) form proper shaped vesicles like all the other samples seen in Figures 4.13, 4.14 and 4.15. It can be concluded that increasing the ceramide ratio up to 40% do not disturb the morphology of the liposomes. Skin lipids consist of 40% ceramides, so higher ceramide including samples are chosen for the further experiments.

4.6. Cholesteryl Sulphate Addition to the Lipid Mixture

Since cholesteryl sulfate is found approximately in a molar ratio of 10% in skin lipid compositions, an increasing concentration of cholesteryl sulfate is added to the two chosen and fixed DMPC: DMPG: Cholesterol: Ceramide mixtures with the ratios of 18:2:6:3 and 45:5:15:14. Total mmole of the lipids is kept constant as 3.82 within 5 mL solvents for each as shown in Table 4.6.

Table 4.6. Mole percentages of the lipids. Samples 1,2 and 3 represent %26 ceramide content where samples 4,5 and 6 represent %39 ceramide from the Table 4.4

Sample	DMPC	DMPG	Cholesterol	Ceramide	Cholesteryl Sulfate
1	49.5	5.50	16.3	25.1	3.5
2	47.7	5.30	15.8	24.2	7
3	51.3	5.10	15.2	23.3	10.5
4	40.9	4.54	13.5	37.7	3.4
5	40.3	4.48	13	36.4	6.8
6	37.9	4.22	12.5	35.1	10.2

Results in Table 4.7 show that 2nd sample is the best to continue with encapsulation studies. It has the narrowest size distribution with an appropriate effective diameter for skin delivery studies and STEM image in Figure 4.18 supports that it formed good shaped vesicles. Sample 4, 5 and 6 are over-sized vesicles for skin delivery applications. Results also indicate that increasing the cholesteryl sulfate ratio in high

ceramide content including formulations yields vesicles in bigger sizes compared to low ceramide content including formulations. No STEM image is obtained for sample 4.

Table 4.7. Size and homogeneity comparisons of the liposomes prepared with the defined cholesteryl sulfate ratios

Sample	Effective Diameter (nm)	PDI
1	217.7	0.089
2	204.7	0.043
3	195.5	0.074
4	285.7	0.217
5	241.1	0.114
6	330.0	0.242

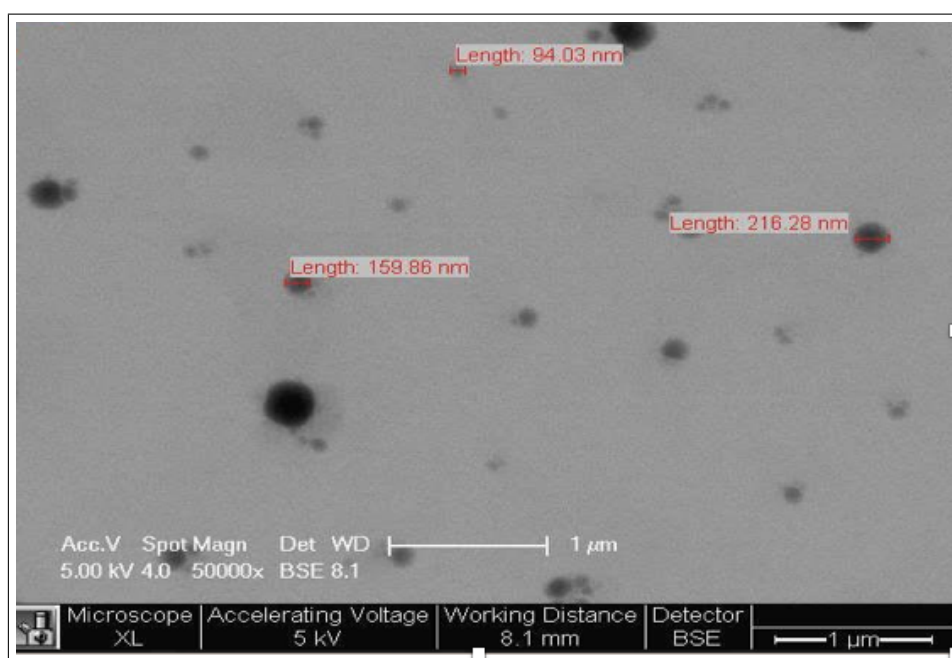


Figure 4.17. STEM image of sample 1

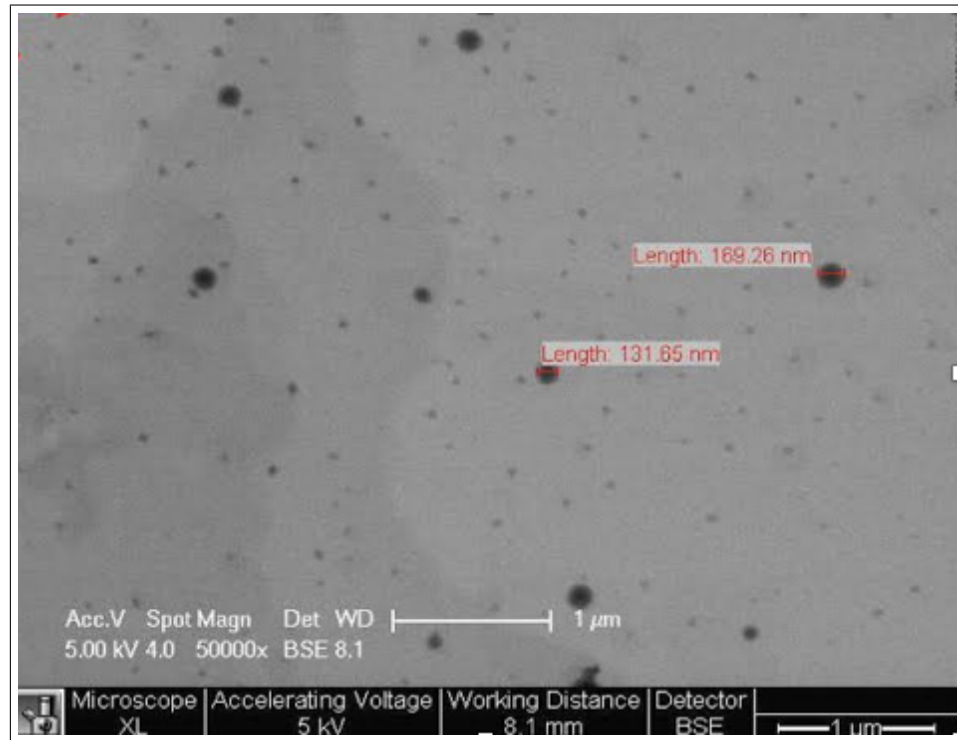


Figure 4.18. STEM image of sample 2

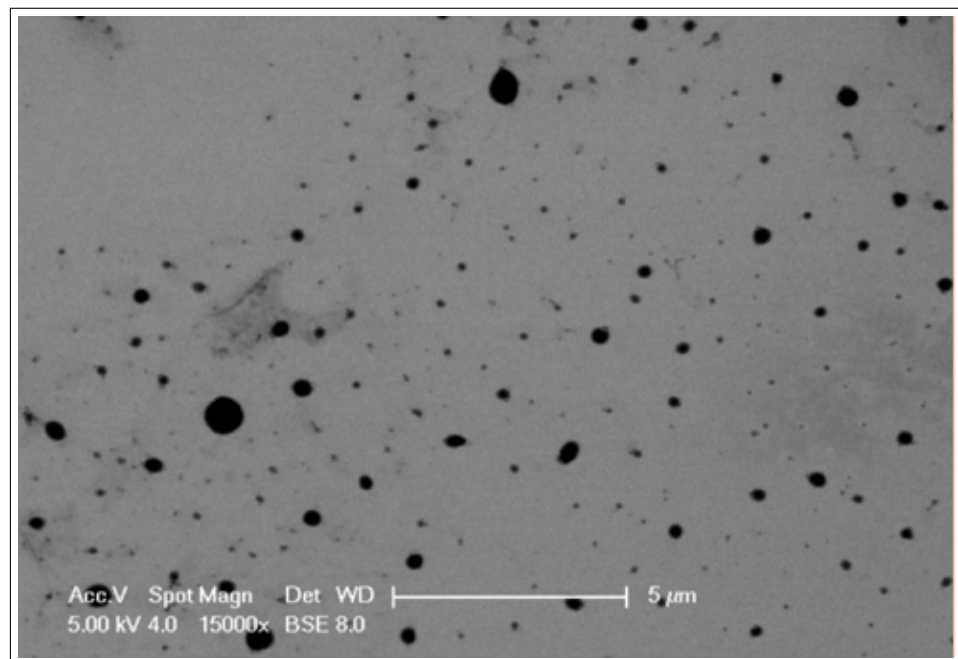


Figure 4.19. STEM image of sample 3

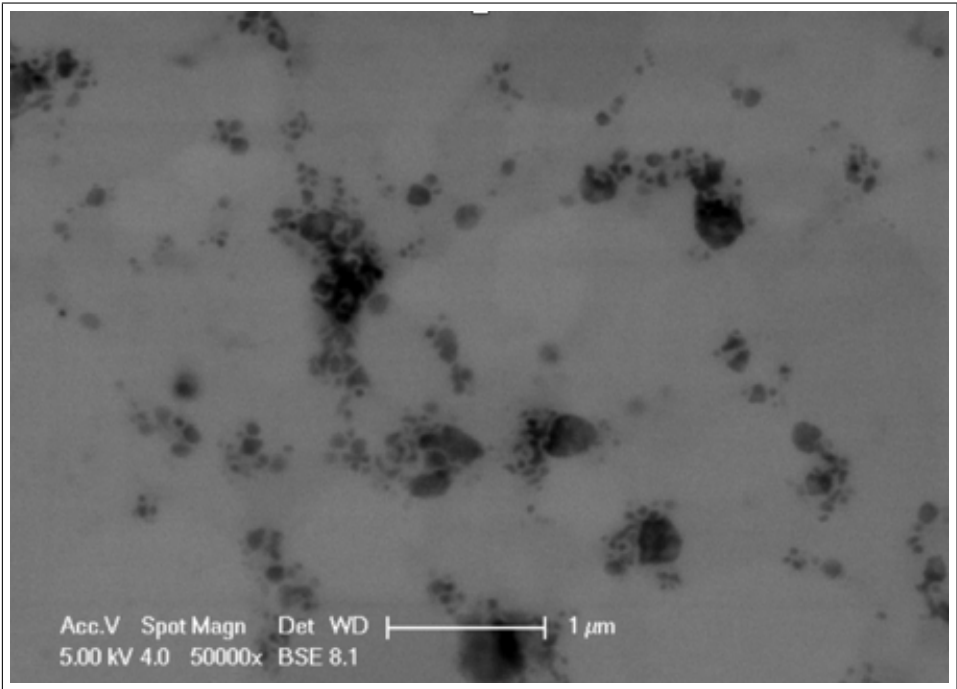


Figure 4.20. STEM image of sample 5

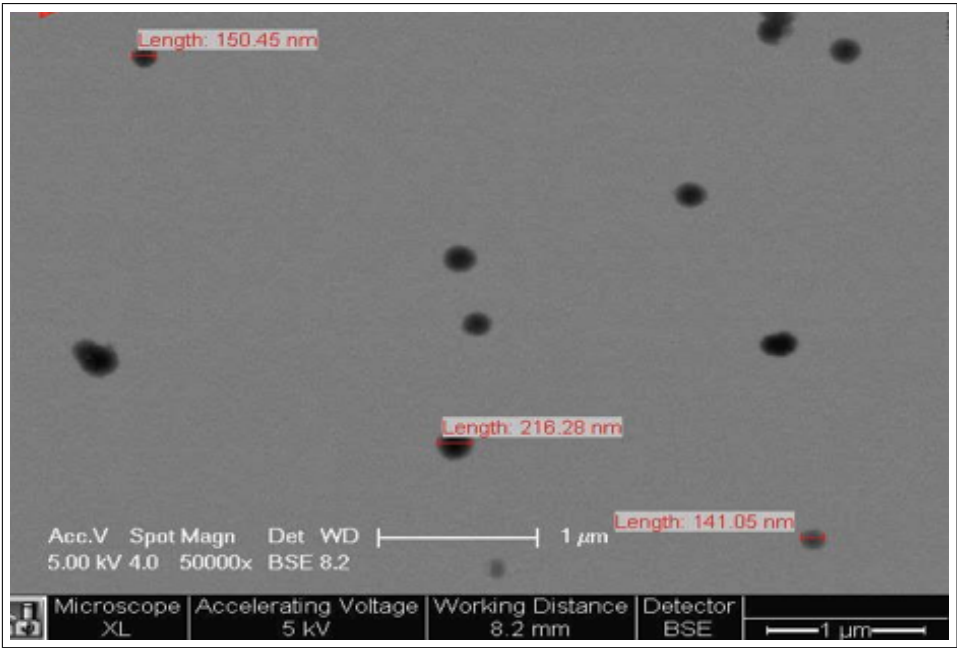


Figure 4.21. STEM image of sample 6

4.7. Filaggrin Encapsulation Study

In this study, to follow up the protein encapsulation yield, UV spectrophotometer is used. Absorbance is calculated by using the Beer-Lambert law. Maximum absorbance wavelength of the filaggrin protein in PBS: Ethanol is determined as 278nm.

Filaggrin is a water soluble protein and encapsulation is done within the hydration process. Filaggrin is purchased in a solution containing 0.05 mol/L Tris-HCl and 0.015 mol/L sodium azide to stabilize the protein at a pH of 7.6 with the concentration of 4 g/mL. 0.2 mL of this solution is dissolved per 1ml hydration medium and added to the lipid films. Absorbance of the protein in 6 mL of hydration medium is measured as 0.753 before encapsulation.

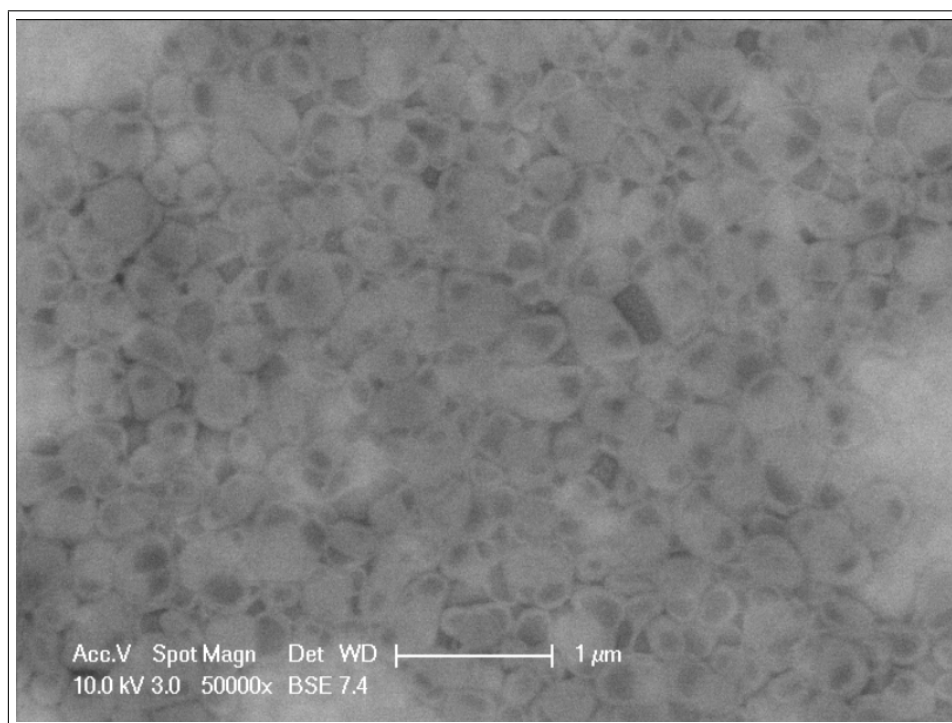


Figure 4.22. STEM image of the filaggrin encapsulated liposomes

After encapsulation, liposomal solutions are extruded and freeze thawed with liquid nitrogen. In Figure 4.22, encapsulated liposomes can be seen. Resultant liposomal

solutions are filtered 3 times via ultracentrifugation process at 60000 rpm in order to remove all the free filaggrin from the exterior medium of the liposomes. Removal of the excess material is followed by absorbance versus wavelength graph with the decreasing absorbance in the amount of filaggrin after each filtration as mentioned in Section 3.2.2. Final solution containing the encapsulated filaggrin only is then broken down by the addition of 1:1 ratio of 0.02 M sodium cholate solution. Absorbance of released filaggrin is measured as 0.106. Final liposomal solution is exposed to four times dilution which of three in filtration steps and one in liposome breakage step. Thus the encapsulation yield is calculated as 56%.

4.8. Encapsulation of Madecososide and Heterosides

Madecososide is a water soluble active reagent within the pH range of 4 and 7, that is very suitable for encapsulation in hydration step. It is the precursor of filaggrin synthesis and used to treat psoriasis and chronic lesions of the skin in cosmetic industry.

Maximum absorbance value of this product in the hydration medium is determined as 205 nm with a UV/Vis spectrophotometer. Encapsulation is performed in hydration step same as described in the filaggrin encapsulation method. 5mg product is dissolved in 10 mL PBS and absorbance is measured as 2.013 for this solution. Encapsulated liposomes are ultra centrifuged at 60000 rpm until no more excess material is found in the exterior medium and filtrated liposomes are broken with sodium cholate. Absorbance of the released madecososide from the broken liposomes is measured as 0.417 which indicates an encapsulation yield of 83%.

Heterosides is also a hydrophilic raw material that helps to improve the skin barrier function by stimulating ceramide formation, filaggrin synthesis and water recycling through the skin. 5 mg of heterosides are dissolved in 10 mL PBS and absorbance is measured as 2.403 for this solution. After the UV/Vis measurements upon removal of excess heterosides and breakage of the resultant liposomes, the absorbance is measured as 0.256 which shows that heterosides can be encapsulated with 42% efficiency.

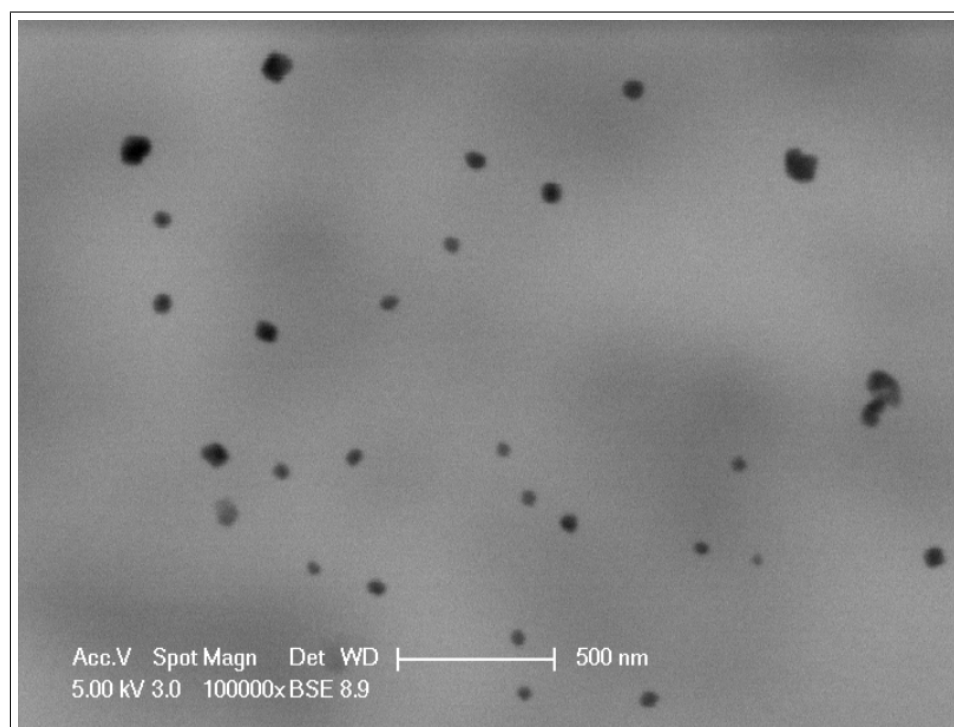


Figure 4.23. STEM image of the madecassoside encapsulated liposomes

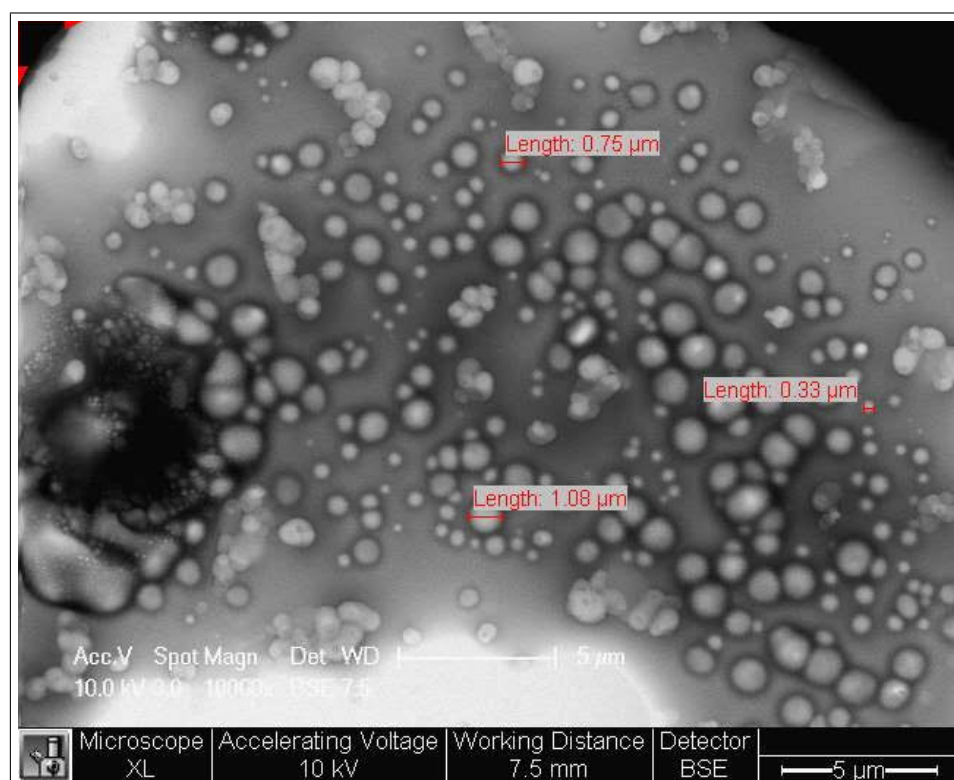


Figure 4.24. STEM image of the heterosides encapsulated liposomes

Table 4.8. Size and homogeneity comparisons of the un-encapsulated and encapsulated liposomes with several raw materials

Sample	Effective Diameter (nm)	PDI
Unencapsulated	160.4	0.084
Filaggrin Encapsulated	168.6	0.038
Madecassoside Encapsulated	173.5	0.053
Heterosides Encapsulated	437.8	0.037

Table 4.8 indicates the results of size and homogeneity comparisons of the un-encapsulated and encapsulated liposomes with several raw materials. Although the structure of the filaggrin protein is the biggest, liposomes encapsulated with it has the smallest effective diameter among all the encapsulated vesicles. The reason behind this phenomenon can be the folding structure of the protein. Filaggrin has so many -OH bonds and histidine molecules on its structure and hydrophilic parts of the lipids may cause attractions between these molecules. Heterosides is a mixture of two glycosides; madecassoside and asiaticoside. Higher effective diameter of heterosides with respect to madecassoside can be explained with the more complicated and bigger structure of the heterosides.

In this study, DMPC, DMPG, cholesterol, ceramide and cholesteryl sulphate lipids are used to prepare skin liposomes with the given ratios for the first time in the literature. Filaggrin is a trending molecule that is in the scope of eczema and atopic dermatitis related studies. Although lots of methods are tried to be introduced to get rid of filaggrin deficiency in patients, encapsulation of the protein in skin liposomes is tried as a novel method in this research. Madecassoside and heterosides also used as encapsulated materials for being effective molecules in the synthesis of filaggrin. All three materials have been successfully encapsulated in the designed liposomes.

5. CONCLUSION

In this thesis, by using the main human skin lipids like ceramide, cholesterol and cholesteryl sulfate, liposomal formulations with a lipid composition and dispersion media applicable for cosmetic applications was introduced.

Effects of sonication and extrusion down-sizing methods are compared in liposome preparation and extrusion method showed better results in liposomal stability.

A new dispersion media including ethanol was also introduced in liposome preparation to avoid the bacterial formation, to increase the skin enhancement properties and stabilities of the produced skin liposomes. DLS and STEM analyses confirmed that designed liposomal formulations with the indicated lipid composition and suspension media generates liposomes in desired vesicle size and shapes with stabilities up to 1 month storage at room temperatures.

The developed liposomes were further investigated in encapsulation studies of filaggrin protein which is the major constituent of the epidermis that determines the dryness level of the human skin. Madecoside and heterosides were also involved in encapsulation studies due their filaggrin synthesis assistance features. Encapsulation yields are followed and calculated upon UV/Vis spectrophotometer measurements. Results shown that all filaggrin, madecoside and heterosides are proper molecules to be encapsulated in the designed skin compatible liposomes. Encapsulation yields of the filaggrin, madecoside and heterosides were %56, %83, %42 respectively.

As a conclusion, producing skin compatible liposomes encapsulated with filaggrin and filaggrin synthesis simulating active raw materials is achievable. These liposomal formulations can be further utilized in eczema curing cosmetic and skin care product manufacturing.

6. FUTURE WORK

For future work, leakage potentials and stabilities of the encapsulated liposomes can be followed over time.

Effect of folding during encapsulation on the structure and activity of filaggrin protein can be investigated with some computational chemistry methods.

Designed and encapsulated liposomes can be further utilized in some cosmeceutical products and transdermal studies can be analyzed.

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APPENDIX A: DLS ANALYSIS RESULTS

The following pages show the DLS analysis results of the samples mentioned in Sections 4.1, 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8.

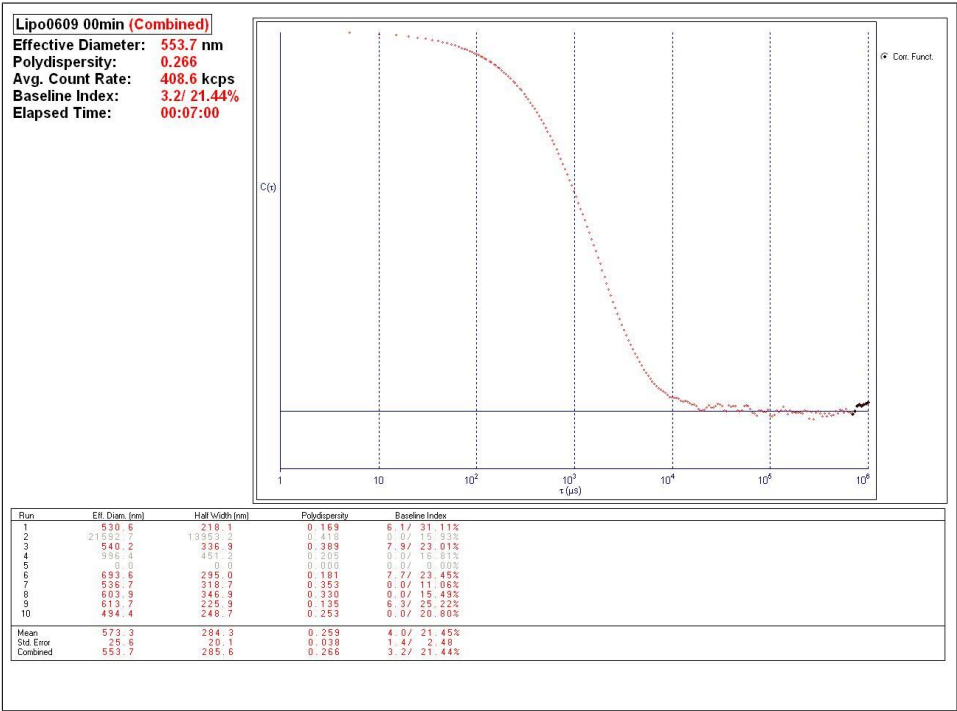


Figure A.1. DLS analysis of neither sonicated or extruded liposomes

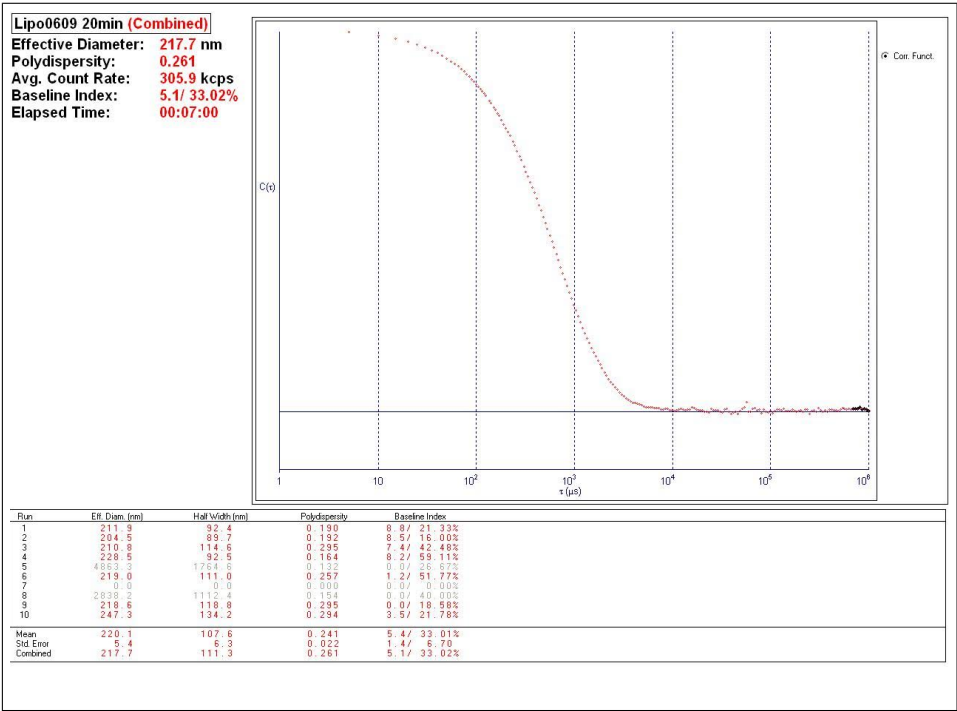


Figure A.2. DLS analysis of 20 min sonicated liposomes

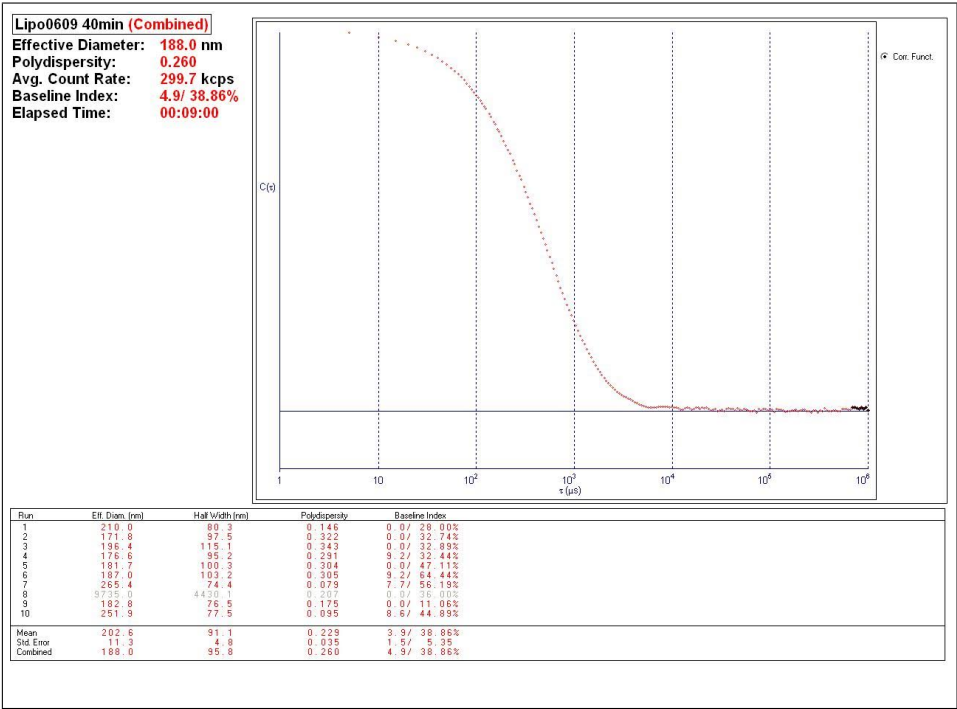


Figure A.3. DLS analysis of 40 min sonicated liposomes

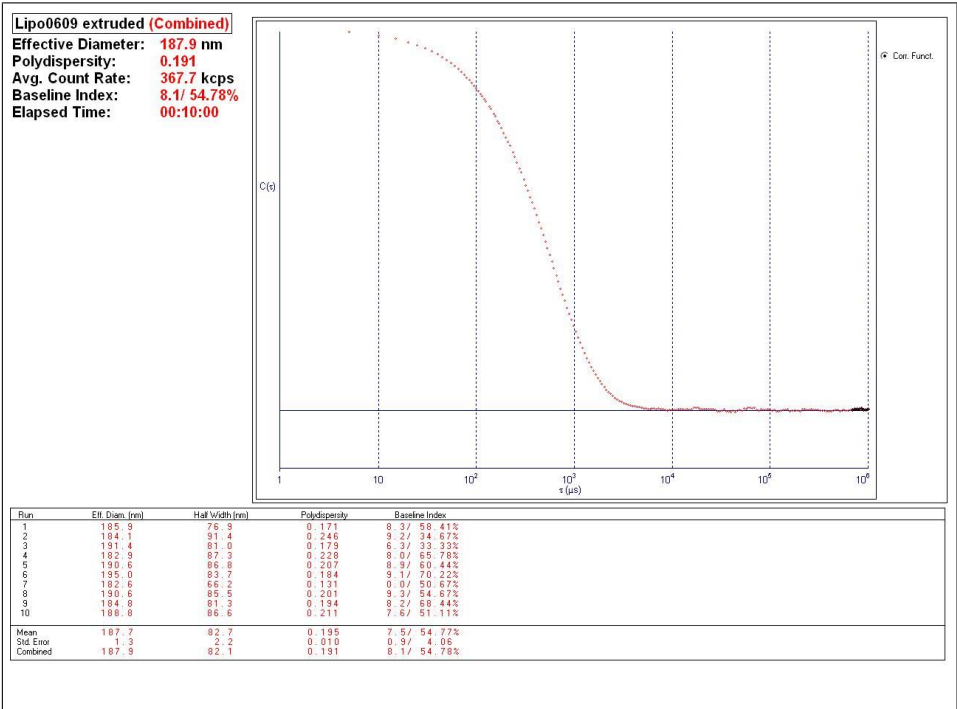


Figure A.4. DLS analysis of extruded liposomes

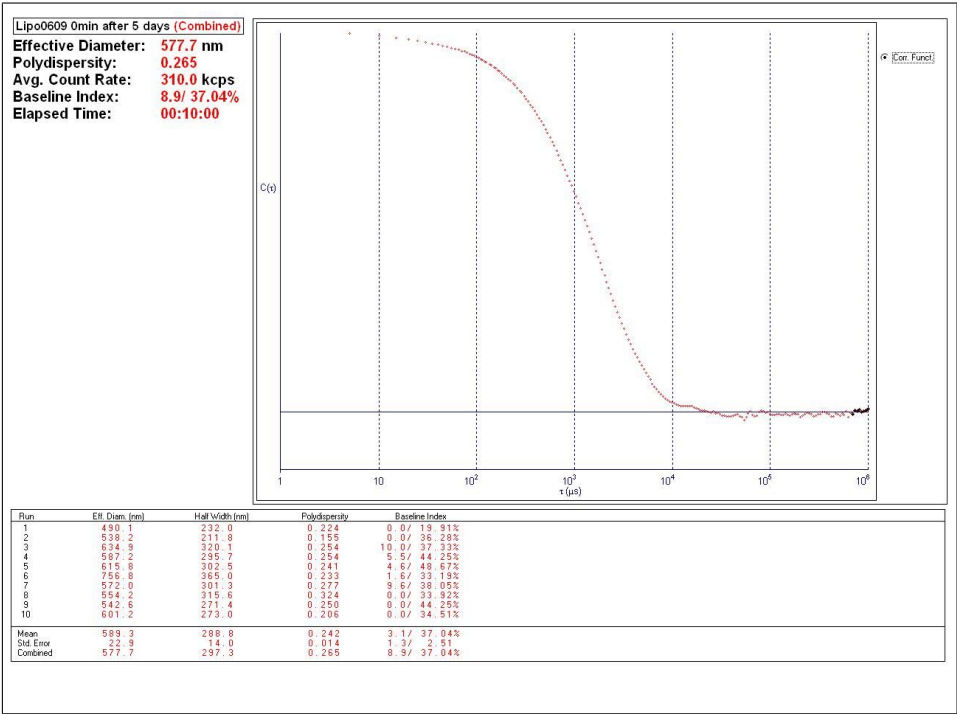


Figure A.5. DLS analysis of neither sonicated or extruded liposomes after 5 days

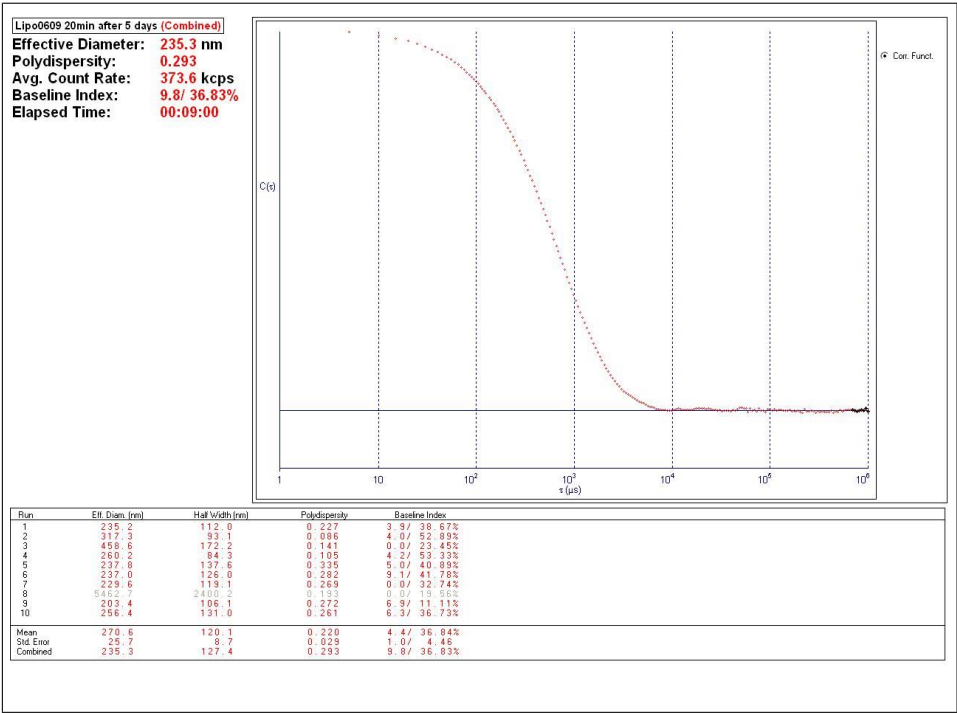


Figure A.6. DLS analysis of 20 min sonicated liposomes after 5 days

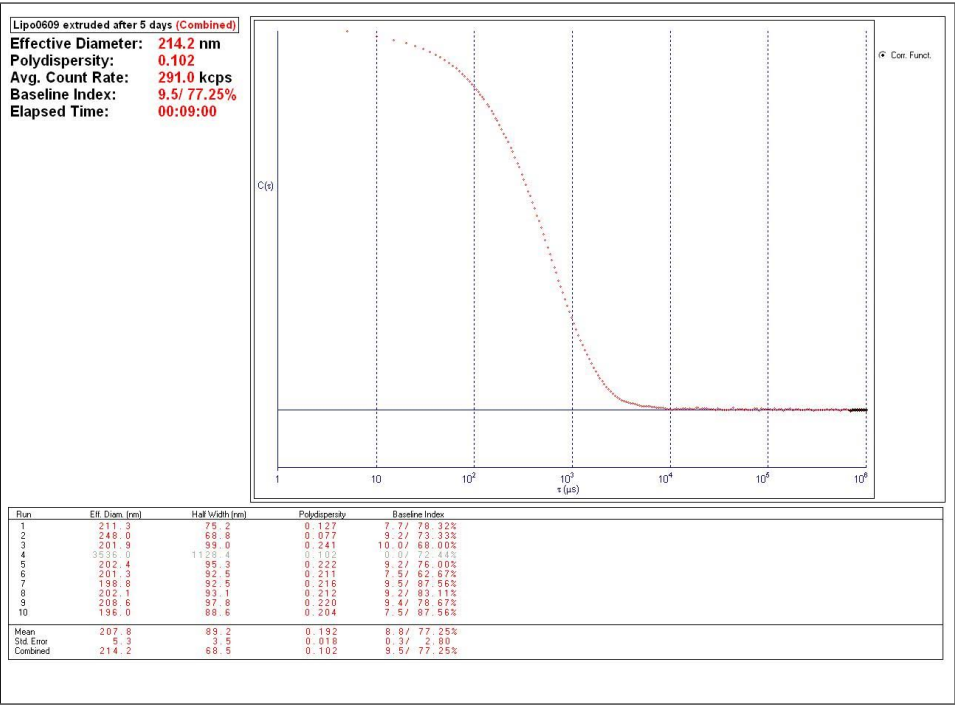


Figure A.7. DLS analysis of extruded liposomes after 5 days

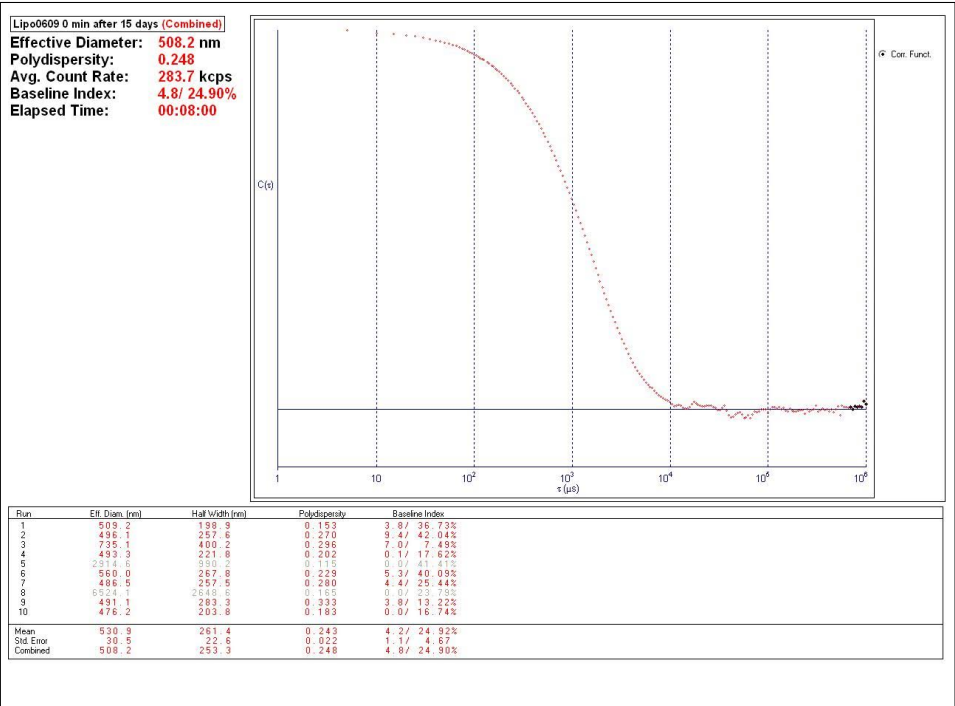


Figure A.8. DLS analysis of neither sonicated or extruded liposomes after 15 days

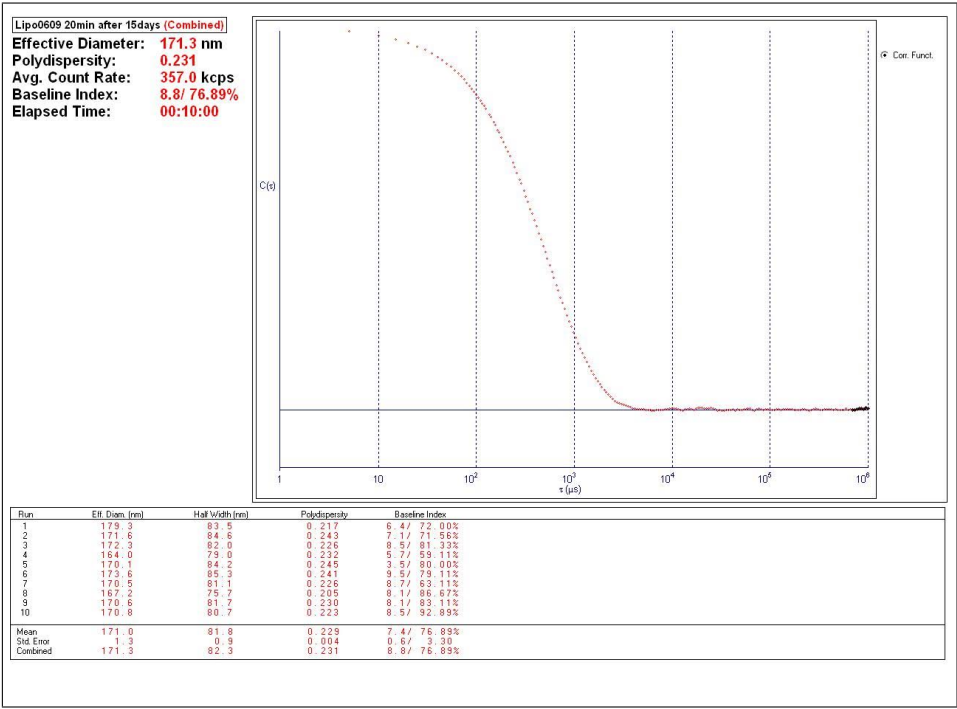


Figure A.9. DLS analysis of 20 min sonicated liposomes after 15 days

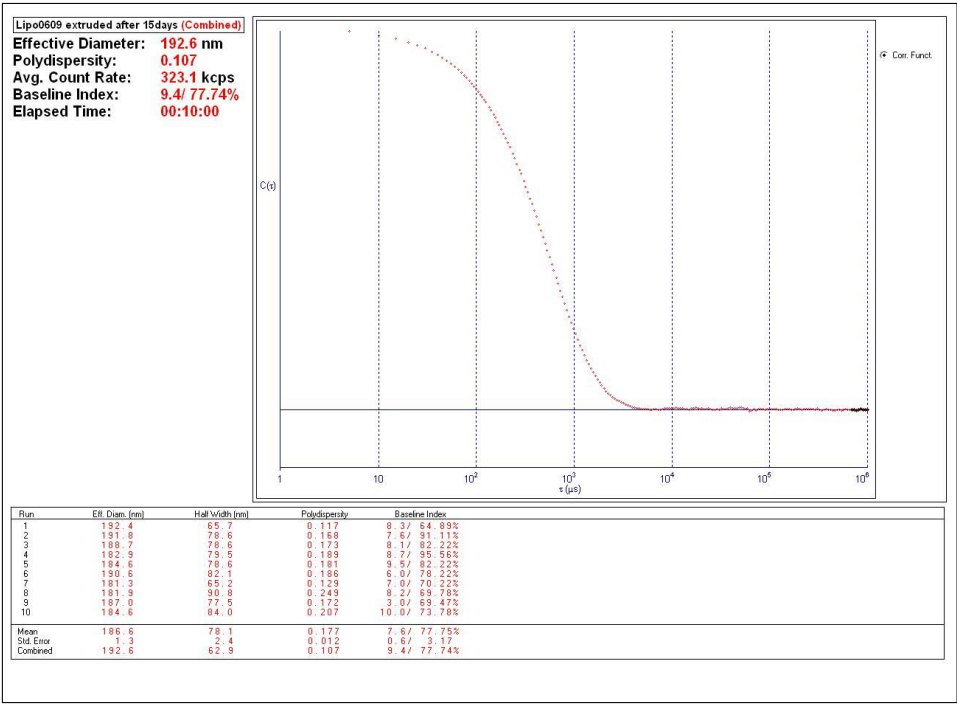


Figure A.10. DLS analysis of extruded liposomes after 15 days

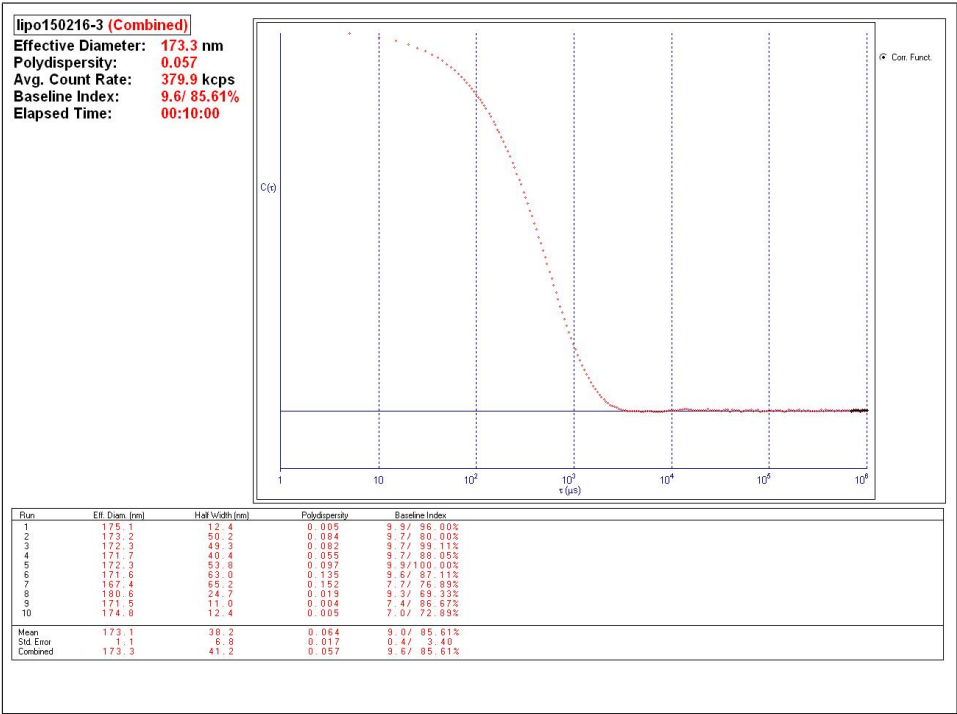


Figure A.11. DLS analysis of liposome prepared with 2:1 chloroform:methanol (v/v)

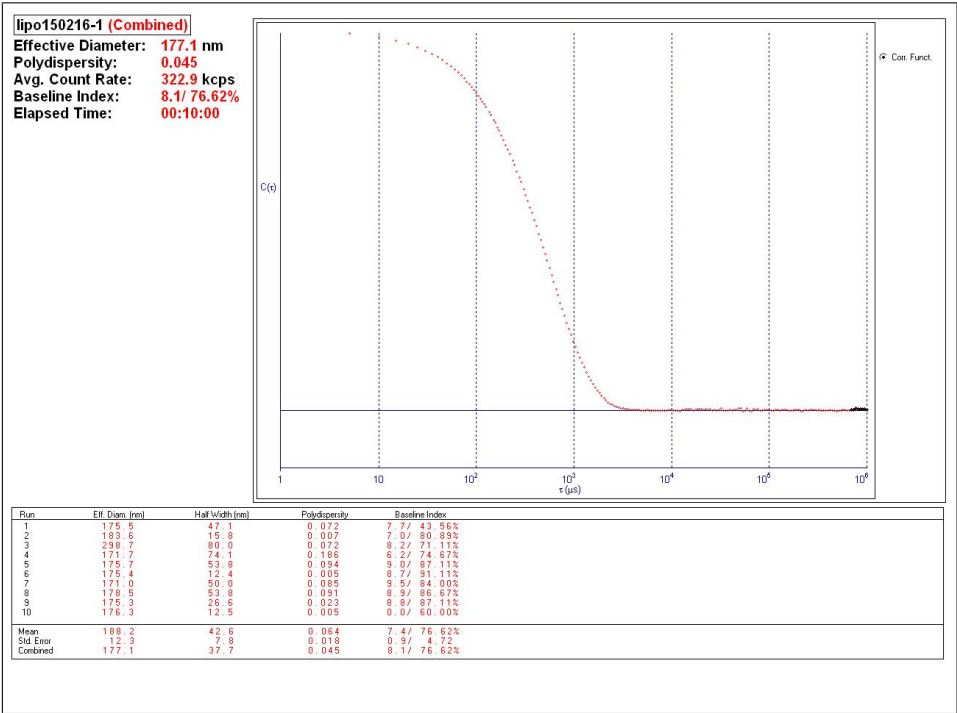


Figure A.12. DLS analysis of liposome prepared with 1:1 chloroform:methanol (v/v)

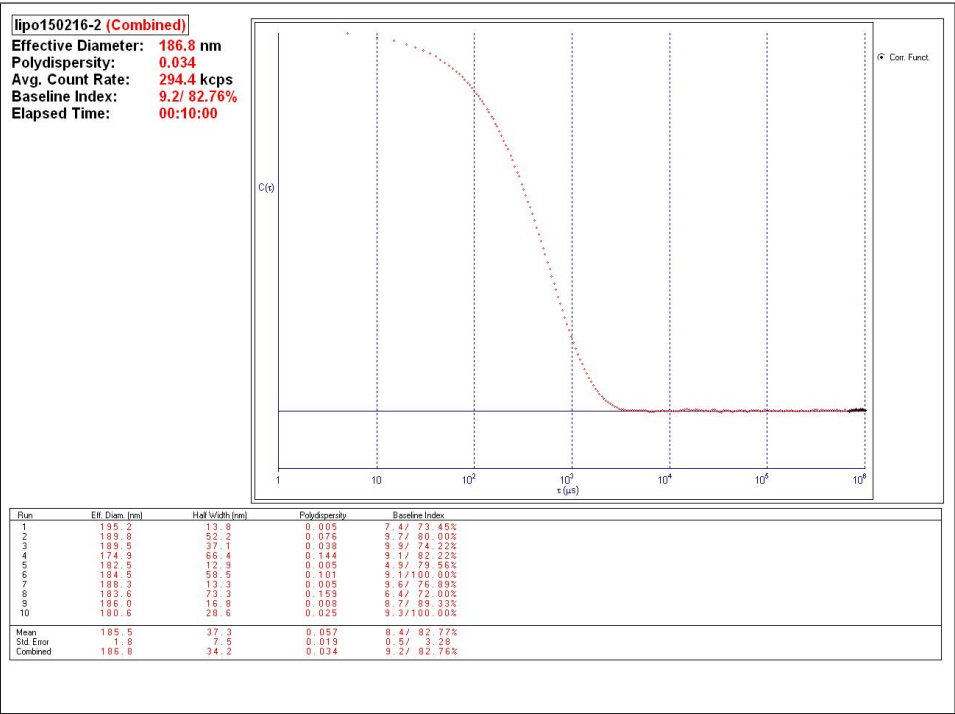


Figure A.13. DLS analysis of liposome prepared with 1:2 chloroform:methanol (v/v)

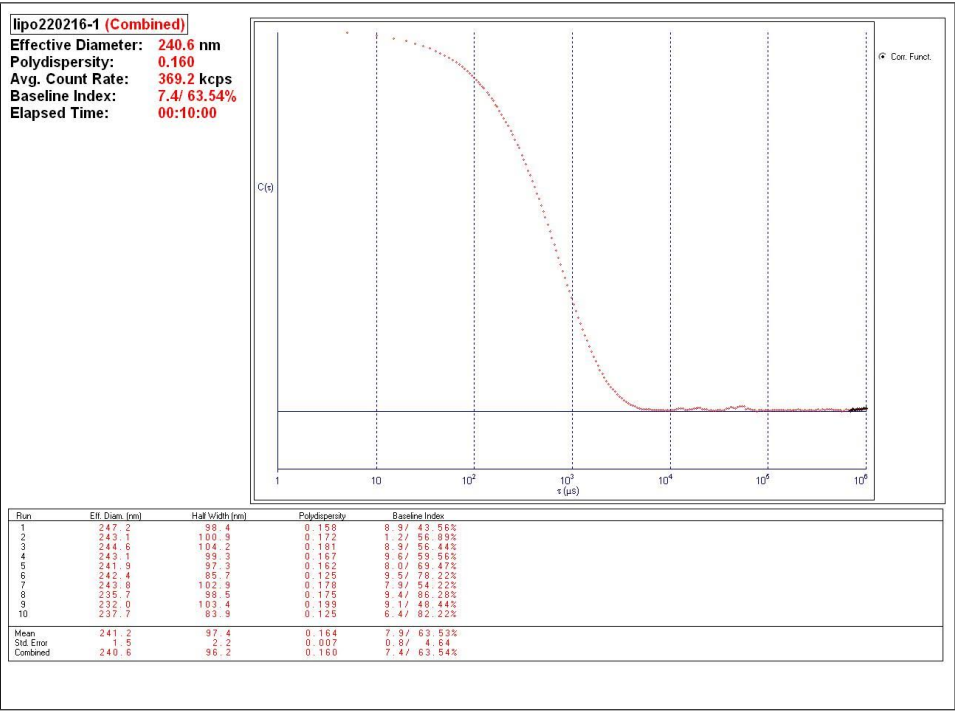


Figure A.14. DLS analysis of liposome prepared with 4:1 PBS:ethanol solution (v/v)

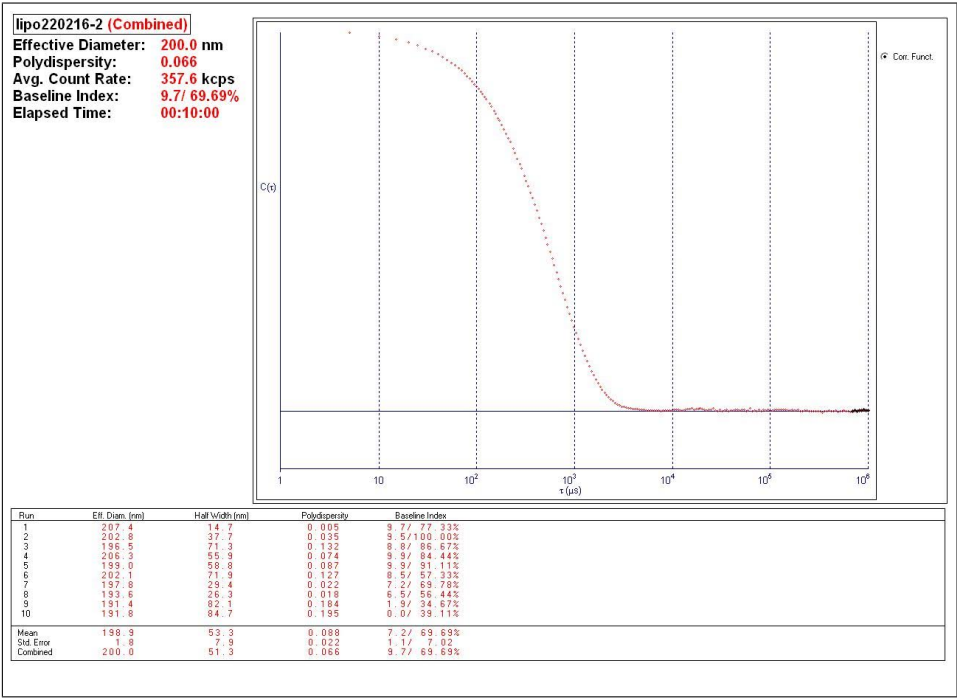


Figure A.15. DLS analysis of liposome prepared with 2:1 PBS:ethanol solution (v/v)

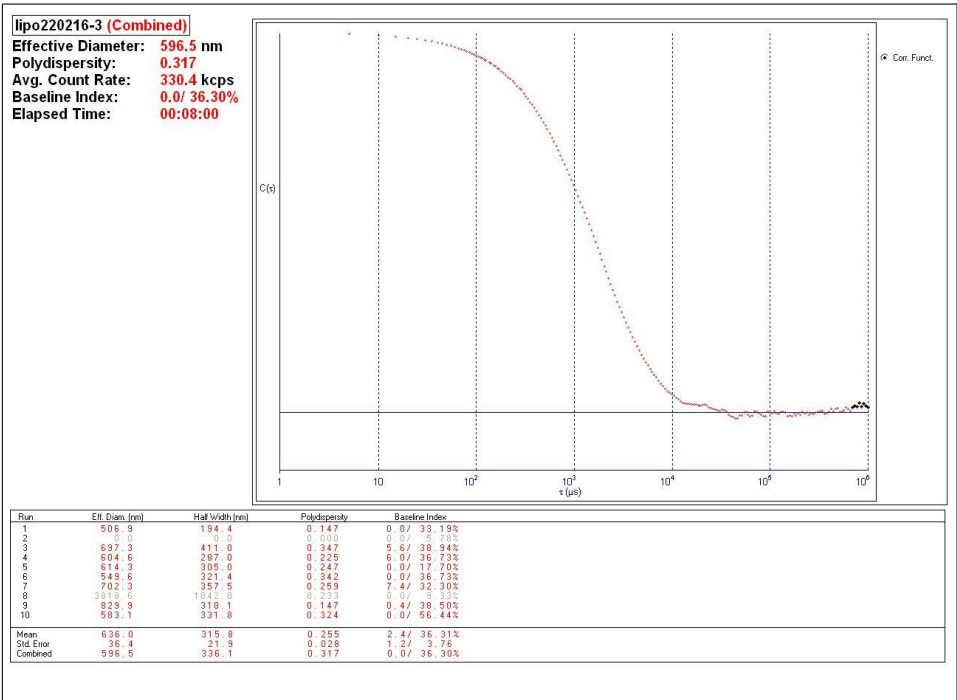


Figure A.16. DLS analysis of liposome prepared with 1:1 PBS:ethanol solution (v/v)

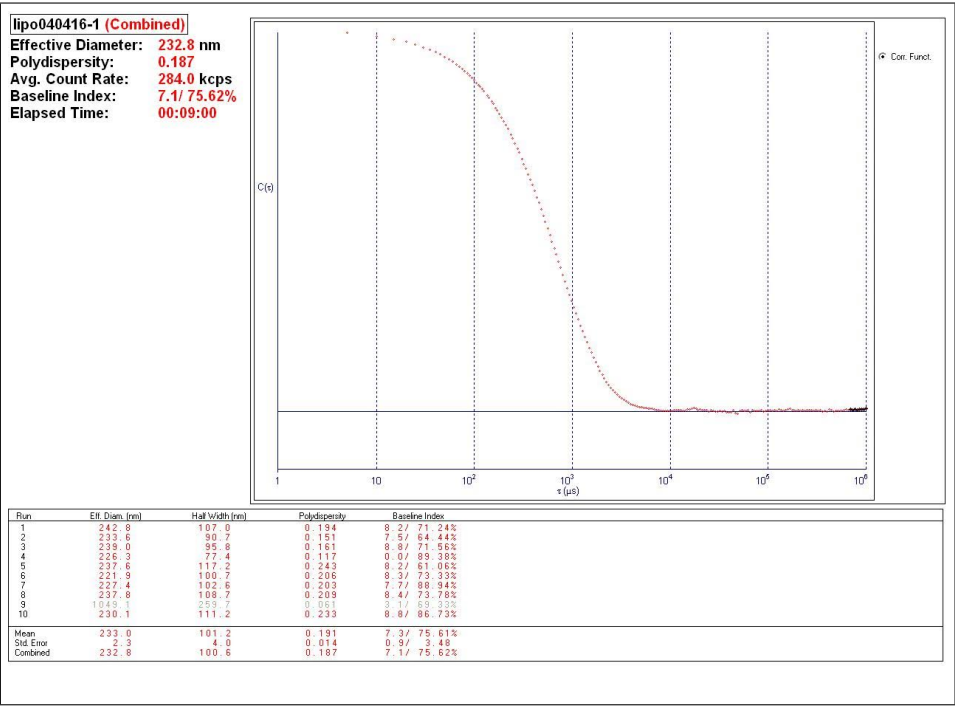


Figure A.17. DLS analysis of sample 1 in Section 4.5

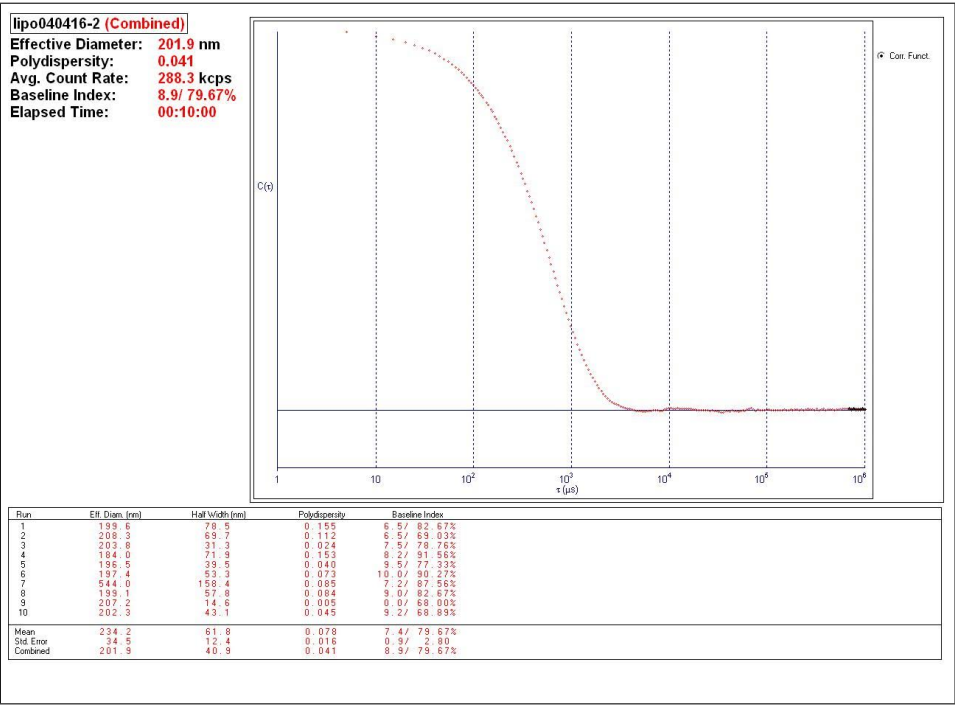


Figure A.18. DLS analysis of sample 2 in Section 4.5

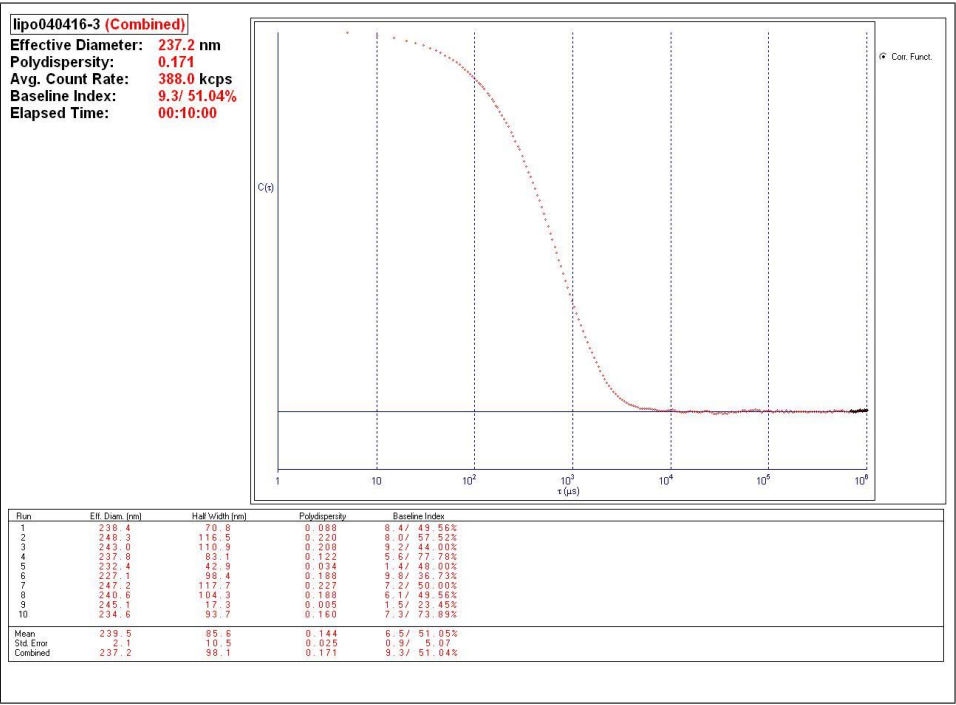


Figure A.19. DLS analysis of sample 3 in Section 4.5

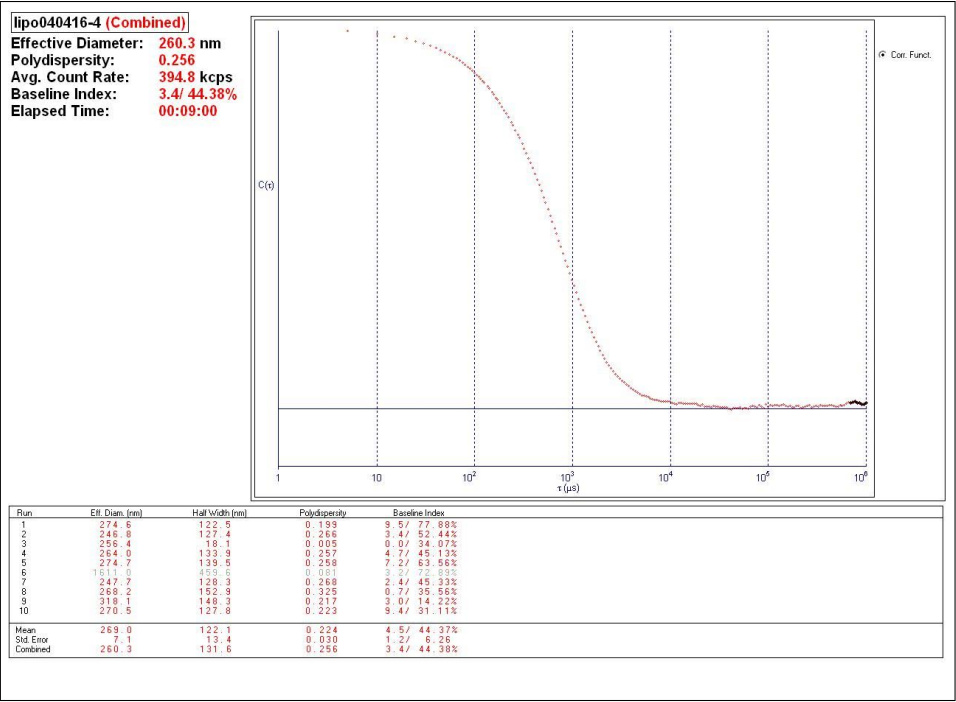


Figure A.20. DLS analysis of sample 4 in Section 4.5

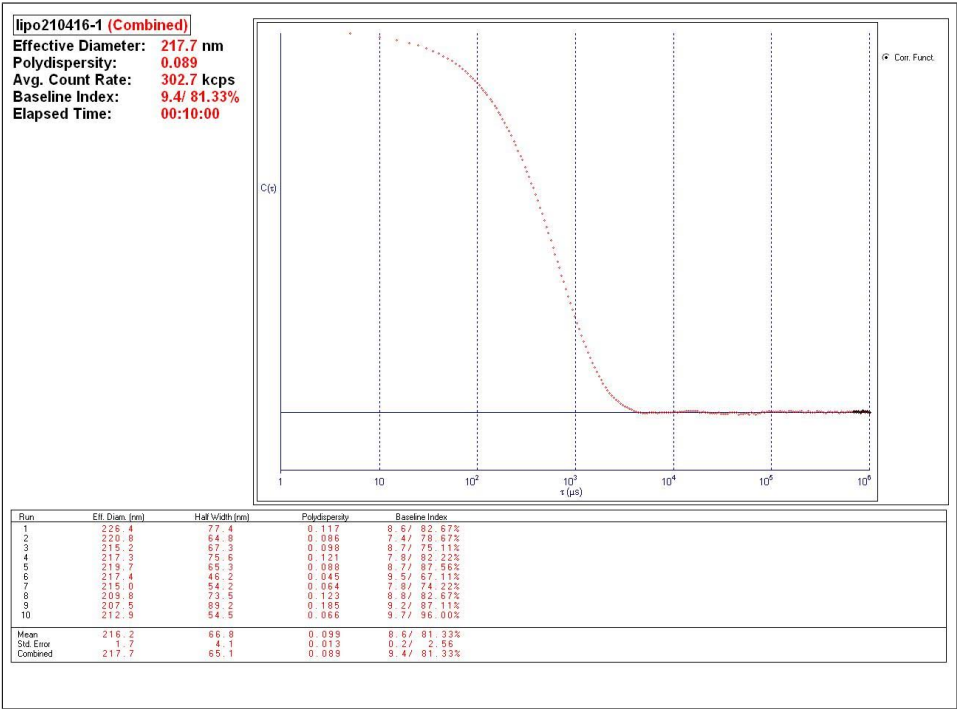


Figure A.21. DLS analysis of sample 1 in Section 4.6

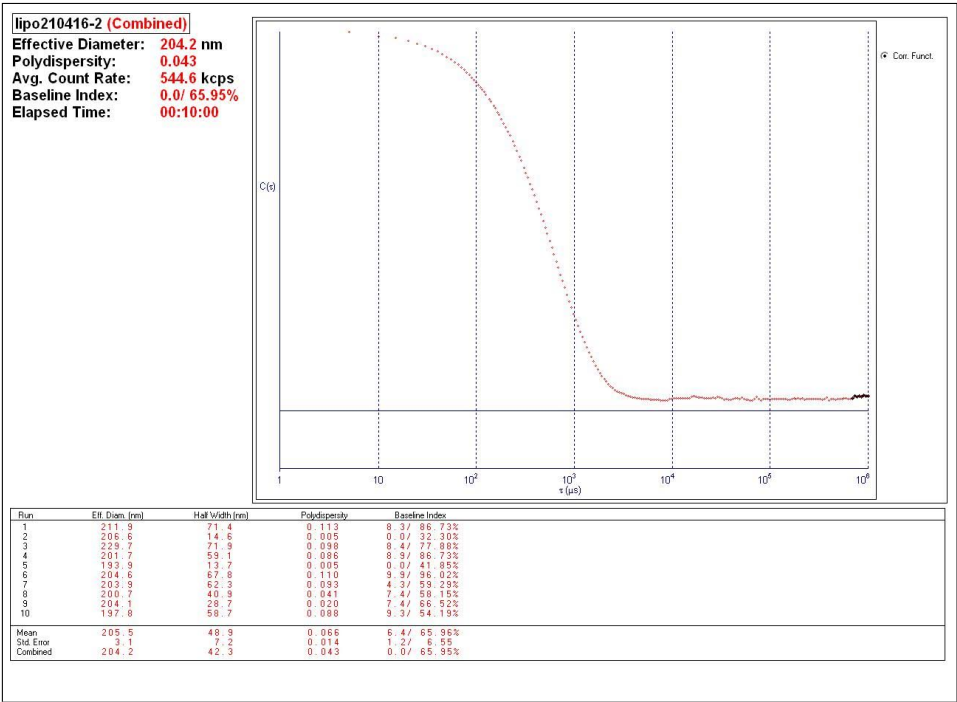


Figure A.22. DLS analysis of sample 2 in Section 4.6

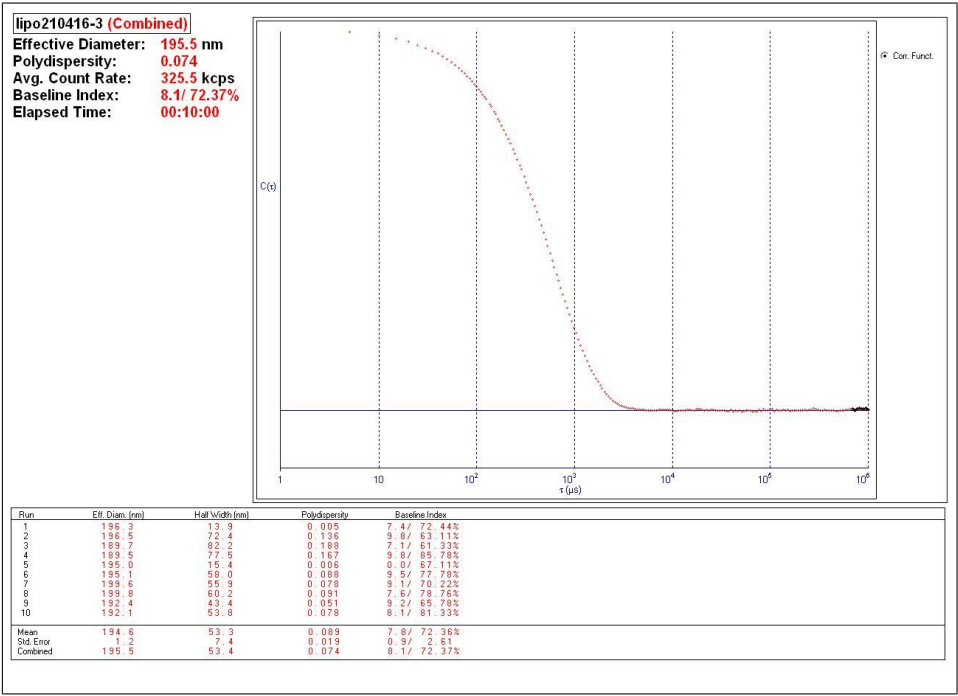


Figure A.23. DLS analysis of sample 3 in Section 4.6

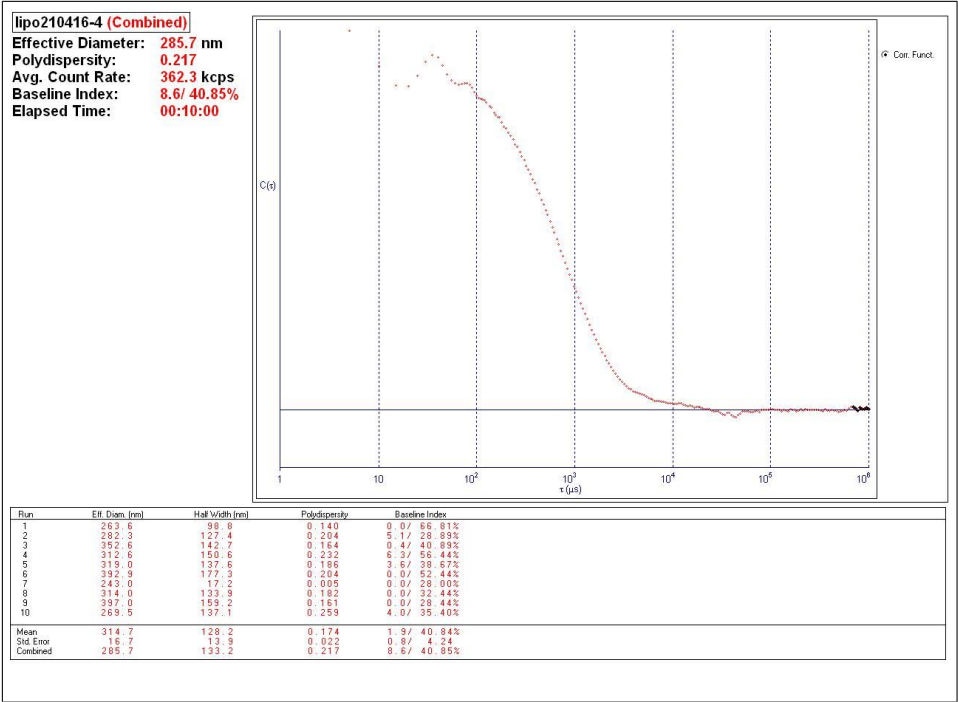


Figure A.24. DLS analysis of sample 4 in Section 4.6

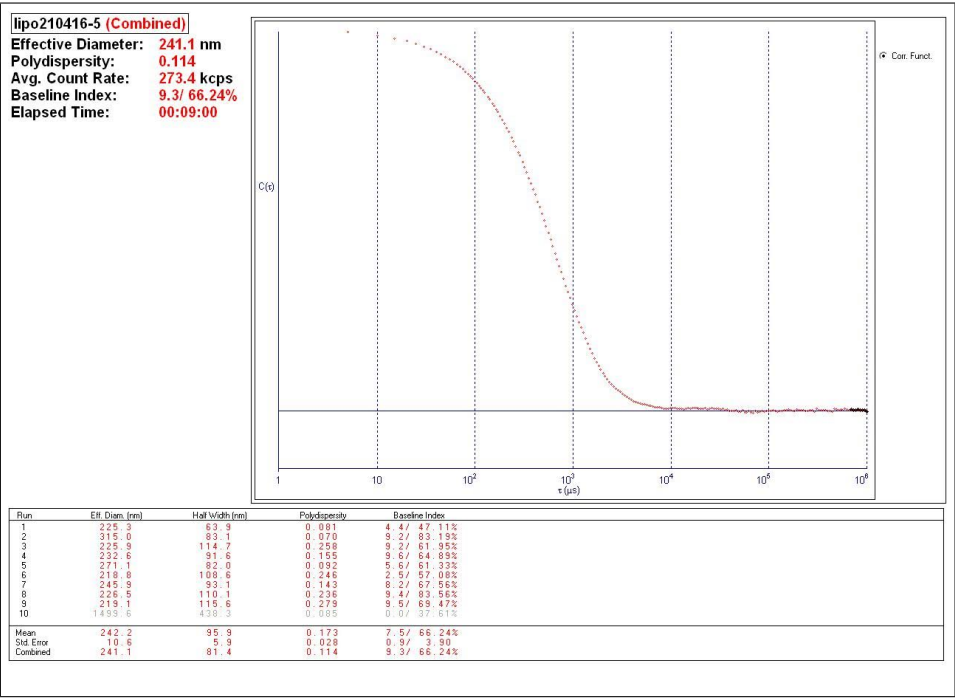


Figure A.25. DLS analysis of sample 5 in Section 4.6

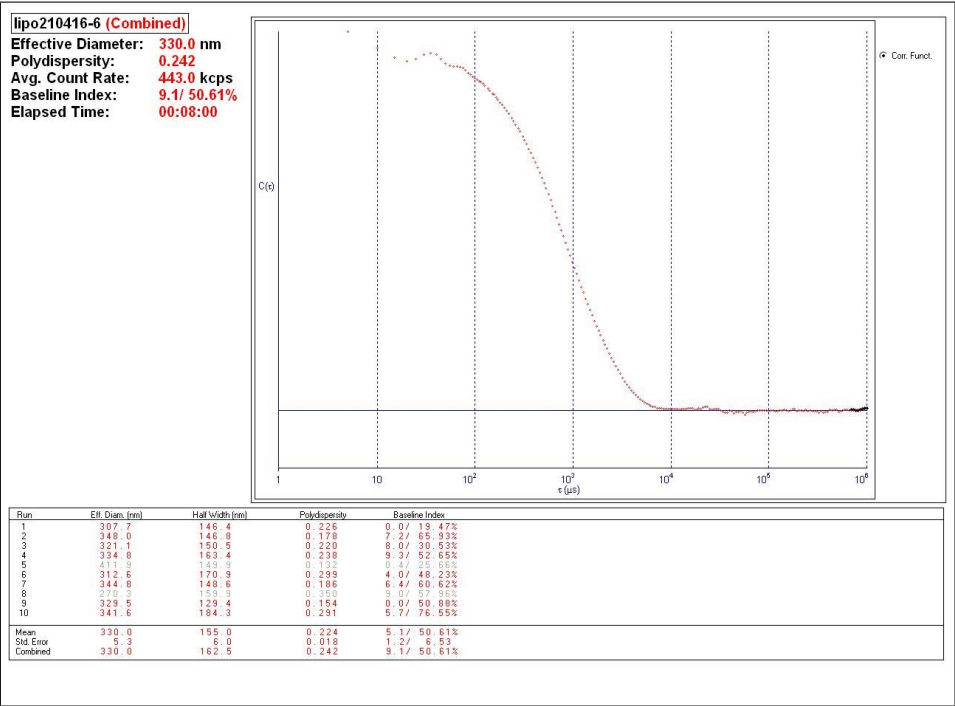


Figure A.26. DLS analysis of sample 6 in Section 4.6

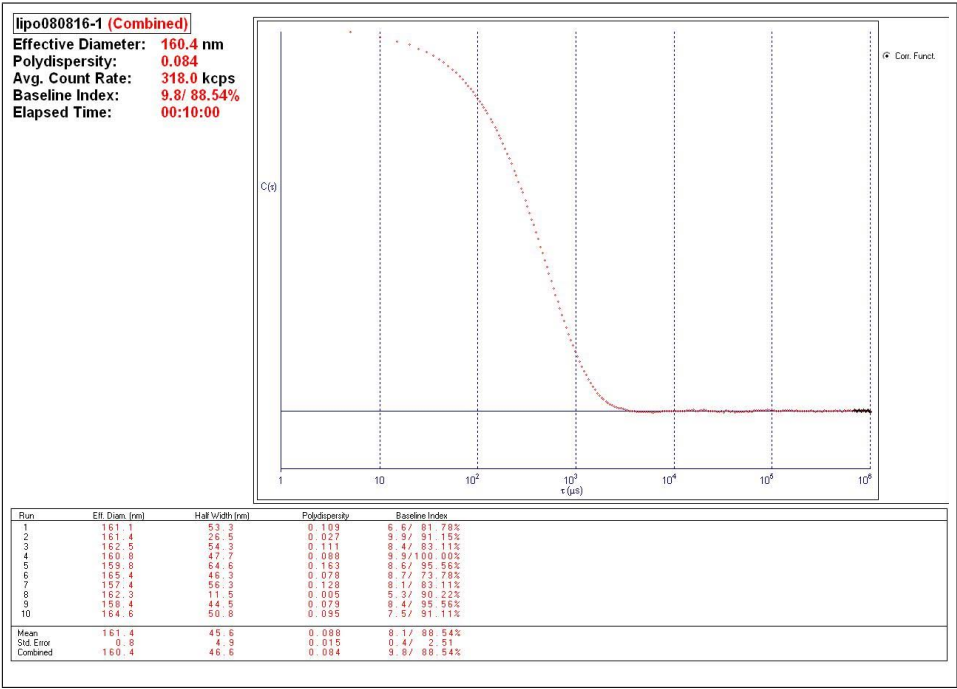


Figure A.27. DLS analysis of un-encapsulated liposome

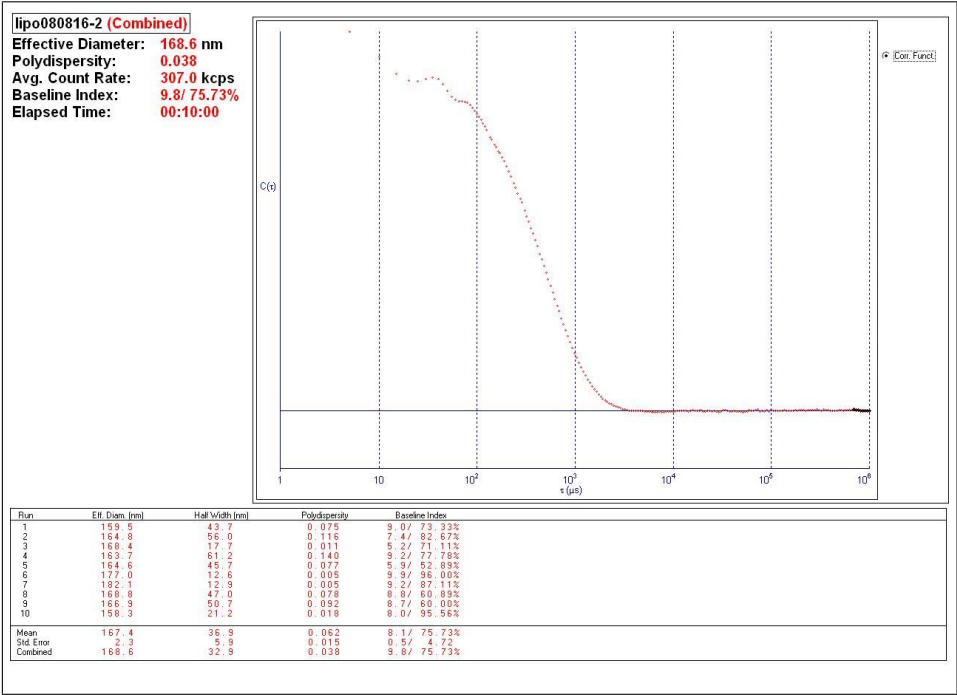


Figure A.28. DLS analysis of filaggrin encapsulated liposome

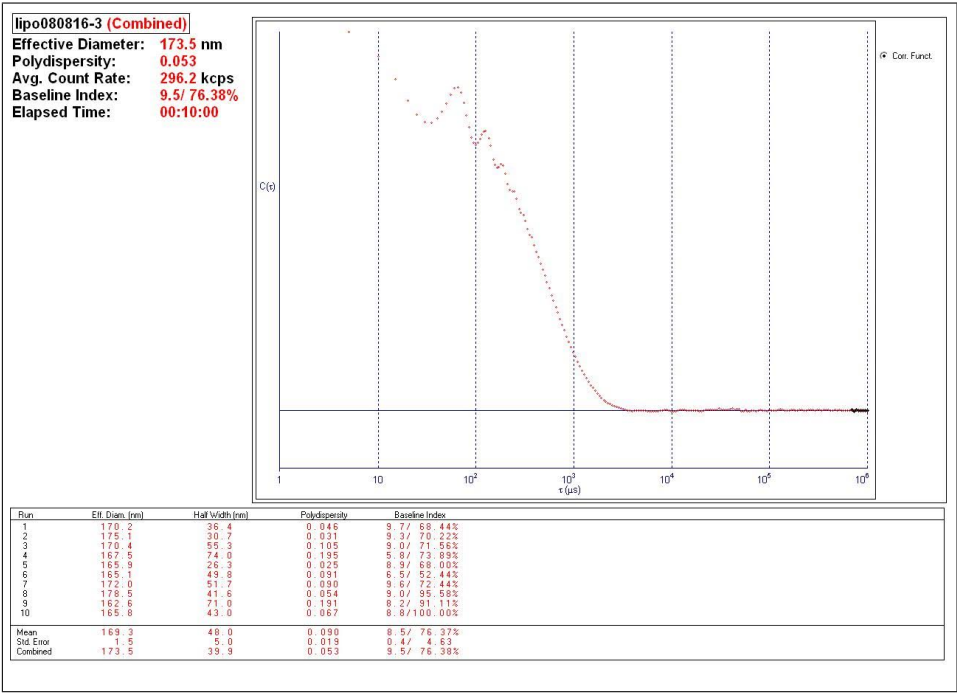


Figure A.29. DLS analysis of madecososide encapsulated liposome

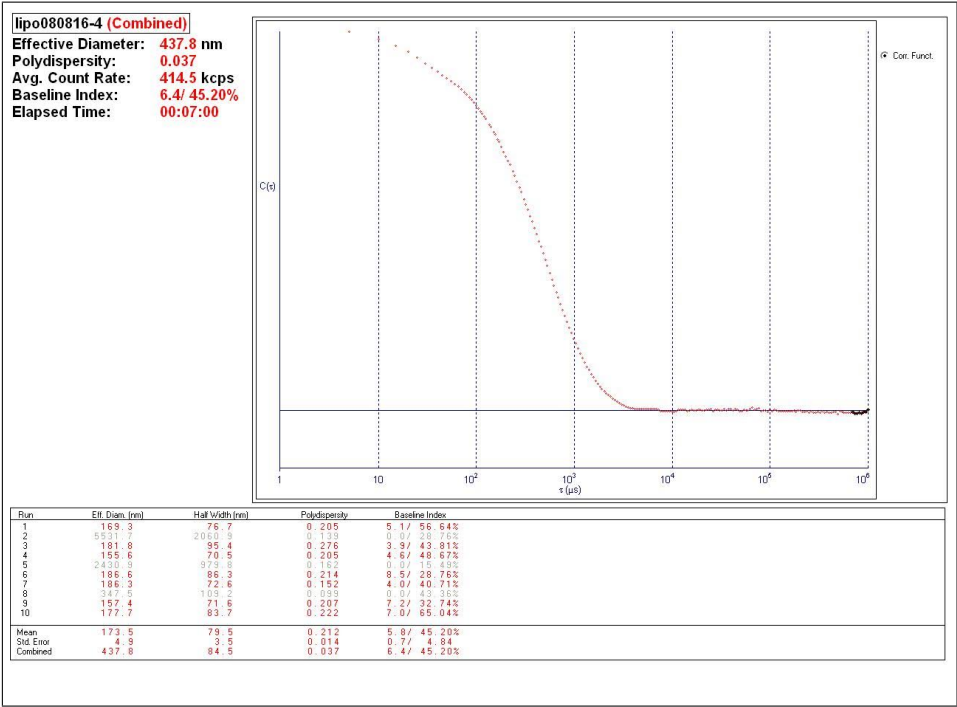


Figure A.30. DLS analysis of heterosides encapsulated liposome