

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE EFFECT OF GROWTH FACTOR LOADED NIOSOMES
ON NEURONAL CELL SURVIVAL

GÜLBAHAR ÖZTÜRK
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE

2021

T.R.

GEBZE TEKNİK ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

**BÜYÜME FAKTÖRÜ YÜKLÜ NİOZOMLARIN
NÖRONAL HÜCRE ÜZERİNE ETKİSİ**

GÜLBAHAR ÖZTÜRK

YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK

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SUMMARY

Growth factors (GF) are a subset of cytokines that have the capability to stimulate protein signaling for cellular growth, proliferation and cellular differentiation, usually in the form of protein or steroid hormones. A new approach to treat neurological diseases with GF, offer the potential to reduce or prevent neuronal degeneration in the brain. However, GF has a limited effect, because relatively large and polar to cross the blood-brain barrier and rapid degradation rate leading to a short biological half-life. For enhancing the efficiency of GF nanoparticles are used for delivery. Niosomes are nano-sized vesicular drug delivery systems consisting of non-ionic surfactants and steroids.

The main purpose of this study is load Neuronal Growth Factor (NGF) into niosomes in order to deliver it to neural cells without degradation and to evaluate the therapeutic efficiency of niosome delivery systems for neuronal cell survival.

Bovine serum albumin (BSA) loaded niosomes was initially involved in optimization studies. BSA and NGF loaded niosomes were prepared using the reverse phase evaporation technique using Span 60. The encapsulation efficiency (EE %), protein release profile, particle size, vesicle morphology, cytotoxicity of empty and BSA and NGF loaded niosomes were evaluated.

Niosome formulation prepared with Span 60 with 50% cholesterol content, the highest BSA and NGF entrapment efficiency rate was obtained as $64.6\% \pm 3.3$ and the average particle size was $185 \pm 1, 0$ nm. Vesicle morphology was evaluated.

In this study, the effect of the components on the niosome structure was demonstrated with using different surfactants and preparing niosomes with different cholesterol percentages. NGF has been shown to be successfully loaded into the niosome and has a slow release profile.

Key Words: Niosome, Growth Factor Delivery, Bovine Serum Albumin, Controlled Release, Drug Delivery.

ÖZET

Büyüme faktörleri (GF), genellikle protein veya steroid hormonları yapısında hücresel büyüme, çoğalma ve hücresel farklılaşma için protein sinyallemesini uyarma yeteneğine sahip bir sitokin alt kümesidir. Nörolojik hastalıkları GF ile tedavi etmeye yönelik yeni bir yaklaşım, beyindeki nöronal dejenerasyonu azaltma veya önleme potansiyeli sunmaktadır. Bununla birlikte, GF kan-beyin bariyerini geçmek için nispeten büyük ve polar olması ve kısa bir biyolojik yarı ömre yol açan hızlı bozunma oranı nedeniyle sınırlı bir etkiye sahiptir. GF'in beyin üzerinde etkinliğini arttırmak için nanaopartiküller kullanılır. Niozomlar, noniyonik yüzey aktif maddeler ve steroidlerden oluşan nano boyutlu veziküler ilaç taşıyıcı sistemleridir.

Bu çalışmada Nöral Büyüme Faktörü (NGF), bozunmadan iletilmek amacıyla niozomlara yüklenme ve nöral hücre üzerindeki terapötik etkisi değerlendirilmek amacıyla yapılmıştır. Bu bağlamda, NGF yüklenmeden önce bir model protein olarak sığır serum albumin (BSA) kullanılmıştır.

Başlangıçta optimizasyon çalışmaları için, BSA yüklü niozomlar kullanıldı. Daha sonra, BSA ve NGF yüklü niozomlar, Span 60 ile ters faz buharlaştırma tekniği kullanılarak hazırlandı. Enkapsülleme verimliliği (% EE), protein salım profili, parçacık boyutu, vezikül morfolojisi, boş ve BSA ve NGF yüklü niozomların sitotoksitesi değerlendirildi.

Niozom formülasyonlarından % 50 kolesterol içeriği ve Span 60 ile hazırlanan formülasyonda, en yüksek BSA ve NGF enkapsülasyon oranı 64.6 ± 3.3 ve ortalama parçacık boyutu $185, 5 \pm 1.0$ nm olarak elde edildi. Vezikül morfolojisi değerlendirildi.

Bu çalışmada farklı sürfaktanlar kullanılarak ve farklı kolesterol yüzdeleriyle niozomlar hazırlanarak bileşenlerin niozom yapısı üzerindeki etkisi incelenmiştir. Niozoma NGF' in başarıyla yüklendiği ve yavaş salım profiline sahip bir sistem olduğu gösterilmiştir.

Anahtar Kelimeler: Niozom, Büyüme Faktörü Taşınımı, Sığır Serum Albümini, Kontrollü Salım, İlaç Taşınımı.

ACKNOWLEDGEMENTS

First and most important, I would like to state my most intimate gratitude to Prof. Işıl AKSAN KURNAZ and Assoc. Dr. Şebnem ERÇELEN CEYLAN for their supervision, guidance and advice during the period of my research.

I am grateful a debt of thanks to my family for standing behind me in all circumstances and to my friends who I know are always there for me and whom I see as my second family. I would like specially thank Esra EKMEKÇİ YİĞİT for all their help and friendship during the thesis process and to my friend Gökçe TANIYAN, who added colour to my thesis with her drawings.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
AFM	: Atomic Force Microscope
BBB	: Blood Brain Barrier
BDNF	: Brain Derived Neurotropic Factor
BSA	: Bovine Serum Albumin
CH	: Cholesterol
CNS	: Central Nervous System
CPP	: Critical Settlement Parameter
DLS	: Dynamic Light Scattering
FDA	: Food And Drug Administration
GI	: Gastrointestinal System
GRAS	: Generally Recognized As Safe
HLB	: The Hydrophilic Lipophilic Balance
HSA	: Human Serum Albumin
LUV	: Large Unilamellar Vesicles
MLV	: Multilamellar Vesicles
NGF	: Nerve Growth Factor
NTs	: Neurotrophins
SEM	: Scanning Electron Microscope
SUV	: Small Unilamellar Vesicles
PALS	: Phase Analysis Of Scattering
PCS	: Photon Correlation Spectroscopy
TEM	: Transmission Electron Microscope
TTS	: Transdermal Therapeutic Systems

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1. INTRODUCTION

The application of nanotechnology to lifesciences, which is a multidisciplinary scientific field that applies engineering and production principles to a molecular level, has led to the emergence of nanoparticles to imitate or change biological processes [1]. The progress of nanoparticle-based drug formulations provided an opportunity to address and treat difficult diseases. Solid and colloidal particles that ranged between 10 nm and <1000 nm, are named nanoparticles. The desired size for nanomedical application is less than 200 nm [2]. Nanoparticles can be evolved carrying imaging agents and therapeutics as well as intelligent systems with hidden properties by changing the materials used, surface properties and size [3].

Many drugs have been developed to treat difficult complex diseases, but some of these drugs have been shown to produce severe side effects in addition to their benefits [4]. On the other hand, although some drugs show very effective results in vitro, it has been observed that they can't resist the endogenous enzymes found in the all gastrointestinal (GI) tract (if taken orally), which renders them dysfunctional in vivo [5]. These nanocarrier systems may have the ability to deliver the active ingredient to the desired tissues in controlled manner. The main purpose of a drug delivery system is the distribution of the drug at the rate desired by the body's needs throughout the treatment and direct the active presence to the targeted domain [6]. In addition to helping reduce drug-related toxicity targeted and controlled release also, provides less dosing to the patient. These advances in nanotechnology have proven to be auspicious in the treatment of many diseases as well as cancer [5]. The Blood Brain barrier (BBB) is a barrier that prevents the passage of most circulating chemicals, cells and molecules. BBB is composed of a complex system of astroglia, endothelial cells, and pericytes [6]. It is a real challenge to pass through the BBB for drugs that need to be delivered to the brain. Nowadays, with the advent of nanoparticles as an attractive tool potentially able to solve the unsolved problem of improving drug transport across the BBB.

Although drug targets have been advanced in designing more effective drug formulations, there is still way to progress drug delivery systems and targeting [3].

One of the systems envisaged for prolonging the presence of the drug in the systemic circulation and reducing the toxicity is considered to be the encapsulation of the drug in vesicular structures. As a result, a number of vesicular drug delivery systems such as pharmacosomes, transfersomes, liposomes and niosomes have been developed [2], [7].

The research will continue until we find a drug that has maximum efficacy and no side effects. Therapeutic efficiency of the substantial drugs is advantageously formulated and developed with drug delivery systems.

In recent years, great importance has been attached to the improvement of new drug delivery systems. An ideal drug delivery system must fulfil two prerequisites. Firstly, it should deliver the active ingredient in proportion to the needs of the body during treatment. After that, it should direct the active content to the effect area. The drug delivery system works by keeping the drug effect at a fixed and effective level to maintain a predetermined rate and minimize harmful side effects in the body. This can be achieved with the novel targeting strategies for drugs to desired domain in the diseased tissue or organ. [2].

There are several types of pharmaceutical drug carriers such as polymeric, macromolecular, particulate and cellular carriers. Particle-type of carriers, including polymeric micelles, nanoparticles, microspheres, lipid particles and vesicular-like liposomes, niosomes, are also acknowledged as colloidal carrier systems [1], [8].

Niosomes (non-ionic surfactant based vesicles nonionic surfactant vesicles), which are analogues of liposomes, are closed bilayer structures formed by non-ionic amphiphiles (surfactants) in the aqueous medium. Niosomes first emerged in Strathclyde University in the early 1970s by researching the potentials of nonionic surfactants synthesized by Guy Vanlerberghe et al. immediately after these cosmetic studies, the use of niosomes as drug delivery systems came to the agenda. Non-ionic surfactants which are play an important role in sustained and targeted drug delivery are bio-compatible molecules. Also, they have a significant impact on niosome stability and encapsulation efficiency. It is possible to encapsulate phytotherapeutic agents, bio macromolecular agents such as peptides and proteins, and both hydrophilic and lipophilic drugs with niosomes [7].

1.1. Purpose, Contribution and Content of the Thesis

NGF is a well known survival factor throughout the development of sympathetic and sensorial neurons. NGF has promising therapeutic features on degenerative and traumatic central nervous system diseases. Since the neurotropic factors have large molecular weights, they cannot easily cross the blood-brain barrier (BBB) and do not completely disperse after systemic administration. For effective passage from BBB, nanocarriers are the encouraging choice. Niosomes are nano-sized vesicular drug delivery systems consisting of non-ionic surfactants and steroids. In this study, we prepared and tested basic features of niosomes as a nanocarrier for NGF delivery. The main purpose of this study is loading NGF into niosomes in order to deliver it to neural cells without degradation and to evaluate the therapeutic efficiency of niosome delivery systems for neuronal cell survival.

2. VESICULAR SYSTEMS

In recent years, vesicles have been a preferred tool for drug delivery in modelling biological hazards and transporting and targeting the active substance. It has been observed that lipid vesicles have an important value in genetic engineering, diagnostic techniques, and immunology and recently in membrane biology. Lipid vesicles also play an important role in the transport of active ingredients and even their targeting [9].

The structures that surround and divide and restrict all cells and organelles are called biological membranes. All biological membranes have a double lipid layer. After the publication of the cell theory in 1839, numerous theoretical membrane structure models emerged. These experimental models helped us to have information about the motion dynamics and static structures of biological membranes. Although lipid vesicles, one of the experimental models of biomembranes, have been improved for main research, many technological innovations have originated from their applications. Over time, lipid vesicles have been developed as a successful tool for controlled release [2], [9].

Traditional chemotherapy drugs used for the treatment of intracellular infections are not effective due to their limited penetration into the cells. This can be overcome by using vesicular delivery systems [10]. Encapsulation of a drug with vesicular structures prolongs the drug's presence in the systemic circulation by sustained release. If selective uptake is available, it can be estimated to reduce toxicity. The phagocytic uptake of the systemically administered drug-loaded vesicular delivery system provides an effective method for delivering the drug directly to the infection site [4]. There is some examples of nanoparticles enhanced drug delivery to the brain. For examination of brain drug delivery, nanoparticles made of Polylactide-co-glycolides embedding pyrazinamide, rifampicin, ethambutol and isoniazid (antituberculosis drugs) were given to mice, sustaining high drug levels up to 8 days in plasma and for up to 9 days in the brain. These concentration results were higher than free drugs [11]. In other study, nanoparticles made of poly-butyl-cyano-acrylate were succesfully used for delivery of necessary functional proteins into central and peripheral nerve cells [12]. Thus, it ensures a reduction of drug

toxicity without any side effects. Vesicular drug delivery reduces the cost of treatment by increasing the bioavailability of the drug, particularly when less dissoluble drugs are considered [2]. Vesicular delivery systems can be used for both hydrophilic and lipophilic drugs. They slow down the drug elimination in body high metabolism rates. Thus, it functions as sustained-release systems. Drug insolubility, imbalance and fast deterioration problems can be solved by this systems. For this purpose a number of vesicular delivery systems such as liposomes, niosomes, pharmacosomes, etc. have been evolved.

Vesicular systems have been used in many different scientific fields recently because they are extremely useful carrier systems. Over time, vesicular delivery systems have been developed as an important drug carriers due to their flexibility to adapt to various targets. There are also some disadvantages of vesicular delivery systems. Such as, limited shelf-life: due to fusion, aggregation, seepage of seized drugs and hydrolysis of encapsulated drugs. Despite some disadvantages, vesicular delivery systems are still an important option in selective targeting and controlled delivery of various drugs. All developers continue to develop the vesicular system to prevent the leakage of vesicle content, oxidation of the content and elimination of vesicles by the natural defence mechanisms of our body. Existing researchers are often exploring the use of different approaches (biotinylation, pegylation, etc.) for cellular targeting [3], [4], [6].

2.1. Liposomes

Liposomes are two-layer spherical lipid vesicles consisting of polar lipids that are used in the food industries, chemical industries, personal care and pharmaceutical to encapsulate both hydrophilic and hydrophobic compounds. It occurs when polar lipids are dispersed in water like polar environments. The main sources of polar lipids in nature include, mainly; egg, soy and sunflower lecithin. These lecithins have been used as an emulsifier or structure modifying agent in foods for many years and are in the Generally Recognized As Safe (aka GRAS) ; category according to the American Food and Drug Administration (FDA) [6]. Liposomes formed by combining lecithins to form a double-layer spherical structure are important in terms

of encapsulating functional components that can be dissolved in both water and oil. It has been shown that the confinement of functional components into liposomes increases the stability of the encapsulated substance, eliminates its interaction with the medium, thereby maintaining its activity for a longer period in environments that would normally deteriorate [13].

Liposomes can be in sizes ranging from 20 nm to 20 μ m in diameter. The vesicles can exist as double or multiple bilayer membranes. These double-layer polar lipid structures are similar to the lipid part of the cell membranes. The primary reason for liposomes to be investigated is the fact that their structure is very similar to that of a typical cell membrane. Its ability to mimic cell membrane behaviour has enabled liposomes to be used in pharmaceutical applications, such as tumour cells, in a specifically targeted drug release [14]. In recent years; liposomes have also been found to be ideal systems for encapsulating many different active drug agents in biological, biochemical, agricultural and food fields. Enzymes, antimicrobial agents and antioxidants are some of the encapsulated substances. Liposomes are also investigated with respect to their potential uses in the food industry, although such applications are not common due to the interaction of liposomes with other components in food as well as the cost of liposome applications, both in terms of raw materials and manufacturing. Still, liposomes can be used in the delivery of antimicrobial agents, flavouring agents and active agents with proven positive effects on health. They have also become more suitable for the transfer of substances in the production of functional foods [15].

The most important feature that distinguishes liposomes from other encapsulation agents is its structural similarity to the cell membrane. Thanks to this feature; some intracellular studies have shown that some bioactive agents can achieve the distribution to and release in specific areas (cells) in the body [3]. This unique structure of liposomes allows these nanoparticles to enter the intercellular compartment as a result of blood transport. Liposomes can interact with the cells, fuse with the cell membrane and release the entire drug into the cell cytoplasm.

This feature can be used not only in pharmaceutical applications but also in food applications to increase the bioavailability of functional agents. It has been observed that the bioavailability of the active agent encapsulated with liposome has

been observed in oral applications. This is due to the similarity of liposomes to the cell membrane; it is shown that the agents encapsulated with liposomes are protected from external effects in the digestive system, preventing their degradation until they enter the blood [8].

Liposomes prevents the degradation reactions that may occur with the effect of the external environment thanks to the interruption of the interactions of the agents encapsulated with liposomes with the system they are in. It has been observed that controlled release of active agents into the environment can be realized, thus, the compounds such as encapsulated antimicrobial, antioxidant, and vitamins are more resistant to processes such as frying, drying, pasteurization, cooking [6]. Vitamin A shows the toxic effect when is consumed in high amounts. Liposomes, which are also used for controlled release in foods, have been shown to eliminate this effect when used in the capsulation of vitamin A [13].

The drug delivery system through the liposome is quite advantageous due to the protection of the drug, the controlled release of the active part of the drug along with the targeted dose, and the aspects associated with cellular uptake through endocytosis [4]. Liposomes also present some problems with hydrolytic degradation, oxidation, and sedimentation and drug leakage. Furthermore, clustering or fusion problems occur during storage. Approaches that can be used to enhance liposome stability include effective formulation and lyophilization [2], [8].

2.2. Niosomes

Niosomes (non-ionic surfactant based vesicles nonionic surfactant vesicles), which are analogues of liposomes, are closed bilayer structures formed by non-ionic amphiphiles (surfactants) in the aqueous medium (Figure 2.1) [7]. Niosomes first emerged in Strathclyde University in the early 1970s by researching the potentials of nonionic surfactants synthesized by Guy Vanlerberghe et al. immediately after these cosmetic studies, the use of niosomes as drug delivery systems came to the agenda [16], [17].

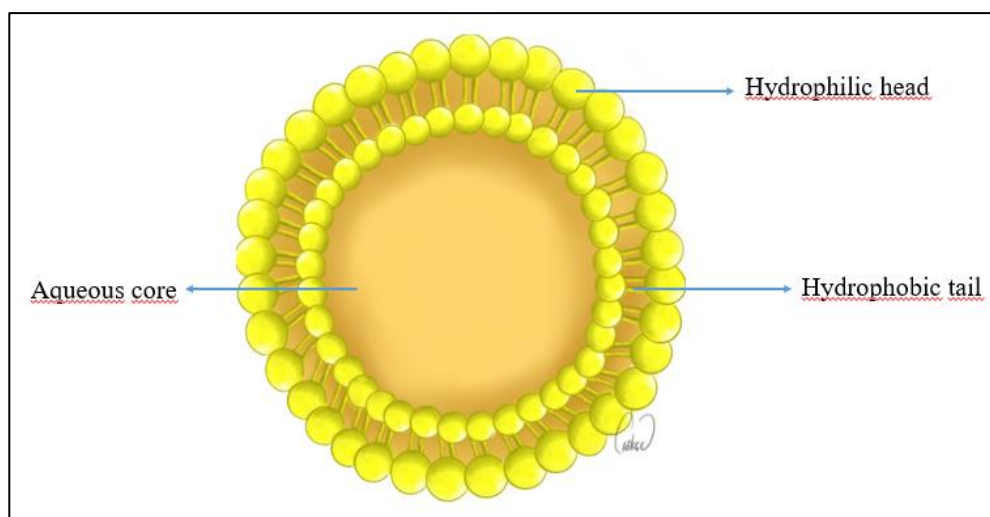


Figure 2.1: Schematic representation of the niosome.

The in vivo behaviour of niosomes is same to liposomes. Niosome structures in spherical, helix, tubular and polyhedral shapes can be obtained by changing factors such as vesicle composition and temperature. Niosomes undergo shape changes when mechanical stress is applied, but niosomes are more stable than liposomes [5]. They do not need to work in an inert atmosphere while processing. Disadvantages in liposomes due to variable phospholipid purity and high cost are eliminated with niosomes [8]. They can be sterilized by filtering through membrane, autoclaving and gamma radiation [4].

Niosomes and liposomes are similar in the way they encapsulate drug. Niosomes are osmotically active and stable and increase the stability of the drugs loaded. The use and storage of niosomes do not require any special conditions. Niosomes are made up of smaller pieces of hydrophilic and hydrophobic parts. As a result, they can contain drug molecules with a wide variety of solubility. Niosomes offer flexibility in structural features such as size, composition, fluidity and can be designed due to the desired situation [4], [18]. Niosomes increase the oral bioavailability of poorly absorbed drugs, as well as increases the penetration of drugs into the skin for topical applications [2].

Niosomal surfactants are non-immunogenic, biodegradable and biocompatible. Niosomes increase the therapeutic performance of drug molecules by delaying their elimination during the circulation, protecting the drug from degradation in the

biological environment and limiting effects on target cells [2], [18].

The formation of closed double layers in niosomes rarely develops spontaneously, and generally physical agitation or heat must be applied to the system. In the resulting structure, the hydrophobic portions of the molecule are protected from the aqueous solvent, and the hydrophilic tips are in contact with the aqueous medium.

Generally physical agitation and heat need to be carefully checked during the preparation of niosome delivery systems. Niosomes can be prepared as monolayer or multilayer. Generally, various auxiliaries are added to the formulation to prepare a stable niosome formula. Structures with different permeability and stability can be obtained by varying the proportions and surfactants used [19].

- Advantages of Niosomes

- They are water-based vesicle mixture offers high patient compliance compared to oily dosage forms.
- Niosomes are suitable for the use of drug molecules with a wide range of solubility due to their hydrophilic, lipophilic and amphiphilic [20].
- Their vesicle size can be controlled by varying the size lamellarity, surface charge, guided volume, and concentration composition.
- The surfactant used is easy to store and use, and an inert environment and low temperature are sufficient to form the niosomes.
- Surfactants are biodegradable, biocompatible, non-toxic and non-immunogenic.
- Niosomes are osmotically stable and active. They also improve the stability of the drug they contain.
- Niosomes act as a depot and they release the drug continuously and in a controlled manner [20].
- They increase the oral bioavailability of poorly soluble drugs [21].
- They can increase the permeability of the drugs through the skin.
- They have bile stable structure in emulsion form.
- Niosomes are economic choice for large-scale production.
- Niosomes can protect the drug from enzyme metabolism.

- Niosomes can protect the active part of the drug from biological circulation [21].
 - The therapeutic effects of drug molecules can be enhanced by delayed circulation clearance [21].
 - Niosomes can restrict the drug delivery rate as aqueous phase niosomal dispersion can be emulsified in the non-aqueous phase and thus normal vesicle can be administered in an external non-aqueous phase [22].
- Disadvantages of Niosomes
 - Limited shelf life: due to fusion, aggregation, leakage of encapsulated drugs and hydrolysis.

Niosomes can be formulated by several different methods to create multilayer vesicles. The most common methods include the formation of lipid layer using hydration method or reverse-phase evaporation method or transmembrane pH gradient retention (remote loading) [19]. Less frequently used methods include handshake, ether injection and sonication. These methods are attributed on if the drug is actively or passively incorporated into vesicles. Drug and lipids used for the passive trap are distributed together with some of the drug held according to hydrophobicity and electrostatic charge. If the drug is hydrophilic, it is trapped in the internal aqueous phase and the hydrophobic drug is trapped primarily in the lipid region. Active capture can be obtained in response to ion gradients placed on niosomal membranes. This allows eclipse of the drug after the niosomal carrier is formulated [9], [19] .

Niosomes are classified according to different features such as trap efficiency, in vitro release rate, vesicle diameter, freeze capture microscopy and photon correlation microscopy. Other features examined include drug stability, toxicity and drug leakage, etc [6], [23].

Same with liposomes, there are 3 main types of niosomes based on their size. These are small unilamellar vesicles (SUV, size $\sim 0.025\text{--}0.05\ \mu\text{m}$), multilamellar vesicles (MLV, size $> 0.05\ \mu\text{m}$), large unilamellar vesicles (LUV, size $> 0.10\ \mu\text{m}$) (Figure 2.2). MLV vesicles require handshaking. Also, they show increase in trapped

volume and balance solute distribution [21]. The 3 varieties differ in their lipid composition. SUVs are usually produced by sonication method. Solvent dilution techniques or ultrasonic electrocapillary emulsification can be used to prepare the SUV. Lipid injections dissolved in an aqueous buffer in an organic solvent can cause spontaneous formation of LUV. However, the method for better preparation of LUV is reverse-phase evaporation or detergent dissolution [6].



Figure 2.2: Types of niosomes SUV, LUV, MLV.

Some researchers prepared niosomes of sorbitan monoesters (Span 20, 40, 60, and 80) by film hydration method without sonication, and showed that vesicles containing Span 60 have highest insulin resistance against proteolytic enzymes [4], [20]. Micromanipulation of the outer layers of niosomes allows the formation of bonds that have liquid state lipid-surfactant lamella nanotubes and can be used as a vesicle transport mode in a flexible microtube [17].

In niosomes, as in liposomes, aggregated drugs may exhibit aggregation, fusion, leakage, or hydrolysis. Therefore, it may limit the shelf life of the niosome solution. It is not efficient, especially if smaller amounts are required for a particular application or dose [18].

2.2.1. Factors Affecting Niosome Formation

- Structure and Properties of Niosome-forming Surfactants

In theory, a certain amphiphilic substance and an aqueous solvent medium is required to form a niosome. In some cases, cholesterol can be added to the formulas or molecules to prevent the vesicles from aggregating with repulsive steric or electrostatic effects and increase its stability [6].

Niosome-forming amphiphiles shown in Table 2.1 must have a hydrophilic head group and hydrophobic tail structures. The hydrophobic portion may consist of one or two alkyl or perfluoroalkyl groups or, in special cases, a single steroidal group [5]. The alkyl chain length is between 12 and 18 carbons. The molecules can contain 1-3 alkyl chains. In perfluoroalkyl surfactants forming vesicles, the chain length can be reduced to 10 carbons. Crown ether amphiphiles containing a steroid, C14 alkyl or C16 alkyl group have also been shown to form vesicles. While many hydrophilic head groups can enter the structure of the vesicle-forming surfactants, the hydrophobic groups are limited in number. Two separate parts of surfactants can be connected by ether, amide or ester bonds [21].

Table 2.1: Examples of surfactants used in niosome formation.

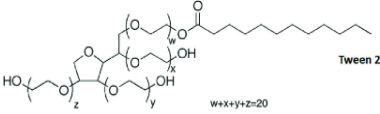
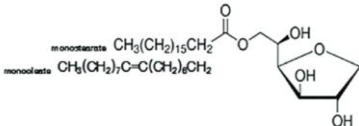
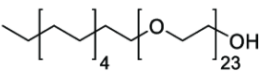
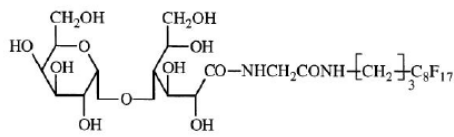
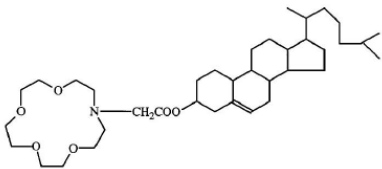
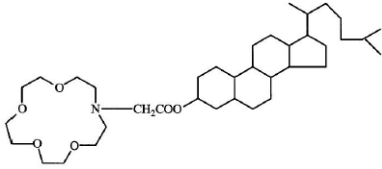
Name of Surfactant	Structure of Surfactant
Tween surfactants (Polyethylene glycol sorbitan esters) (Example: Tween 20)	
Span surfactants (Sorbitan esters)	
Brij surfactants (Polyethylene glycol ethers)	

Table 2.1: Continuation

Fluorinated surfactant	
Crown ether surfactant	
Crown ether surfactant (cholesteryl derivative)	

There is an equilibrium between the hydrophilic and lipophilic ends of the surfactants. This is referred to as the hydrophilic lipophilic balance (HLB). HLB values of surfactants are parameters that show whether they can form vesicles. For SPAN surfactants, 4-8 HLB was found suitable for vesicle formation. "Polysorbate 20" can form niosomes in the presence of cholesterol.

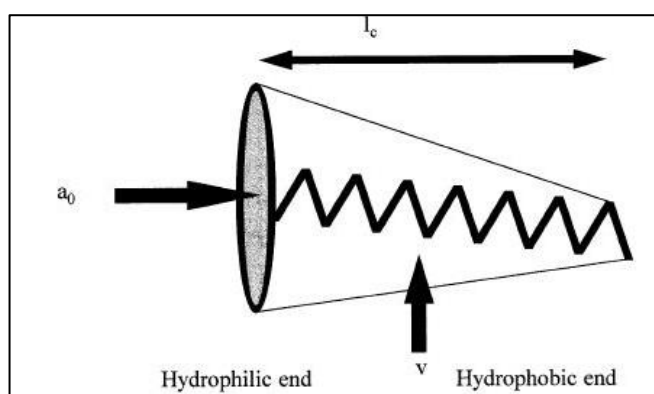


Figure 2.3: Schematic representation of an amphiphilic substance.

Determination of the ability of surfactants to form vesicles is not only related to HLB value. Their chemical structure and some other factors play important role. The critical settlement parameter (CPP) introduced by Israelachvili in 1985 defines the vesicle forming properties of surfactants and is calculated using Equation 2.1 (Figure 2.3), [19].

$$CPP = \frac{v}{lca_0} \quad (2.1)$$

v: Hydrophobic group volume

l_c: Critical hydrophobic group length

a₀: Area of hydrophilic head group

CPP value;

- Between 0, 5-1 tends to form a surfactant vesicle.
 - If <0.5 (where the area of the hydrophilic head group is more), global micelles are formed.
 - If > 1 (where the hydrophobic group volume is greater), reverse micelles are formed.
- Auxiliary Substances Added to the Niosome Structure

The most commonly used excipient in the niosome structure is cholesterol. The double layered membrane of the vesicle is properly ordered and can be gel or liquid crystalline (lamellar phase). The main difference between gel and liquid is the order of surfactant. Gel structure is a more uniform, while the liquid crystal structure is less uniform. While the liquid crystalline double-layer material diffuses sideways, gel chains in the gel structure crystallize or are less mobile. Liquid crystal structure occurs at higher temperatures than gel structure. As the temperature increases in the system, it increases the entropy by increasing the enthalpy time and reduces the free energy and provides phase transition [19].

Cholesterol prevents the transition from gel phase to liquid phase and prevents the active substance removal from niosomes. Cholesterol is usually inserted to the

formulations as a surfactant / cholesterol in a 1: 1 ratio. Even if cholesterol is added, the phase transition property of the surfactant that forms the vesicle affects its dispersion properties. Dispersion properties include membrane permeability, encapsulation efficiency, and hardness of the double layer, rehydrationability and toxicity of freeze-dried niosomes. Generally, niosomes need to be stabilized by adding a charged substance such as stearyl amine or disethyl phosphate.

- **Characteristics of the Encapsulated Active Substance**

A homogeneous dispersion may not be obtained for both substances if two active substances of hydrophilic or hydrophobic properties are loaded into niosome formulations of the same structure. The properties of the active substance loaded are important in terms of being able to be loaded into the niosome. The active substances in the hydrophobic structure are located between the alkyl chains and the active substances in the hydrophilic core [21].

- **Surfactant and Lipid Content**

During the preparation of niosomes, the amount of surfactants and lipids in the organic solution are generally equal and 10-30 mM (1-2.5%). Changing the surfactant in the hydration stage, water ratio changes the microstructure and therefore the properties of the system changes. Increase in the surfactant / lipid ratio enhances the total amount of active encapsulated substance [24].

- **Hydration Temperature**

Hydration temperature should generally be above the gel-liquid phase transition temperature in order to obtain proper niosomes and thus increasing encapsulation efficiency of drugs [21].

2.2.2. Niosome Preparation Methods

While preparing the niosomes, energy must enter the system. The basic principle in niosome preparation methods is to hydrate the surfactant / lipid mixture at high temperature and then optionally reduce the size of the vesicles in order to

obtain colloidal dispersion [25]. Preparation methods for niosomes are listed below

- Ether injection method
- Hand shaking method / thin film hydration method
- Micro fluidization
- Multiple membrane extrusion method
- Reverse phase evaporation technique
- Sonication
- Transmembrane pH gradient drug uptake
- The bubble method
- Formation from pro-niosomes

- Ether Injection Method

The surfactant / cholesterol mixture is dissolved in diethyl ether and slowly injected into the aqueous phase at 60 °C with the aid of a needle. As the ether evaporates, large MLVs are formed. By changing the injection and mixing speed, niosomes with different size and homogeneity can be obtained. In this method, petroleum ether, ethylmethyl ether or dichloromethane can be used instead of ether or can be used in combination with methanol. Small and uniform vesicles with high loading efficiency can be obtained with this method. The main disadvantage of this method is that the ether is generally not fully removed from the medium. As a result, disruptions can occur in the structure of substances loaded into the vesicle structure, such as proteins [26].

- Thin Film Hydration Method

The surfactant / cholesterol mixture is dissolved in a round bottom flask with an organic solvent such as chloroform, methanol or diethyl ether. Organic solvent is evaporated in a rotavapor by applying vacuum. Dry surfactant film is mixed with the aqueous phase at 50-60 °C [27] (Figure 2.4). This method is also known as the hand shaking method. It is used in the preparation of multi-layer vesicles (MLV). The vesicles prepared are large in diameter and their capacity to load water-soluble active substances is rather low [4], [27].

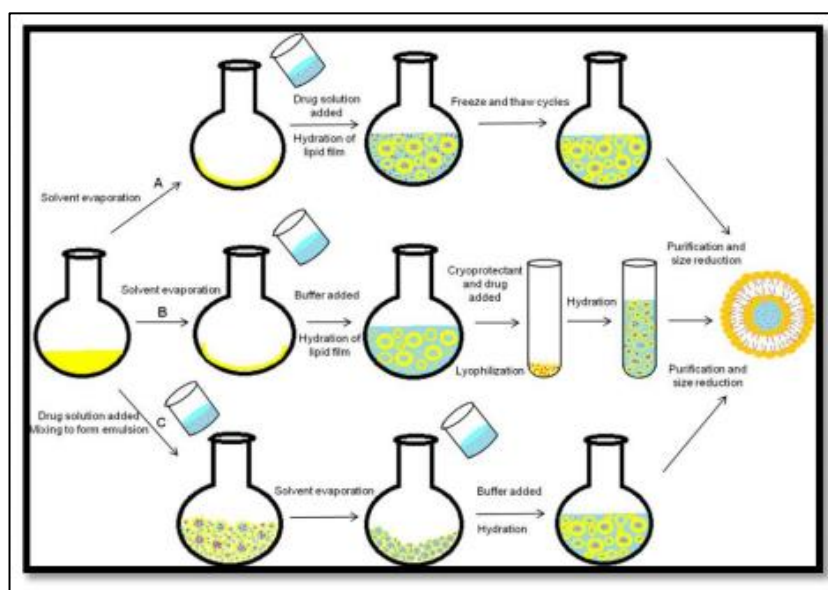


Figure 2.4: Hydrophilic drug encapsulation by thin film layer hydration.

- Microfluidization

Microfluidization is a high pressure (5000-30000 psi) homogenization technique. Microfluidization is intended to prepare vesicles with a particular particle size distribution. The basis of the method lies in contacting two fluids at very high pressure [28].

- Multiple Membrane Extrusion Method

It is a suitable method to prepare vesicles of the desired particle size. Polycarbonate membranes are based on placing them in series up to 8 passages. Thin film is made from the mixture of surfactant, cholesterol and dicetyl phosphate by evaporation method. This thin film is then rehydrated. For rehydration, an aqueous solution containing the drug is used. Finally, the present solution is extruded through the poly carbonate membrane (0.1 μm nucleofor) at desired passages [18].

- Reverse Phase Evaporation Technique

The surfactant / cholesterol mixture is dissolved with chloroform. The aqueous phase in which the active substance is dissolved is added to this mixture, and after the two phases are sonicated at 4-5 $^{\circ}\text{C}$, chloroform is removed under reduced

pressure. The mixture forms a gel, which is then hydrated to form vesicles. With this method, it can be ensured that the active substances are loaded into large MLVs. A disadvantage of this method is that it causes denaturation of macromolecules [4], [16].

- **Sonication**

The aqueous phase is added on the surfactant-cholesterol solution in the glass vial. The mixture is mixed with the probe sonicator for a certain period of time. The vesicles obtained are small and monolayer (SUV). Drug loading efficiency is lower compared to ether injection method [29].

- **Transmembrane pH Gradient Drug Uptake**

In this method, niosomes are prepared by use of a factor that allows retention in the vesicle, such as the presence of ammonium sulfate or pH difference in the membrane of the vesicle. It is ensured that the active substances in the amine structure can be loaded into the niosomes using ammonium sulphate. Ammonium sulfate loaded niosomes are obtained by sonicating the dry surfactant / lipid dispersion with ammonium sulfate solution or hydrating the surfactant / lipid film with ammonium sulfate solution. High loading efficiency can be achieved with this method and this is a suitable method for loading low / high molecular weight active substances [19].

In addition to the use of ammonium sulfate, the active substance can be trapped in the vesicles by using a pH difference. Other researchers using the similar method have obtained a film by dissolving the surfactant / cholesterol mixture in the organic solvent and blowing the organic solvent under low pressure. They hydrated this film with citric acid using a vortex process and prepared the vesicles. The MLVs obtained were thawed. Then, the aqueous phase was added and vortexed. The pH was raised to 7.0 by the addition of disodium hydrogen phosphate. The resulting suspension was heated to 60 °C in 10 minutes and niosomes were formed [4], [19].

- **The Bubble Method**

This technique was developed to prepare liposomes and niosomes without

using organic solvent and high shear forces. Some researchers have created stable liposome / niosome dispersions by treating inert gas bubbles with unhydrated (phospho) lipids or nonionic surfactant / cholesterol mixtures [19]. The particle size in the dispersions was between 0.2-0.5 μ m. In the experimental setup, the 3 necked round bottom balloon is placed in a water bath with temperature control. A water-cooled flow column was placed around the neck in the middle of the balloon. Temperature control is provided by placing a thermometer in another neck. Nitrogen gas is given from the 3rd inlet. With this preparation technique, liposomes / niosomes can be produced in one step and using only one carrier container [29].

- **Formation of Niosomes from Pro-Niosomes**

Another method of preparation for niosomes is the coating of a water-soluble carrier such as sorbitol with surfactant. As a result of the coating, a formulation is obtained in the form of dry powder. Each water-soluble carrier particle is covered with a dry surfactant film. These formulations are called pro-niosomes. Niosomes are obtained by adding the aqueous phase to the proniosomes above the average phase transition temperature and mixing the system [4], [18].

2.2.3. Methods to Remove Active Substance That are Free in Niosome Solution

As a result of hydration of the surfactant / lipid films, encapsulation of all the active substance is generally not possible. In order to ensure the optimization of production, the active substance that cannot be loaded must be removed. In the case of half of the active substance is encapsulated and half free in niosome solution, biphasic biodistribution can be achieved. In this case, the starting dose of the treatment is rapidly removed and then the maintenance dose is released [9], [18]. The techniques used to remove the active substance that is not loaded into the niosomes are given below

- **Dialysis**

Aqueous niosomal dispersion is placed on dialysis membrane and dialyzed in

solution such as phosphate buffer, saline or glucose solution. This is a suitable method for removing active substances dissolved in water [19].

- Gel Filtration

Gel filtration of the niosomal dispersion is performed using Sephadex columns. Free active substance and niosomes are separated by phosphate buffer or saline column as mobile phase [9].

- Centrifugation

The niosomal suspension is centrifuged at a speed below 7000 g and precipitated. The precipitate is washed and resuspended with buffer. Thus niosomal suspension which is free of active substance is contained [18].

- Ultracentrifugation

This method is used to precipitate niosomes with particle size below 1 μm which cannot be deposited by centrifuge. The niosome precipitate obtained by applying centrifugation at approximately 150 000 g for 90 minutes is re-suspended and used [18].

Advantages and disadvantages of the methods for removing free active substance in niosome solution are given in Table 2.2. Considering the industrial size production, the step of separating the active substance that is not loaded by increasing the loading efficiency as much as possible can be completely removed from production; alternatively formulations for providing the first dose of the substance that cannot be loaded as mentioned before can be developed. However, the consistency of the amount of active substance retained should be determined [19].

Table 2.2: Comparison of the techniques used to remove the active substance not loaded into the niosomes.

Decomposition Method	Advantages	Disadvantages
Dialysis	• Suitable for below 10 μm vesicles. • It is suitable for high viscosity systems. • Cheap.	• The process is completed in a very long time (5-24 hours). • The dialyzed suspension should be in large volume. (It may not be suitable for substances that need to be disposed of in a special way.) • Causes dilution of niosome dispersion because of requirement of high dialysis volume. • The formula should be concentrated by ultrafiltration again.
Centrifuge (< 7000g)	• It is a fast method (30 minutes). • Cheap. • It allows to obtain concentrated niosome suspension.	• It cannot cause the niosomes under micron to collapse. • It can cause fragile systems to fail.
Ultracentrifugation (~ 150 000g)	• It allows niosomes to be collapsed above and below the micron. • It allows the niosome suspension to be concentrated.	• Ultracentrifuge device is expensive. • The centrifuge time is long (1-1.5 hours). • It can cause fragile systems to fail. • It may cause aggregate formation.

2.2.4. Particle Size Reduction Methods in Niosomes

Niosomes are usually micron in size when they are first prepared. Particle size plays an important role in the biodistribution of vesicles. For example, phospholipid vesicles below 200 nm are affected in the liver, but not in the spleen. Due to the importance of the particle size, the step of reducing the particle size is also added to the preparation procedures of the niosomes [1]. There are several methods to reduce the particle sizes of niosomes

- Sonication (Probe sonication, water bath sonication)
- Filtration
- Combined use of sonication and filtration
- Microfluidisation

In this study, both probe sonication and water bath sonication were tried as particle reduction techniques. In the probe sonication technique, the temperature increased excessively that causing the niosome and protein structure to deteriorate. On the contrary, water bath sonication was not increase temperature. The water bath sonication technique was effective in particle reduction and not deteriorate the niosome and protein structure. At the same time, due to the trimodal structure of the niosome, filtration was applied to obtain a suitable particle size after sonication.

2.2.5. Characterization Methods for Niosomes

- Determination of Active Substance Loading Effectiveness to Niosomes

In order to determine the active substance loaded to niosomes, it is necessary to disrupt the niosome structure and ensure that the active substance loaded is revealed. Isopropyl alcohol, a minimum amount of Triton X-100 and absolute ethanol can be used to break down the niosome structure. After the addition of the niosome disintegrating agent to the suspended formulation, a mixing process such as sonication can be applied in order to obtain a clear solution [19]. In some studies,

active substance loaded niosomes are precipitated by ultracentrifuge or centrifuge. The amount of active substance loaded is calculated by determining the amount of active substance that cannot be loaded to the niosomes by measuring the active substance amount in the supernatant [30].

- **Determination of Particle Size and Distribution of Niosomes**

Particle sizes of niosomes can vary over a wide range depending on the method of preparation. Micro and nano-sized niosomes can be prepared. Depending on the size of the niosomes, particle size measurement methods also vary. Laser light diffraction method is used for micro dimensions. Photon correlation spectroscopy (PCS), or dynamic light scattering (DLS) method, and multi-angle laser light scattering method are used for nano-dimensions [22]. Particle size and distribution of niosomes can be examined with various imaging methods. While optical microscopy is used for micro-sized niosomes, imaging-permeable electron microscopy (Transmission electron microscope-TEM), Cryo-TEM, scanning electron microscope (SEM) and atomic force microscope (Atomic force microscope-AFM) can be used in nano-sized niosomes [20].

- **Zeta Potential Determination of Niosomes**

Zeta potential indicates the electrostatic charge of the particle surface in a suspension. High zeta potential indicates high surface charge and high electrostatic repulsion between particles, thereby resisting aggregation.

Phase analysis of scattering (PALS) technique is performed by determining the zeta potentials of niosomes. In this technique, zeta potentials are measured by applying electric fields to the dispersions. The charged molecules in the dispersion proceed at a rate commensurate with the magnitude of their zeta potentials against the opposite charged electrode. The speed of molecules is measured with laser Doppler anemometry. The frequency shift or phase shift occurring in the laser beam due to this movement of the particles is measured as particle mobility. This mobility is calculated by Smoluchowski or Huckel theories, taking into account the viscosity of the dispersion medium. Since it is difficult to directly measure the charge of the particles, zeta potential is determined by measuring the speed of the particles in the

indirectly applied electrical field [20].

- Stability of Niosomes

A stable niosome dispersion should have a fixed particle size, and the active substance it encapsulates should be compatible with the niosome material, and should not leak over time. There should be no collapse in membrane components that do not have water solubility. Niosomes should not show vesicular aggregation, fusion and swelling. Ideally, niosomes should be kept in dry powder and prepared by treating them with water when is used. In such a case, the hydrated dispersion is expected to have the same properties as the original dispersion [17]. There are 4 different forces that are basically effective on the stability of vesicles. These forces can be listed as follows

- Van der Waals forces between surfactant molecules,
- Driving forces resulting from electrostatic interactions between charged groups in the structure of surfactant molecules,
- Entropic driving forces originating from the head groups of surfactants,
- Short acting driving forces [16].
- Electrostatic propulsion forces occur with the addition of charged surfactants to the double layer and increase system stability [16].

The Stability of Niosomes Can Be Analyzed Mainly Under 3 Sections

- Physical Stability
- Chemical Stability
- Biological Stability

Physical stability problems may occur in niosomes that are increased particle size, aggregation and sedimentation of niosomes, leakage of encapsulated active substance from niosomes. Depending on the osmotic pressure difference, the swelling or shrinkage of the vesicles and the change of the vesicle shape are also observed stability problems [7], [8].

Niosomes contain non-ionic surfactants that are chemically more stable than

liposomes with phospholipids in their structure. Phospholipids often result stability problems such as lipid peroxidation and hydrolysis. Less stability problem has been observed in niosomes due to lipid peroxidation [7].

In biological stability studies, the stability of small monolayer vesicles in plasma was found to be better than in other forms. It has been found that doxorubicin-loaded Span 60 niosomes conjugated with plasma copolymer remain stable in the plasma for a long time without releasing the active substance encapsulated [31]. Some researchers have shown that dyestuff-laden niosomes incubated with human serum at 37 °C and neutral pH only release the dyestuff as low as 5%, and thus are stable [18].

Different factors are known to affect niosome stability as discussed. Firstly, since surfactants used in the preparation of niosomes determine the structure of the membrane, they directly affect its stability. The release of the encapsulated active substance through the vesicles over time depends on the fluidity of the membrane and the addition of cholesterol to the formulation generally reduces this leakage [17], [18]. In addition, detergents (soluble surfactants) in high concentrations are incompatible with niosomal systems. It may cause niosomes to dissolve and form mixed micelles and aggregates [5].

Secondly, the active substance held in the niosome also plays an important role in the stability of the system. As a result of conjugating the active substance with a polymer, it was observed that the stability of the system also increased as a macromolecule was formed that would not pass through the niosome membrane. Depending on the chemical properties of the active substance, the active substance can be loaded on the niosomes using ammonium sulfate. In this method, since the active substance forms a gel in the vesicle, the stability of the system against leakage from the membrane increases [6], [31].

Thirdly, storage temperature of the dispersions also affects niosome stability and therefore should be kept under control. Changes in temperature may cause changes in the basic characteristics of the system and cause the active substance to be released. These properties of niosomes can also be used to create thermoresponsive nanocarriers [5], [31].

Lastly, vesicular nanocarriers consisting of double layers such as niosomes are

not considered thermodynamically stable. The vesicle is thought to be in a metastable state with excessive energy. As a result, it is predicted that these particles turn into double layer piles over time. In order to obtain highly stable vesicles, this conversion should be as slow as possible and an appropriate shelf life should be ensured [31], [32].

2.2.6. Analysis of Active Substance Release from Niosomes

Two methods are generally used in determining the effluent discharge from niosomes. According to the diffusion technique from the membrane, the niosome formulation placed on the dialysis membrane is placed in the release medium and mixed and samples are taken from the external environment at certain intervals. In another method, niosomes diluted with release medium are taken into small test tubes and incubated in shaker. One tube is taken for each sample time and centrifuged and the amount of active substance left in the precipitate is determined [5].

2.2.7. Cytotoxicity of Niosomes

The number of studies examining the toxicity of niosomes is very limited. Non-ionic surfactants such as Span 60, 80 and Tween 60, 65, 80, which are generally in the niosome structure, are food additives approved by the American Medicines and Food Authority (FDA) [7]. Therefore, they are safe in terms of toxicity. When the effect of different surfactant types on toxicity is examined, the surfactants in the ester structure were found to be less toxic than those in the ether structure. This is because the ester bonds undergo enzymatic degradation [33].

2.2.8. Use of Niosomes in Pharmaceutical Field

Various research groups have investigated the effectiveness of vesicles by applying niosome formulations by loading different active substances in various ways such as oral, parenteral, topical, vaginal, and pulmonary [8].

2.2.8.1. Oral Administration of Niosomes

Oral bioavailability of orally administered active ingredients may be limited for some reason. These reasons are as follows; low solubility of the active substance in the gastrointestinal (GI) system, metabolism of cytochrome enzymes in the intestinal wall, release back into the intestinal lumen with a P-gp pump, inability to absorb and degradation by the cytochrome enzymes in the liver.

In order for an active substance administered orally to be absorbed, it must dissolve after it is dispersed in the GI system, come to the region where it is absorbed without being disintegrated (stomach, intestine) and pass into the blood. Particulate drug delivery systems (ITS) can be used to increase the bioavailability of active substances with limited oral bioavailability [18]. ITS can encapsulate active substances of different solubility by increasing their stability and absorption from the intestine in the GI system [34].

Due to the difference in physiological conditions in the GI system, particulate systems must meet some criteria. First of all, they need to be resistant to degradation in the GI system and thus be able to protect the active substances they contain from degradation. Second, in order for the active ingredients in the particles to be therapeutically effective, the particles must be effectively absorbed in the GI system. Many studies for today show that only 2-3% of the received dose of submicron particles can be absorbed [18]. Three possible ways are identified

- Paracellular transition: It is a transition made from tight junction between epithelial cells.
- Transcellular transition: It involves the passage through epithelial cells, namely enterocytes. This transition occurs by passive diffusion into the cells, endocytosis and exit in the basolateral part (transcytosis). Carriers and pumps may also be included in a transport process that requires passive diffusion to the basolateral portion following removal from the apical portion.
- Transition through M-cells: After the particles in the intestinal lumen settle on the apical surfaces of the M-cells in the intestinal structure, these cells are removed by transcytosis and enter the mesenteric lymph ducts.

Factors to Consider Before Intestinal Absorption of Particles Are

- Physical stability of carriers in the GI system,
- Chemical stability of the carrier and active substance,
- GI system transition times,
- Time of stay in the area where the particles are taken,
- Interactions with GI content,
- Transition from mucus,
- Adhesion to epithelial surfaces,
- Stimulus for cellular uptake,
- The diameter of the particles to be absorbed from the GI system [small colloidal-submicron-particles has been shown to be absorbed from enterocytes by transcellular pathway (endocytosis) while larger-several micron-particles are absorbed through M-cells [19], [34].
- Structure and surface properties of particles.

2.2.8.2. Parenteral Applications of Niosomes

Studies on parenteral administration of niosomes are particularly focused on anti-cancer active substances. The main purpose of these studies is to target anticancer active substances directly to tumor cells in order to prevent their side effects on healthy tissues. Niosome formulations have been prepared in order to increase the circulation times of the active substances in the blood [5], [31].

2.2.8.3. Topical Applications of Niosomes

The stratum corneum layer of the epidermis in the structure of the skin prevents percutaneous absorption of many drugs. Patch technology is used in the vast majority of transdermal therapeutic systems (TTS). However, application in this way is suitable for active substances with a molecular weight of less than 500 Dal. Topical application of molecules larger than 500 Dal is more difficult. This problem is tried to be overcome by using complex devices such as particle guns, iontophoretic

devices. In addition, studies are carried out to increase the penetration of active substances from the skin by using drug carrier systems such as lipid-based carrier systems [35]. Although liposomes come to the fore in this area, the use of niosomes has been on the agenda long ago and has developed in parallel with liposomes [5].

Niosomes increase the residence time of the active substance they trap in the stratum corneum and epidermis and penetration of the active substances. They reduce the side effect by releasing the active substance in a controlled manner. The mechanism by which niosome-forming surfactants increase skin penetration. The researchers stated that surfactants are partitioned in the stratum corneum layer and increase penetration by increasing the fluidity of the intracellular fluid matrix structure or by increasing interaction with keratin filaments.

The first use of niosomes has been to topically moisturize and soften the upper layers of the skin for cosmetic purposes. There are several cosmetic niosome products in the market. However, these products are no longer in the market due to the stability problem.

The mechanism of the transition of niosomes to the stratum corneum layer is not fully known. It has been suggested that the vesicles penetrate into the stratum corneum without disintegration or diffuse molecularly dispersed surfactant molecules to form vesicles by mixing with the intracellular lipid matrix. The fact that the niosome is in the gel or liquid crystal phase affects its absorption from the skin. It has been found that a small amount of niosomes in the gel phase pass into the living layers of the epidermis, the amount in the dermis increases after prolonged incubation, but the amount of passage is always less than in the liquid phase vesicles [5].

2.2.8.4. Ocular Applications of Niosomes

The use of a vesicular drug carrier in ophthalmic applications ensures the controlled and long-term release of the active substance on the surface of the cornea. It also prevents the active substances from being metabolised by enzymes on the surface of tears or cornea [36]. The partition and transition to the cornea of the active substance trapped in the niosomes is better. It enables the active substance to show

its effect locally in the effect area [5].

2.2.8.5. Vaginal Applications of Niosomes

The active substance in many peptide-protein structures is administered parenterally due to its low oral bioavailability. The vaginal route is an advantageous way of applying drugs especially in the treatment of gynecological diseases due to its large surface area, rich blood circulation and high permeability. Vaginal bioavailability of peptide-proteins is limited due to low absorption from the vaginal epithelium and destruction by vaginal enzymes. Researchers have conducted studies on niosomal drug delivery to increase the bioavailability of active substances [5].

2.3. Bovine Serum Albumin (BSA)

Bovine Serum Albumin (BSA) was preferred as the material for the particle matrix. BSA is non-toxic and can be used as a nanocarrier in food and drug applications. In addition, BSA can be degraded in vivo, and nanoparticles can be easily adapted to the human body. There are preliminary studies try to encapsulate BSA in niosomes [28], [37]. The reason for the use of this protein in encapsulation is due to its similarity to Human Serum Albumin (HSA), a transport protein in human blood. Serum albumin is used as a typical drug carrier because it's unique properties. Albumin is biodegradable, biocompatible and stable. It also has low toxicity and does not show immunogenicity. The initial temperature for the BSA under construction conditions is 58 °C and the denaturation temperature is 62 °C. The amino acid sequence of human albumin has 76% similarity with bovine. BSA is a zwitterion and contains 5.82 isoelectrics, pH negatively charged at 7.4 [28], [38].

The average zeta potential of a BSA solution in ultrapure water at PH 6.1 is 28.9mV. BSA consists of only one polypeptide chain. It has a helical structure and a molecular mass of 66,430 Da. Both BSA and human albumin can bind and transport molecules such as metabolites, fatty acids, hormones, medicines in the blood [37].

BSA and HSA contain 3 homologous areas where each area has two

subdomains (A and B) [39]. BSA is an abundant protein and is often used for research instead of HSA because of its high stability. BSA turns out to be chemically inert in many biological and chemical reactions. It is also easy to purify BSA. It shows superior ligand binding properties. It can bind enormous amounts of ligands reversibly [40]. BSA is a beef blood protein, a by-product in the cattle industry. Therefore, BSA's cost is lower than HSA. One of the interesting features of BSA is that it undergoes reversible conformational changes when the pH drops from 7 to 2. BSA is a polyampholyte with a pH dependent charge. This feature provides reversible binding sites for loading and releasing drugs in the body [38].

2.4. Nerve Growth Factor (NGF)

Neurotrophic factors are defined as target-controlled, anti-apoptotic molecules that enable the maintenance of embryonic or adult neuronal cells. The word "trophic" is derived from the Greek "trophe", meaning food or food intake. Neurotrophism; an ability to invade and live in neural tissue. This term is usually used to describe the ability of viruses to infect nerve tissue. Today, it is thought that neurotrophins function in the development of the central nervous system in the embryo, as well as regulate the plasticity of the adult nervous system and maintain its structural integrity. NGF is a well known survival factor throughout the development of sympathetic and sensorial neurons. While most of the sympathetic neurons are NGF survival factors in adulthood, sensory neurons are not dependent on NGF as a survival factor in the postnatal period [41].

NGF, the prototype of neurotrophic factors, was discovered by Rita Levi-Montalcini in 1950 when she was investigating the stimulating properties of peripheral and sympathetic neurons in growth differentiation. A few decades after the discovery of NGF, a number of molecules with similar structures and functions have been discovered, all of which have been called neurotrophins (NTs) as polypeptide growth factor families. After, NGF was identified, the second NT to be identified was the Brain Derived Neurotrophic Factor (BDNF) [42]. Although BDNF is mainly located in the brain, its targets are in the central and peripheral nervous system and more than 50% of its molecular structure is identical to NGF. NGF

family includes, Neurotropic factor-3 (Neurotrophin factor-3, NT-3), Neurotropic factor-4 (Neurotrophin factor-4), then Neurotropic factor-6 (Neurotrophin factor-6, NT-6) and Neurotropic factor-7 (It consists of Neurotrophin factor-7, NT-7). Neurotropic factor-4 is also known as Neurotropic factor-5 (Neurotrophin factor-5, NT-5). Neurotropic factor-6 and Neurotropic factor-7 genes were detected only in fish and are probably not found in mammals and birds. NGF, BDNF, NT-3, NT-4 are all known to be essential for the development of the vertebrate nervous system [14], [41].

2.4.1. Nerve Growth Factor Structure

The adult male mouse submandibular gland is the standard source of isolation for NGF proteins. Two types of NGF proteins were isolated from this gland: low molecular weight 2, 5 S NGF (β NGF) and high molecular weight 7S NGF. The 2, 5 S NGF mediates all known biological functions of NGF. The activity of the 2, 5 S NGF has been demonstrated in tumor tissues and various serpent serums before the mouse submandibular gland source [42].

The NGF gene is located in the short arm of chromosome 1 in humans and encodes the precursor called pre-pro-NGF. NGF 2. 5 S purified form has a molecular weight of 26 kDa. Two peptide chains have been identified for the 2. 5 S NGF. Chain A consists of 118 amino acid residues. Chain B is very similar to chain A but lacks the first eight amino acid residues at the N terminal. The beta subunit is responsible for all known biological activities. NGF dimer shows a protein structure in which the two chains are noncovalently separated on a wide surface, are stable and in a form mediated by the effects of NGF at physiological concentrations. In the submandibular gland, the mouse protein is a combination of alpha (α), beta (β) and gamma (γ) polypeptides and 1-2 grams of zinc ions that contribute to stability. 7S NGF weighs 130-140 kDa and takes its name from the sedimentation coefficient. The 7S complex has not been shown in other mouse tissues or other species; it is thought that the probable reason for this is due to the kallikrein family, which has very limited release of alpha and gamma subunits [42]. Alpha and gamma subunits are from the serine protease family, although they are composed of amino acid

sequences that are very similar to each other, only gamma subunit is catalytically active. Based on other serine proteases, 3D models were developed and preliminary structures were created directly from x-ray crystallography. Gamma subunit interacts with the carboxyterminal sequence of the beta subunit through its catalytic center. It is assumed that the Gamma subunit separates the C-terminal structure from the beta subunit and forms the enzyme product complex (Figure 2.5) [41], [42].

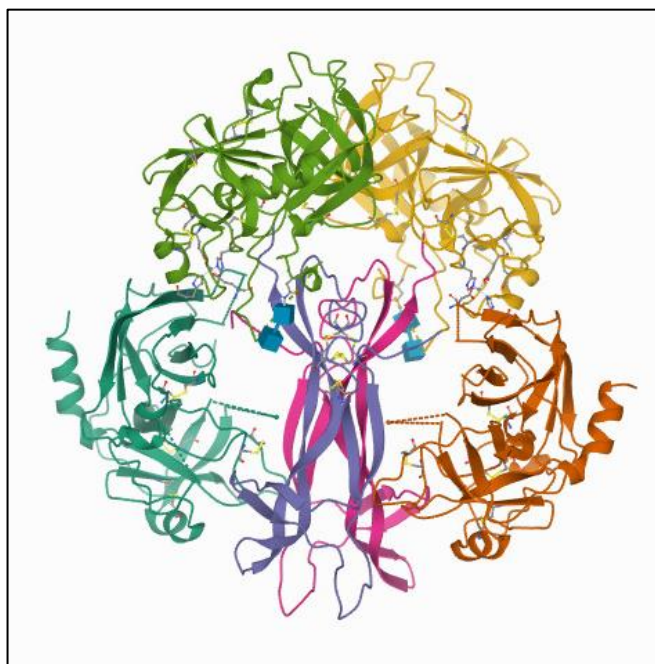


Figure 2.5: Crystal structure of 7S NGF: A complex of nerve growth factor with four binding proteins (Serine proteinases), (PDB code 1SGF).

2.4.2. Regulation of NGF Synthesis in the Brain

Under physiological conditions, NGF mRNA and NGF protein are synthesized in central nervous system (CNS) neurons; while the hippocampus is at high levels in the cerebral cortex and bulbous olfactory, it is relatively low in the corpus striatum and cerebellum. It is found in the highest level in the hippocampus. However, in rodents, hippocampal and cortical NGF protein levels were increased after fimbria-fornix transection. Increased cholinergic activity stimulates NGF synthesis. Hypoxic - ischemic damage, endotoxin administration, excitatory lesions also lead to changes in brain NGF levels. In addition, NGF levels have been reported to increase after

seizures and in the traumatic brain. Therefore, it is thought that the increase in NGF synthesis after CNS injury may be an endogenous protective mechanism to maintain neuronal survival in damaged brain regions. Therefore, introducing NGF in the early phase of acute CNS injuries such as stroke and trauma can prevent the spread of neuronal damage. Various drugs have been developed in order to increase the regulation of NGF synthesis. However, the action of NGF-inducing agents with different pathways in different cell types demonstrates the complexity of NGF regulation. In in vitro studies in the hypothalamus and cerebral cortex, KCl depolarization, kainic acid, glutamate and Ca^{2+} have been shown to increase NGF mRNA levels. On the other hand, glial cells can also synthesize NGF. The location of cellular localization of NGF synthesis under physiological and pathological conditions remains unclear [29], [42].

2.4.3. Neuroprotective Effects of NGF

It is assumed that neurotrophins act by target-controlled retrograde signals and regulate survival in the peripheral nervous system during development. The absence of sympathetic and cutaneous sensory neurons at the birth of a mutant mouse without functional NGF or TrkA NGF receptor genes supports this view. In accordance with the expression of TrkA in sympathetic neurons, cell death occurs in TrkA mutants, sympathetic ganglia. Following peripheral nerve damage, Schwann cells proliferate and differentiate at the distal of the damage to myelin and nonmyelin. Proliferating Schwann cells play an important role in axon regeneration and nerve repair. Reactive Schwann cells cause NGF and P75 NTR upregulation and exogenous NGF was shown to prevent Schwann cell migration [41], [43].

Contrary to the situation in the peripheral nervous system, the biological role of neurotrophins in the central nervous system is to regulate biochemical and morphological differentiation rather than regulating developmental survival. Although the differentiation of basal forebrain and cholinergic neurons of the Trk / NGF double mutant mouse was affected, survival of these neurons was not significantly affected in the perinatal period. Neurotrophins are found in the central nervous system at a high level in the hippocampus and cerebral cortex. NGF protects

cholinergic neurons in the forebrain from axotomy-induced neurodegeneration and atrophy. NGF infusion has been shown to alleviate behavioral deficits associated with cholinergic activity.

These data show that NGF has a protective effect on neurons in brain regions where cerebral ischemia and chronic neurodegenerative diseases such as Alzheimer's disease occur. Therefore, neurotropic factors are of particular interest as NGF neuroprotective agents. However, the therapeutic use of these compositions seems complicated due to their chemical properties. Since the neurotropic factors have large molecular weights, they cannot easily cross the blood-brain barrier and do not completely disperse after systemic administration. With the systemic application of NGF in a low molecular weight form, various studies are conducted to ensure the stimulation of its synthesis in the affected brain areas [29], [43].

In literature, NGF and some other growth factors have been preclinically used in models of Alzheimer's disease and traumatic brain injury, with affirmative effects on neurological deficits. Furthermore, in patients with Alzheimer's disease or traumatic brain injury, NGF levels correlated with neurological outcomes [44]. Recently, many researchers have studied to increase NGF transport to brain with nanoparticles. Our main purpose in this study is to increase the efficiency of NGF by loading it into the niosome.

3. MATERIALS and METHODS

3.1. Materials

- Cell Lines and Cell Culture Solutions

SH-SY5Y human neuroblastoma cell lines (ATCC number: CRL-2266) were used in cell culture experiments. Dulbecco's Modified Eagle Medium (DMEM) (1X) with 4,5 g/L Glucose, L-glutamine and pyruvate (Gibco, 41966-029), Penicilin/Streptomycin (10000U-ml Penicillin / 10mg/ml Streptomycin) (PAN Biotech, P06-07100) 0.05% Trypsin-EDTA Solution (1X) (Gibco, 25300-054), Dulbecco's Phosphate Buffered Saline (1X) (DPBS) (Gibco, 14190-94), Fetal Bovine Serum (FBS) (Gibco, 10270), Dimethylsulfoxide (DMSO).

- Chemical Materials and Equipments

Recombinant human NGF (Thermo Fisher, 11050HNAC25), BSA (Thermo Fisher, B14), FM4-64 (Thermo Fisher, T13320), BCA protein assay kit (Thermo Fisher, 23225), Span 20 (Sigma Aldrich, 1338-39-2), Span 60 (Sigma Aldrich, 1338-41-6), Span 80 (Sigma Aldrich, 1338-43-8), T25, T75, T150 tissue culture flasks (TRP), 96- well plate (NUNC), 100 mm, 150 mm tissue culture plate (TRP), petri dish (100 mm) (Lamtek), 5 ml, 10 ml ,25 ml, 50 ml serological pipettes (CAPP), micro pipettes (Eppendorf), 15 ml and 50 ml Falcon (CAPP), microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml), cryotubes (1ml, 2ml), hemocytometer (Marienfeld), Vortex (JeioTech), UV Spektrofotometre (Schimadzu 1202 UV Visible), Zetasizer Nano ZS (Malvern Instruments), Rotary Evaporator, Water Bath (JeioTech), Fluorescence microscope (Leica-DMi1), Mikroskop (Leica DM 4000B).

3.2. Methods

3.2.1. Optimization of Niosome Preparation Method

Preformulation studies were carried out without use of NGF in order to see the formation of vesicles and to select the appropriate parameters to be used in the preparation of the formulation before the NGF loading stage in the niosomes. BSA used as a model protein for loading to niosomes and physicochemical and biophysical characterization were performed.

The preparation of BSA-loaded niosomes is schematically shown in Figure 3.1. Niosomes were prepared based on the reverse-phase evaporation method. Briefly, cholesterol and surfactant were dissolved in diethyl ether in the determined proportions. BSA was dissolved in distilled water (1 mg/ml). In the next step, these solutions were added to a flask and then vortexed for 60 seconds. The mixture collected in the bottle was emulsified with water bath sonicator at 10 °C for 5 minutes. As a final step, the emulsion was rotated at 40 °C with a rotation speed of 60 rpm to remove the organic solvent in rotary evaporator [45].

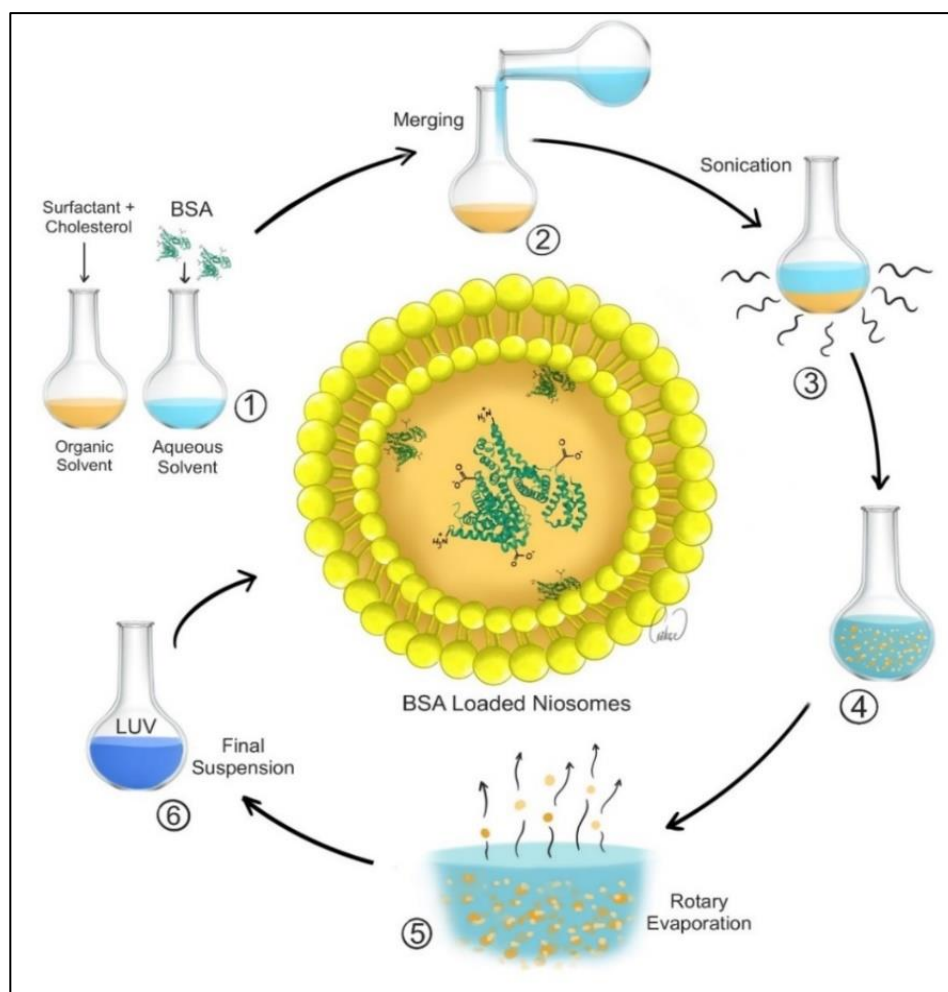


Figure 3.1: Schematic illustration of preparation method of niosome.

3.2.1.1. Preparation and Characterization of BSA Loaded Niosome Formulation Using Different Surfactants

In order to examine the effects of surfactant structure on the physicochemical properties of niosomes, by using Span 20, Span 60, Span 80, niosomes were prepared with equal amounts of lipid by reverse phase method. In our study, the surfactant-cholesterol ratio was selected as 20 mM: 20 mM. The composition of the niosomal formulations prepared by different surfactants is given in Table 3.1.

Table 3.1: Composition of niosomal formulations prepared by different surfactants.

	Formulations	Formulation 1	Formulation 2	Formulation 3
Organic Phase	Span 60	20 mM 34.448 g	-	-
	Span 20	-	20 mM 27.72 g	-
	Span 80	-	-	20 mM 34.28 g
	Cholesterol	20 mM 30.93 g	20 mM 30.93 g	20 mM 30.93 g
	Diethyl ether	5 ml	5 ml	5 ml
Water Phase	BSA	4 mg	4 mg	4 mg
	Distilled Water	4 ml	4 ml	4 ml

Niosomes loaded with BSA were prepared with reverse-phase evaporation method. In order to separate the free BSA, from the environment, centrifugation was applied at 16 000 g for 50 minutes at 4 °C. The niosome precipitate obtained was dispersed in water.

3.2.1.1.1. Determination of Entrapment Efficiency

The encapsulation efficiency of BSA is expressed as the proportion between the BSA concentration in niosomes and the total BSA concentration used for niosome preparation. The obtained niosome suspension contains BSA-loaded niosomes in the pellet portion, while BSA contains BSA in free form in the supernatant. In order to separate the free BSA, we used centrifugation at 16,000×g, 4 °C, and 50 min. The BCA protein assay kit was used to determine the concentration of free BSA in the supernatant. The encapsulation efficiency of niosomes calculated by Equation 3.1.

$$(\text{EE } \%) = \frac{WT-WF}{WT} \times 100 \quad (3.1)$$

WT: Initial amount of BSA used in the preparation of niosomes (mg).

WF: The amount of free BSA present in the supernatant (mg).

3.2.1.1.2. Measurement of Particle Size of Niosomes

Particle size distribution and polydispersity index of niosomal suspensions were determined by using zetasizer. Analyzes were performed with He-Ne laser (633 nm) at 25 °C using a scattering angle of 175°. 40 µL of the niosome suspension was distilled and sterilized water to 4 ml and filtered through a 0.2 µm filter. Particle size measurement was repeated at least 3 times for each sample.

3.2.1.2. Determination of Reproducibility of Formulation F1

The F1 formulation with high entrapment efficiency was selected from 3 formulations (F1-F3) prepared using different surfactant types. In order to determine the reproducibility of the production method used in the study, the F1 formulation was produced many times using the same production method and production parameters. In the result, the formulations prepared by analyzing the entrapment efficiency and particle size were compared.

3.2.1.3. Development of Niosomal Formulations Using Different Span 60 and Cholesterol Amounts

In order to observe the effect of different molar concentrations of the substances in the formulation based on the F1 formulation prepared with Span 60, the formulations given the compositions in Table 3.2 were created. BSA loaded niosomes were prepared using reverse phase evaporation method as described in Section 3.2.1.

Table 3.2: BSA loaded niosomes components prepared with different cholesterol percentages (S: Codes of formulations that are prepared for different surfactant/CH amounts).

	Materials	F1-S1 (20 % CH)	F1-S2 (40 % CH)	F1-S3 (50 % CH)	F1-S4 (60 % CH)	F1-S5 (80 % CH)
Organic Phase	Span 60	6 μ M 11.024 mg	4.8 μ M 8.268 mg	6.25 μ M 10.77 mg	3.2 μ M 5.512 mg	1.6 μ M 2.756 mg
	Cholesterol	1.6 μ M 2.472 mg	3.2 μ M 4.948 mg	6.25 μ M 9.67 mg	4.8 μ M 7.424 mg	6.4 μ M 9.896 mg
	Diethyl Ether	5 ml	5 ml	5 ml	5 ml	5 ml
Water Phase	BSA	4 mg	4 mg	4 mg	4 mg	4 mg
	Pure Water (dH ₂ O)	4 ml	4 ml	4 ml	4 ml	4 ml

3.2.1.4. Particle Size Reduction of Niosomes

The niosomes obtained in our study have been undergone for further particle reduction process. For this purpose, niosomes were prepared in the F1S1 formulation and size reduction was performed with 2 different methods to select the most appropriate agitation method. The formulations given in Table 3.3 were prepared by sonication of the probe sonicator and water bath sonicator shown in the table for different durations. Sonication application time was determined by analyzing the particle size of the obtained niosomes. The shapes of the vesicles obtained were examined with an optical microscope. Thus, the most appropriate agitation method to be used in the hydration stage has been selected.

Table 3.3: Optimization of niosome size with different reduction techniques and durations (P: Codes of formulations that are sonicated for different durations).

Formulations	P1	P2	P3	P4	P5
Probe sonication time (min)	-	-		3	5
Water bath sonication time (min)	5	10	15	-	-

As a result of optimization experiments, the F1-S3 formulations prepared with 6.25 mM: 6.25mM Span 60: CH ratio of the formulation with the highest encapsulation efficiency and optimum nanoparticle size was found to be the most suitable. In addition, water bath sonication was chosen as the most appropriate agitation method and the most suitable time was decided to be 15 minutes (P3). Therefore, the focus will be on F1S3 formulation and P3 sonication in other stages of the study.

3.2.2. Characterization of Niosomes

3.2.2.1. BSA Standard Curve

In order to obtain the conversion formulation from the BSA OD concentration standard curve, five samples with the determined BSA concentrations were prepared and their absorbances were measured. BCA protein assay kit was used to determine the concentration of BSA.

3.2.2.2. Investigation of the Release of BSA in Final Formulations

BSA-loaded niosomes were separated from free BSA contained in the supernatant (centrifuged 16,000 x g, 50 min, + 4 °C) prior to release studies and

pellets were redispersed in diluted water. Samples were incubated at 25 °C. The released BSA was measured at 1, 2, 24, 48, 72, 96, 120, 144, 168 hours. At each measurement, samples were centrifuged (16,000 x g, 50 minutes, +4 °C) and the amount of free protein in the supernatant was measured with the BCA protein assay kit.

3.2.2.3. Particle Size of F1-S3 Niosomes

BSA loaded and blank niosomes were prepared with the F1-S3 formulation, which was the most appropriate. Analyzes were performed with He-Ne laser (633 nm) at 25 °C using a scattering angle of 175 ° using Zeta Sizer. 40 µL of the niosome suspension was diluted with pure water to 4 ml and passed through a 0.2 µm filter.

3.2.2.4. Thermal Stability of Niosomes

The newly prepared BSA loaded niosomes were first calculated with the encapsulation efficiency, then incubated at two storage temperatures of 4 and 25 °C at RH. After the samples are centrifuged (16,000 x g, 50 minutes), the amount of protein in the supernatant part is measured at 24 and 72 h with the BCA protein assay kit.

3.2.2.5. Morphology Analysis

In order to confirm the findings obtained for the size of niosomes, the morphology of niosomes was visualized using a scanning electron microscope (SEM).

3.2.2.6. Fluorescence Images of Niosomes by FM4-64

BSA loaded niosomes were incubated with FM4-64 which is a fluorescent probe as membrane marker. After 30 min incubation at dark, fluorescence images of

niosomes visualized with fluorescence inverted microscope.

3.2.2.7. Cell Viability Assay of Blank Niosome

The cytotoxicity of blank niosomes in the human neuroblastoma SH-SY5Y cell line after incubation was assessed with XTT assay. Since the niosomes are contained in dH₂O, a blank was created for each of them by blindly administering the same amount of dH₂O to the cells.

3.2.3. Preparation and Characterization of BSA and NGF Loaded Niosomes

3.2.3.1. Preparation of BSA and NGF Loaded Niosome

For preparation of BSA and NGF loaded niosomes, reverse-phase evaporation procedure that schematically illustrated in Figure 3.1, was used. In optimization studies, the BSA and NGF loaded niosome characterization and formulation has been explained. These characteristic features composed of function of CH content and protein entrapment efficiency. In this study, 500 ng of NGF and 4 mg of BSA dissolved in deionized water in a flask. In another flask, 5 ml of diethyl ether was used as dissolvent for Span 60 and CH. After that, 50% concentrated CH was added and mixed to dissolve. And then, both solutions were mixed in another flask and swirled for 60 second. Afterthat, the present mixture was emulsified under sonication in a water bath at 10 °C for 5 min. Next step was removing the diethyl ether solvent. For this step, the mixture was rotary evaporated at 40 °C with a rotating speed of 60 rpm. The last step, the mixture was centrifugated at 16 000 g for 50 minutes at 4 °C.

3.2.3.2. Determination of Entrapment Efficiency of BSA and NGF Loaded Niosome

The centrifugation at 16,000 × g for 50 minutes at 4 °C and Equation 3.1 were used for the entrapment efficiency (EE%) calculation of unloaded BSA and NGF

mixture. Spectrophotometer was the determination device for BSA and NGF concentration.

3.2.3.3. The Study of Protein Release from Niosome System of BSA and NGF Loaded Niosome

BSA and NGF loaded niosomes were separated from unencapsulated free protein contained in the supernatant (centrifuged 16,000 x g, 50 min, + 4 °C) prior to release studies. Pellets were resuspended in distilled water. Samples were incubated at 25 °C. The released protein was measured at 24, 48, 120 hours. At each measurement, samples were centrifuged (16,000 x g, 50 minutes, +4 °C) and the amount of free protein in the supernatant was measured with the BCA protein assay kit.

3.2.3.4. Investigation of Particle Size of BSA and NGF Loaded Niosome

Particle size distribution and polydispersity index of BSA and NGF loaded niosomes were determined in niosomal suspensions using the zetasizer device, which measures with the principle of dynamic light scattering (DLS). Analyzes were carried out using a 175° scattering angle with a He-Ne laser (633 nm) at 25°C. 40 µL of niosome suspension was, diluted to 4 ml with ultrapure water and filtered through 0.2 µm filter. Particle size measurement was repeated 3 times for each sample.

3.2.3.5. Imaging Results of BSA and NGF Loaded Niosome by Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is used to determine the morphology of niosomes. SEM photographs were taken at room temperature. Before making observations, lyophilized samples were attached to metal grids with double-sided adhesive tape and gold-plated with Emitech K550X sputter coater for 1 min under vacuum.

3.2.3.6. In Vitro Cytotoxicity of BSA and NGF Loaded Niosome

The biocompatibility of niosomes was evaluated by cell viability measurements with NSC-34 cell line. Approximately 1×10^3 NSC-34 cells were seeded in complete growth medium in 96-well plates 24 hours before treatment. Cells were incubated with the blank niosome, the BSA loaded niosome and the BSA and NGF loaded niosome and compared with the cell exposed to distilled water as control, respectively. The cells were then incubated for 24 hours at 37 °C in a humidified 5% CO₂ atmosphere. Then, 10 µL of MTT reagent was added to each well, including controls. Cells were incubated at cell culture incubator for 4 hours. Plates were then gently shaken and the absorbance measured at 570 nm using an ELISA reader. Since the niosomes are dissolved in distilled water, the same amount of distilled water was blindly applied to the cells to obtain the same conditions at each well.

4. RESULTS

4.1. Preparation and Characterization of BSA Loaded Niosome Formulation Using Different Surfactants

4.1.1. Determination of Entrapment Efficiency of F1- F3 Formulations

Entrapment efficiency of BSA loaded to niosomes were calculated. Percentages of BSA loaded in F1-F3 formulations and HLB values of surfactants used in preparing formulations are given in Table 4.1. Loading efficiency (EE %) of BSA ranges from 25.3% to 39.6%.

Table 4.1: Encapsulation efficiency of BSA loaded in niosome formulations prepared with different surfactants (Number of experiments=3, HLB: Hydrophilic lipophilic balance, CH: Cholesterol, SD: Standard deviation).

Formulations	Surfactants	HLB Value	CH: Surfactant (mM)	Entrapment Efficiency
F1	Span 60	8.6	20:20	25 % $\pm$ 2.6
F2	Span 20	4.7	20:20	39 % $\pm$ 0.8
F3	Span 80	4.3	20:20	36 % $\pm$ 1.0

4.1.2. Investigation of Particle Size of F1- F3 Formulations

Average particle sizes and polydispersity indices of the prepared F1-F3 formulations measured with Zetasizer device are given in Table 4.2. Polydispersity index values (PDI) are between 0.43-0.5. The Z mean value in the formulations is between 158.6 and 601.8 nm.

Table 4.2: Particle size and PDI value of BSA loaded in niosome formulations prepared with different surfactants (Number of experiments=3, CH: Cholesterol, SD: Standard deviation, Z-mean: Average particle size, PDI: Polydispersity index).

Formulations	Surfactants	CH: Surfactant (mM)	PDI $\pm$ SD	Z-mean $\pm$ SD (nm)
F1	Span 60	20:20	0,500 $\pm$ 0,0200	601 $\pm$ 36.1
F2	Span 20	20:20	0.491 $\pm$ 0,0100	205 $\pm$ 12.0
F3	Span 80	20:20	0.430 $\pm$ 0,0100	158 $\pm$ 8.2

4.1.3. The Effect of Different Amounts of Span 60 and Cholesterol on the Niosomes Loaded with BSA

4.1.3.1 Determination of Entrapment Efficiency of F1S1-F1S3 Formulations

Cholesterol is a component of niosome, increasing the stability and mobility of the niosomal vesicles. By preparing niosomes with different CH percentages, the effect of CH on encapsulation efficiency was investigated. The data are shown in both a bar graph and in Table 4.3 (Figure 4.1). As can be seen, niosomes containing 50% CH, the highest EE% at 42 % $\pm$ 1.4. Compared to other reports, this entrapment efficiency was considered a suitable percentage for protein-loaded particles.

Table 4.3: Encapsulation efficiency of BSA loaded in niosome formulations prepared with different proportions of surfactant/CH (Number of experiments=3, CH: Cholesterol).

Formulations	Cholesterol (%)	CH: Surfactant (mM)	Entrapment Efficiency
F1-S1	20	1.6: 6.0	31 % $\pm$ 6.4
F1-S2	40	3.2:4.8	30 % $\pm$ 2.4
F1-S3	50	6.25:6.25	42 % $\pm$ 1.4
F1-S4	60	4.8:3.2	29 % $\pm$ 2.0
F1-S5	80	6.3:1.6	31 % $\pm$ 1.2

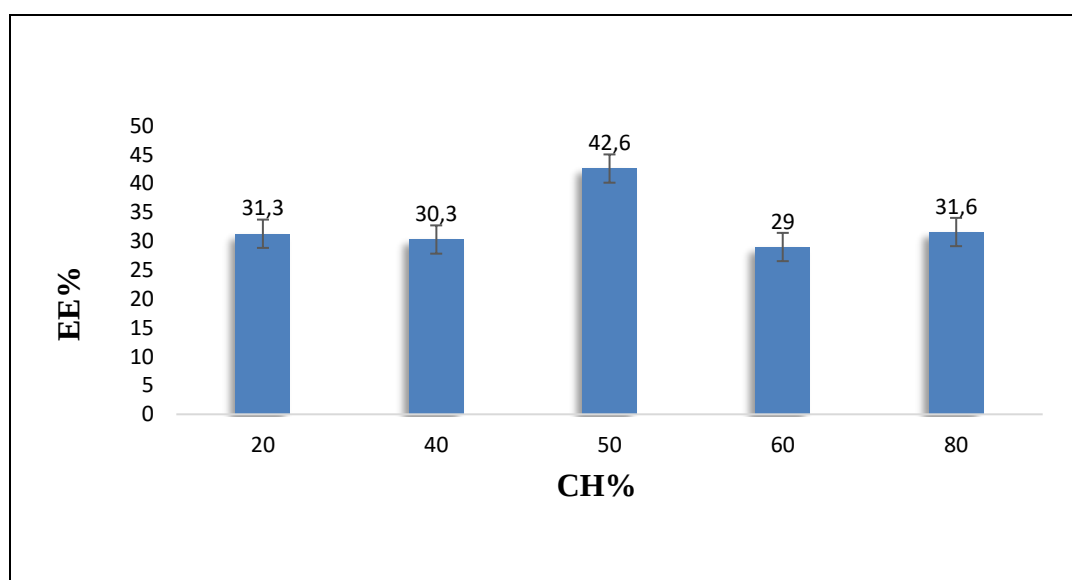


Figure 4.1: The diagram of the correlation of CH percentage and entrapment efficiency (Number of experiments = 3, CH: Cholesterol, EE%: Entrapment efficiency).

4.1.3.2. Investigation of Particle Size of F1-S1 and F1-S3 Formulations

Formulations F1S1-F1S5 were prepared based on the F1 formulation containing Span 60. In these formulations, the amount of BSA was kept constant and the amounts of Span 60 and cholesterol were changed. The following experiments were made on the obtained niosomes. Particle size and polydispersity indexes of

prepared formulations measured with Zetasizer device are given in Table 4.4.

According to the literature, suitable nanoparticle size for tissue engineering application is in the range of 100–500 nm [9]. Samples with different CH percentages showed lower PDI and almost all samples were of suitable size according to requirements. Therefore, the resulting size (205.6 nm) and particle size distribution (0.43) for niosomes with 50% CH content represented the optimal condition for niosome preparation.

Table 4.4: Particle size and PDI value of BSA loaded in niosome formulations prepared with different proportions of surfactant/CH (Number of experiments=3, CH: Cholesterol, SD: Standard deviation, Z-mean: Average particle size, PDI: Polydispersity index).

Formulations	Cholesterol (%)	PDI $\pm$ SD	Z-mean $\pm$ SD (nm)
F1-S1	20	0,500 $\pm$ 0,0200	241 $\pm$ 6.4
F1-S2	40	0.491 $\pm$ 0,0100	220 $\pm$ 5.2
F1-S3	50	0.430 $\pm$ 0,0100	205 $\pm$ 4.0
F1-S4	60	0.360 $\pm$ 0,0100	196 $\pm$ 8.7
F1-S5	80	0.306 $\pm$ 0,0200	184 $\pm$ 7.2

As can be seen in the Table 4.5 as a result of the optimization studies, it was decided to continue with the F1-S3 formulation and the P3 agitation method for further experiments.

Table 4.5: Table of contents of the final formulation (Number of experiments=3, CH: Cholesterol, SD: Standard deviation, Z-mean: Average particle size).

F1-S3 Formulations	CH: Span 60 (mM)	Water bath sonication (min)	Entrapment Efficiency	Zmean $\pm$ SD (nm)
	6.25:6.25	15	42.6 % $\pm$ 1.4	205 $\pm$ 4.0

4.2. Characterization of Niosomes

4.2.1. BSA Standard Curve

Taking into account that the spectrophotometer reports the level of solution concentrations according to the UV absorption level, the standard curve is drawn from the obtained OD of the standard concentration solutions. Five samples with defined BSA concentrations were prepared and measured with the BCA protein assay kit. Their absorbances were measured at 562 nm to obtain the transformation formula from the BSA OD concentration standard curve (Figure 4.2).

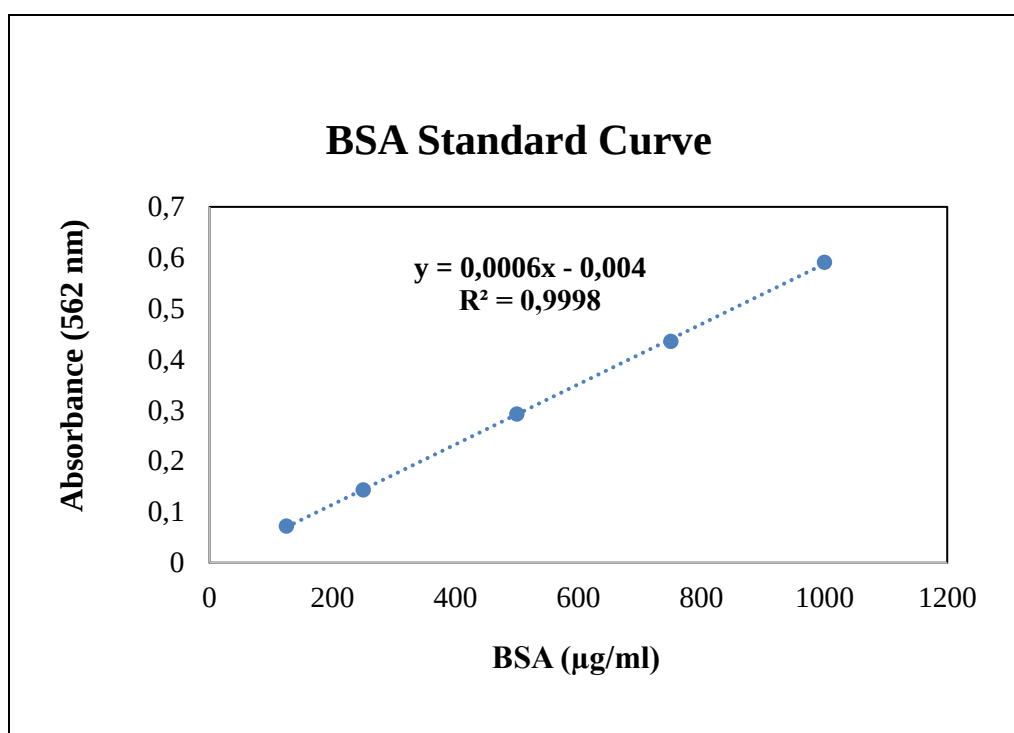


Figure 4.2: BSA standard curve.

4.2.2. Determination of Entrapment Efficiency of F1-S3 Formulation

For determination of BSA entrapment efficiency (EE %), unloaded BSA mixture was separated by centrifuge ($16,000 \times g$, 4°C , and 50 min). BSA concentration was determined by Spectrophotometer and EE% was calculated using

Equation 3.1. The encapsulation efficiency of BSA loaded niosomes was calculated approximately as $45\% \pm 5.7$.

4.2.3. The Study of Protein Release from Niosome Formulations F1-S3

In Figure 4.3, 30-50% of the loaded BSA was released on the first day due to the rapid diffusion of BSA molecules present on the surface. Also, it can be seen niosomes containing 50% of CH showed slow release. 30% of BSA was released after 6 days. In addition, other proteins such as insulin encapsulated in the niosomal vesicle showed a similar sustained-release profile [28].

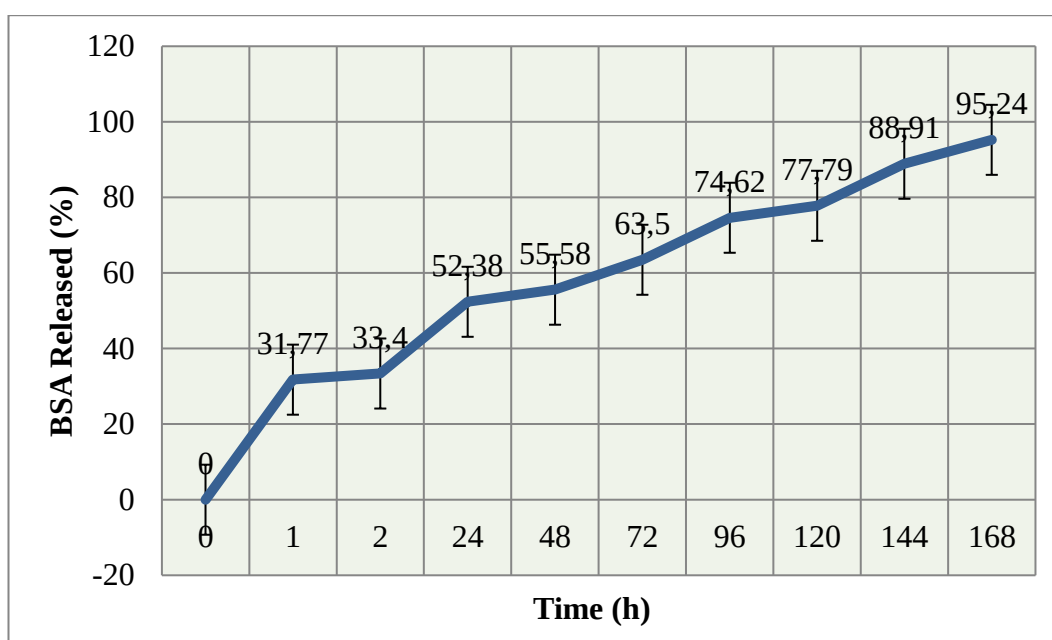


Figure 4.3: The release diagram of BSA loaded niosomes with Span 60 50 % CH (Number of experiments=3).

4.2.4. Particle Size Optimization of Niosomes

Probe sonication (3, 5 min) and water bath sonication (5, 10, 15 min) were applied to the F1-S3 formulations niosomes to obtained changes of particle sizes. Average particle sizes of these niosomes coded as P1-P5 are given in Figure 4.4.

The effect of sonication was evaluated as an important parameter on niosome properties. The mean particle size was decreased by prolonging sonication time. However, when the sonication time was increased more, disruption was observed in the structure of the niosomes. Therefore, in our study, it was observed that the optimum time for water bath sonication is 15 min and for the probe sonication process duration is defined as 5 min. In order to obtain smaller particle size, the most appropriate sonication application time was determined as 15 minutes for water bath sonication. This formulation will be referred to as P3 niosomes later in the study.

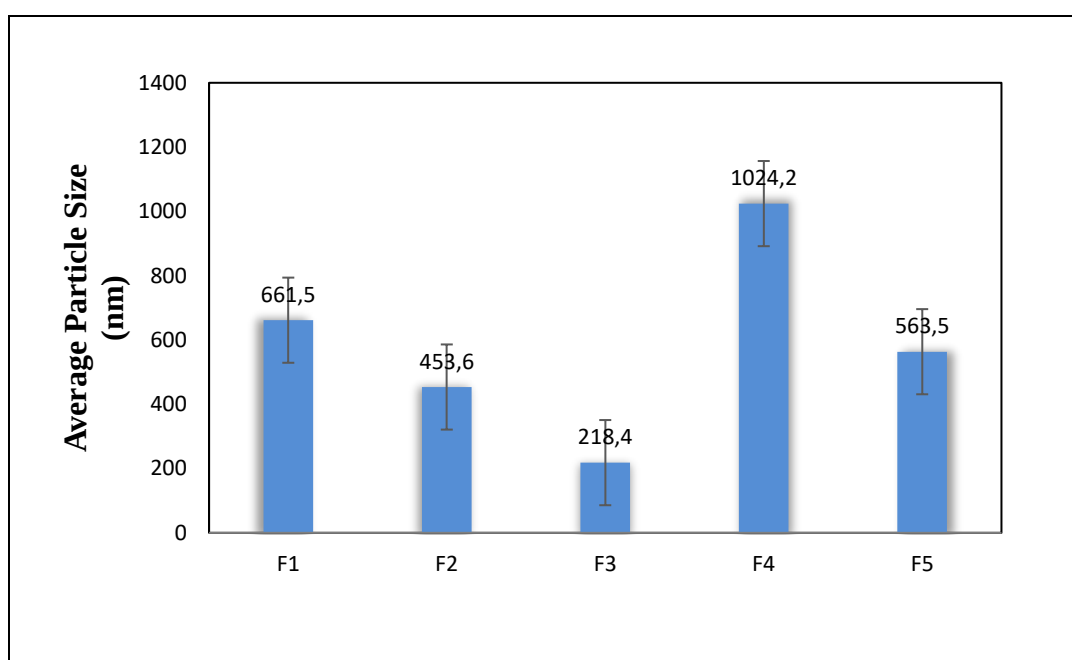


Figure 4.4: Average particle sizes of niosomes by probe sonication and water bath sonication applied at different durations. 5 min water bath sonication (F1), 10 min water bath sonication (F2), 15 min water bath sonication (F3), 3 min probe sonication (F4), 5 min probe sonication (F5), (Number of experiments=3).

Optical microscope images of the niosomes of the F1-S3 formulation prepared to develop the niosome formulations are given in Figure 4.5. It has been shown that vesicular structures can be obtained by using the reverse phase evaporation method with Span 60. As a result of prolonging the vortex application time and / or sonication in an ultrasonic water bath, the niosomes were fragmented into smaller spherical vesicles (Figure 4.6).

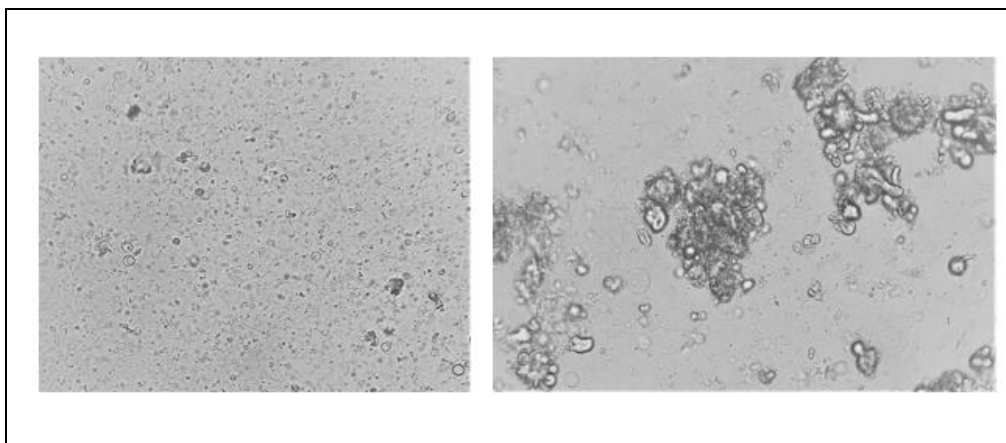


Figure 4.5: Optical microscope images of BSA loaded niosomes (20X, 40X).

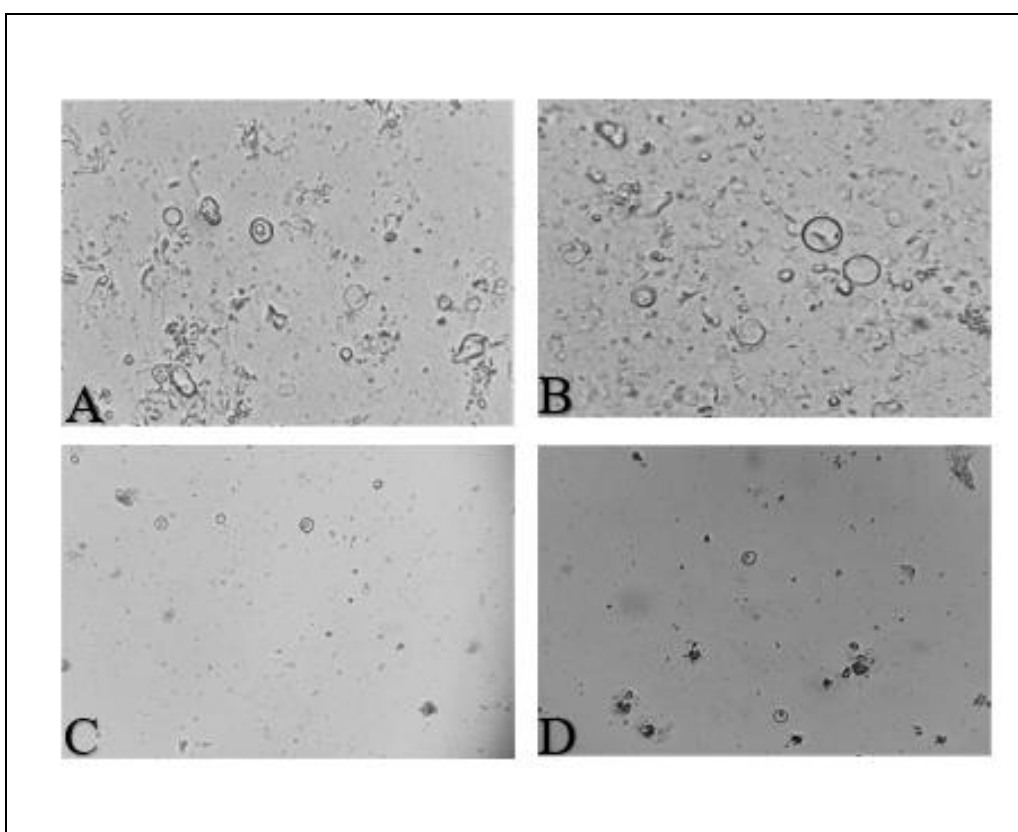


Figure 4.6: 10 min vortex application (A), 10 min vortex application followed by sonication in ultrasonic bath for 15 min (B), 10 min vortex application followed by 15 min sonication in ultrasonic bath Span 60 niosomes diluted With distilled water microscope images (C,D) (X40).

4.2.5. Investigation of Particle Size of Niosomes of F1-S3 Niosomes

Particle size and polydispersity indices measured with Zeta-sizer device after the preparation of blank niosome and BSA loaded niosome are given in Figure 4.7.

The polydispersity index (PDI) value is a measure of the molecular mass distribution for polymers. For nanoparticles, it is an indicator of homogeneity in particle size distribution. It takes values in the range of 0-1. It is said that the larger the PDI value, the less homogeneous particle size distribution [10].

Particle size of niosomes are shown in Figure 4.7 (A). Blank niosome nanoparticles have a restricted size distribution with an average particle size of 184 ± 7.2 nm and a polydispersity index of 0.306.

In Figure 4.7 (B), size of BSA loaded niosome nanoparticles are given. The average particle size of BSA loaded nanoparticles was found to be 218 ± 4.0 nm and the polydispersity index is as 0.43.

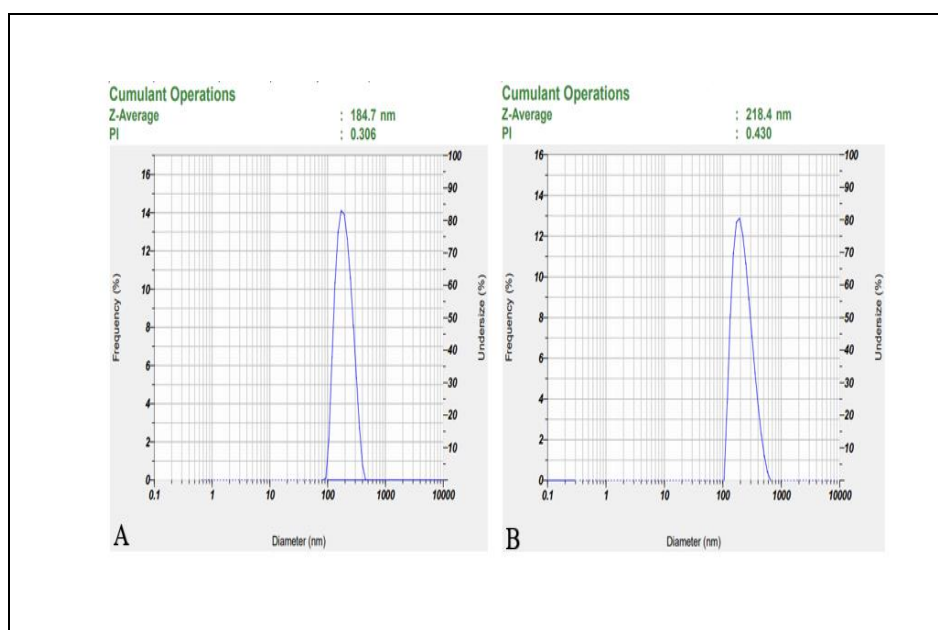


Figure 4.7: The size diagram of blank niosome (A) and BSA loaded niosome (B).

4.2.6. Thermal Stability of Niosomes

In order to characterize the thermal stability of niosomes, they were incubated

at + 4 °C and + 25 °C for four days. As seen in the Table 4.6, we can say that the amount of free BSA in the supernatant of the BSA-loaded niosome stored at + 25 °C was released faster than the niosome stored at +4 °C. Thus, we can say that BSA releases faster than the niosome at 25 °C. This result shows us that the increasing temperature accelerates BSA release from the niosome system. It also shows that, unlike liposomes, niosomes are rather stable at room temperature.

Table 4.6: The effect of temperature on BSA release from niosomes after 24 and 72 hours (Number of experiments=3, h: hour).

Temperature	0 h	24 h	72 h
+4 °C	701.3 ±0.8 µg	746.3±0.8 µg	756.9±0.7 µg
+25 °C	701.3 ±0.8 µg	769.8±1.7 µg	834.3±1.3 µg

4.2.7. Morphology Analysis of Niosomes

Scanning electron microscopy (SEM) is used to determine the morphology of niosomes. SEM photographs were taken at room temperature. Before making observations, lyophilized samples were attached to metal grids with double-sided adhesive tape and gold-plated with Emitech K550X sputter coater for 1 minute under vacuum.

4.2.7.1. Images of Blank Niosome by Scanning Electron Microscopy (SEM)

When the photographs of the BSA-free niosome formulation are examined in Figure 4.8, it is seen that the niosomes are spherical in shaped, small in size and independent from each other.

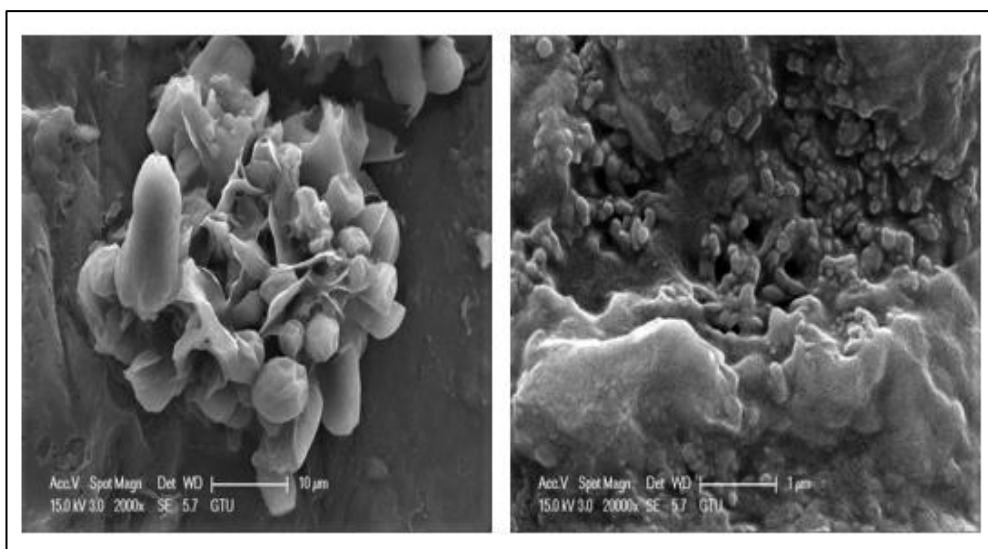


Figure 4.8: SEM electron photomicrographs of blank niosomes.

4.2.7.2. Images of BSA Loaded Niosomes by Scanning Electron Microscopy (SEM)

SEM, which is based on the principle of imaging the high-energy electrons passing through the particles, enables the imaging of much smaller sizes compared to the optical microscope. Blank and BSA loaded niosomes imaged with SEM were monitored. The images obtained are given in Figure 4.9. SEM images have been another indication that niosomes have been successfully created. Afterwards the niosome solution was diluted 2 times to get clearer image (Figure 4.10). In order to examine the effect of sonication, images were taken after 15 minutes of water bath sonication on niosome morphology was applied (Figure 4.11).

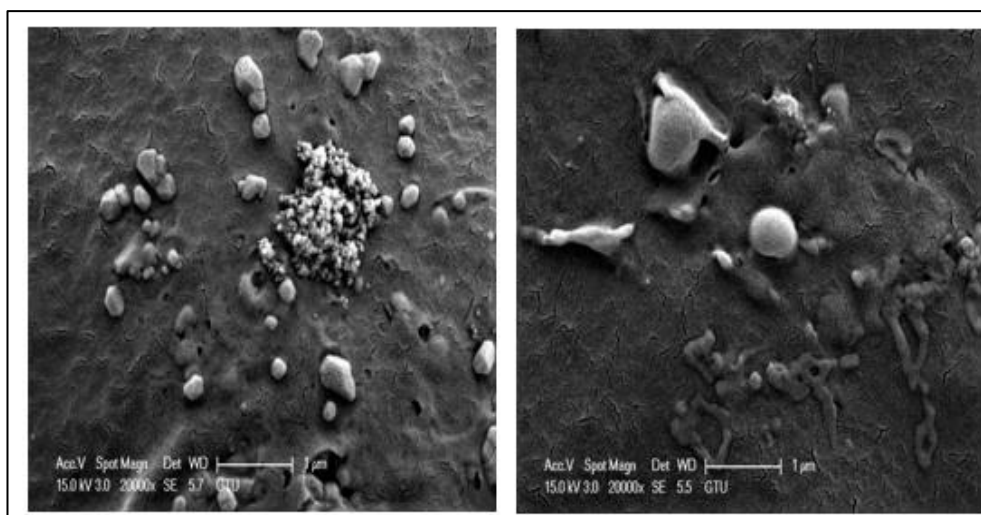


Figure 4.9: SEM electron photomicrographs of BSA loaded niosome.

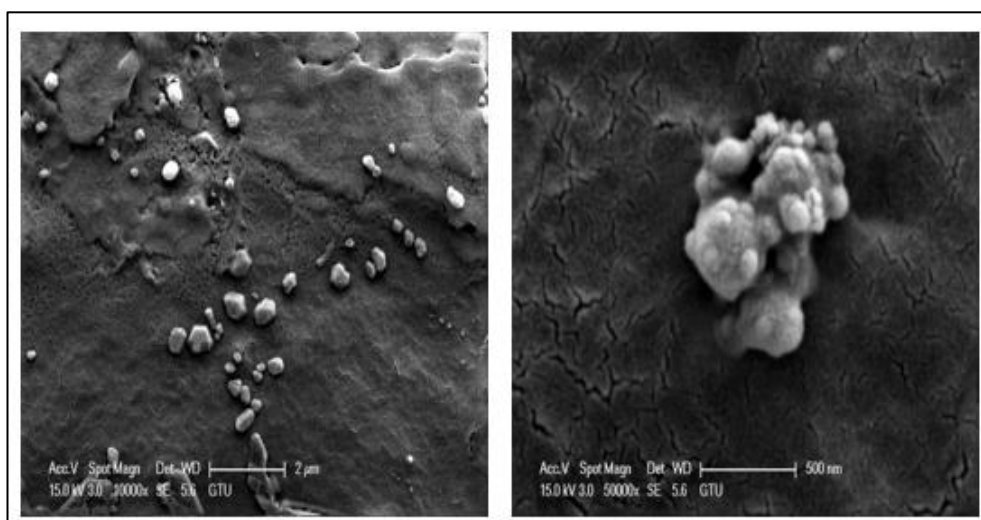


Figure 4.10: SEM electron photomicrographs of 50 % diluted BSA loaded niosomes.

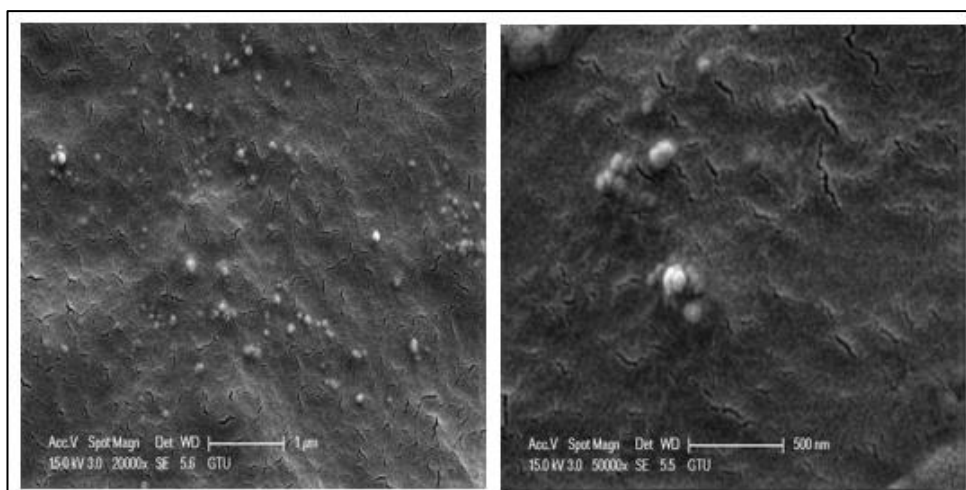


Figure 4.11: SEM electron photomicrographs of sonicated BSA loaded niosome after 15 min water bath.

4.2.8. Fluorescence Images of Niosomes

Fluorescence images of BSA loaded niosomes visualized with fluorescence inverted microscope. The spherical and lamellar structure is clearly seen from the images (Figure 4.12).

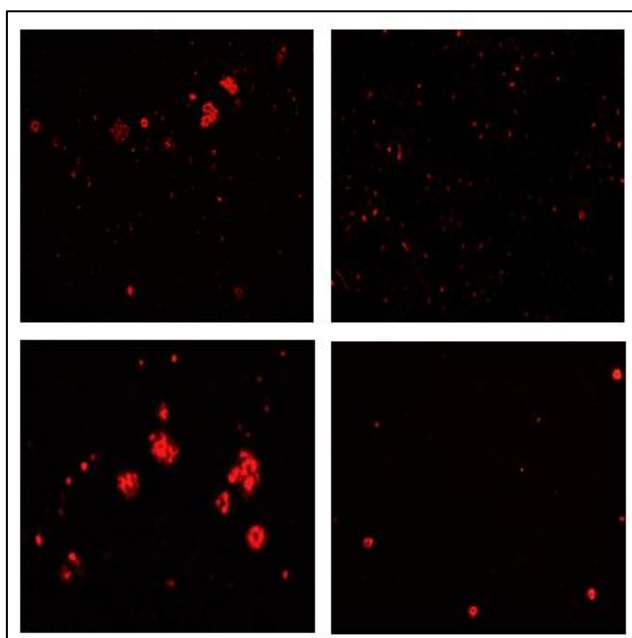


Figure 4.12: Fluorescent images of BSA loaded niosomes.

4.2.9. In Vitro Cytotoxicity of Blank Niosomes

The cytotoxicity of blank niosomes in the human neuroblastoma SH-SY5Y cell line after 5 h of incubation was assessed with XTT assay. As a result of the XTT experiment, it is seen that the cell viability rate decreases with increasing amounts of niosome. Blank niosomes with 50DF, 200DF, 500DF did not induce cytotoxicity on cells, suggesting the niosomes had high biocompatibility and were suitable for drug delivery system (Figure 4.13).

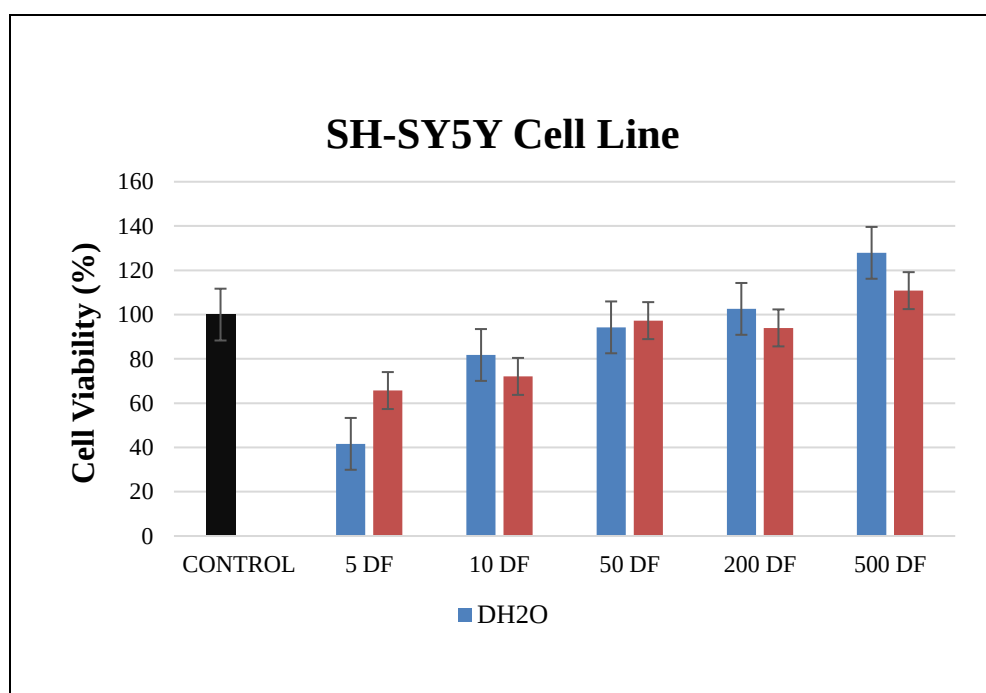


Figure 4.13: Cytotoxicity of blank niosome (Number of experiments=3, DF: Diluted factor, dH₂O: Distilled water).

4.3. Preparation and Characterization of BSA and NGF Loaded Niosomes

4.3.1. Determination of Entrapment Efficiency of BSA and NGF Loaded Niosome

BSA and NGF concentration was determined by Spectrophotometer and EE%

was calculated using Equation 3.1. The encapsulation efficiency of BSA and NGF loaded niosomes was calculated approximately $64 \% \pm 3.3$. While the encapsulation efficiency of BSA loaded niosomes was approximately 45 %, the encapsulation efficiency of BSA and NGF loaded niosomes determined higher.

4.3.2. The Study of Protein Release from Niosome System of BSA and NGF Loaded Niosome

BSA and NGF loaded niosomes release kinetic was studied. In the first 24 hours, due to the rapid diffusion of BSA and NGF molecules on the surface, approximately 40-50 % of encapsulated protein was released. Approximately 30% of protein was slowly released at between 2nd and 5th days. The release diagram of BSA and NGF was shown up in the Figure 4.14.

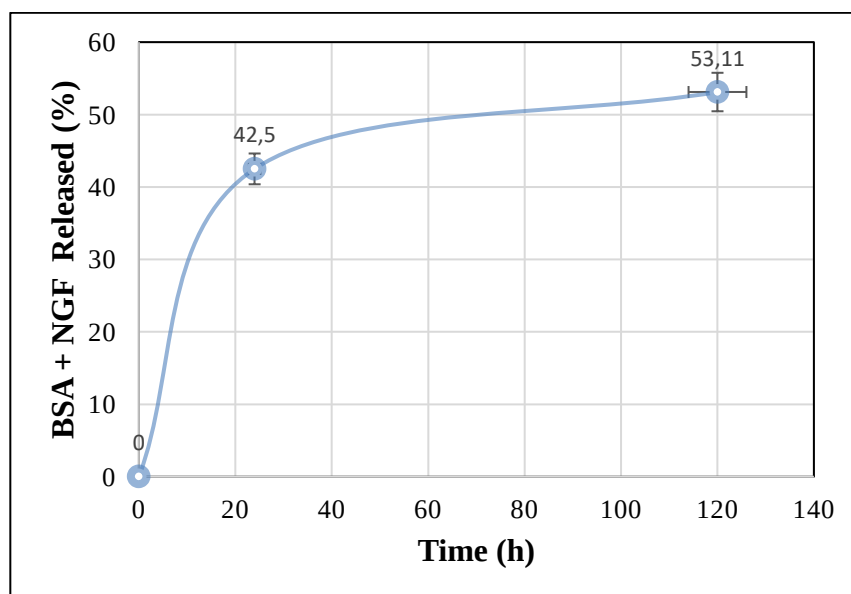


Figure 4.14: The release diagram of BSA and NGF loaded niosomes (Number of experiments=3).

4.3.3. Investigation of Particle Size of BSA and NGF Loaded Niosome

The average particle size of BSA and NGF loaded niosomes was found as 185

± 1.0 nm and the polydispersity index to be 0.1 (Figure 4.15).

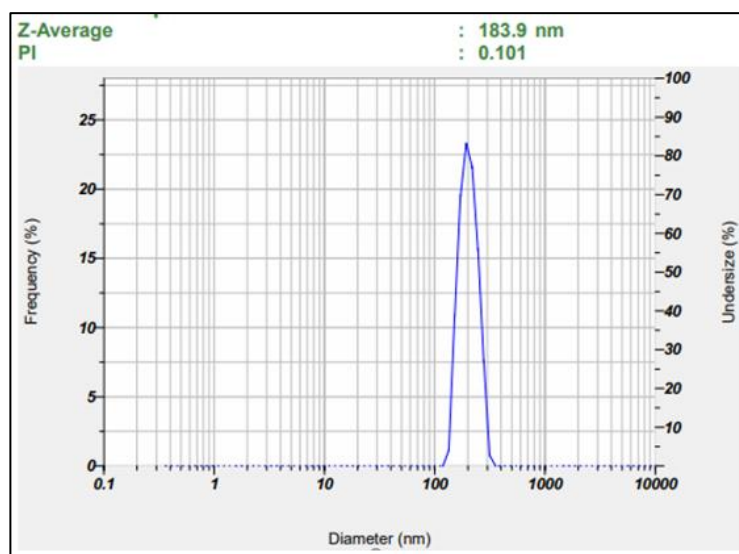


Figure 4.15: The size diagram of BSA and NGF loaded niosome.

4.3.4. Imaging Results of BSA and NGF Loaded Niosome by Scanning Electron Microscopy (SEM)

Image of BSA and NGF loaded niosomes obtained from SEM are given in Figure 4.16. SEM image indicated niosomes have been successfully created. The spherical structure is clearly seen from the image.

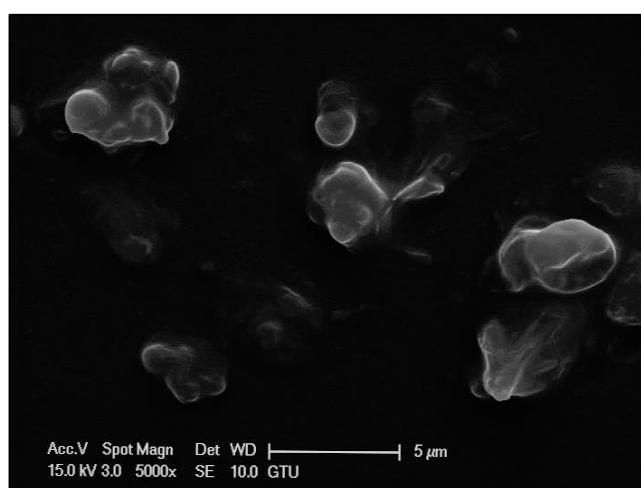


Figure 4.16: SEM electron photomicrographs of BSA and NGF loaded niosomes.

4.3.5. In Vitro Cytotoxicity of BSA and NGF Loaded Niosome

In order to examine the cytotoxic effects of niosome on NSC-34 cell line, MTT assay was applied. We can see in Figure 4.17 that cytotoxicity results of equal amount blank niosome, BSA loaded niosome and BSA and NGF loaded niosome.

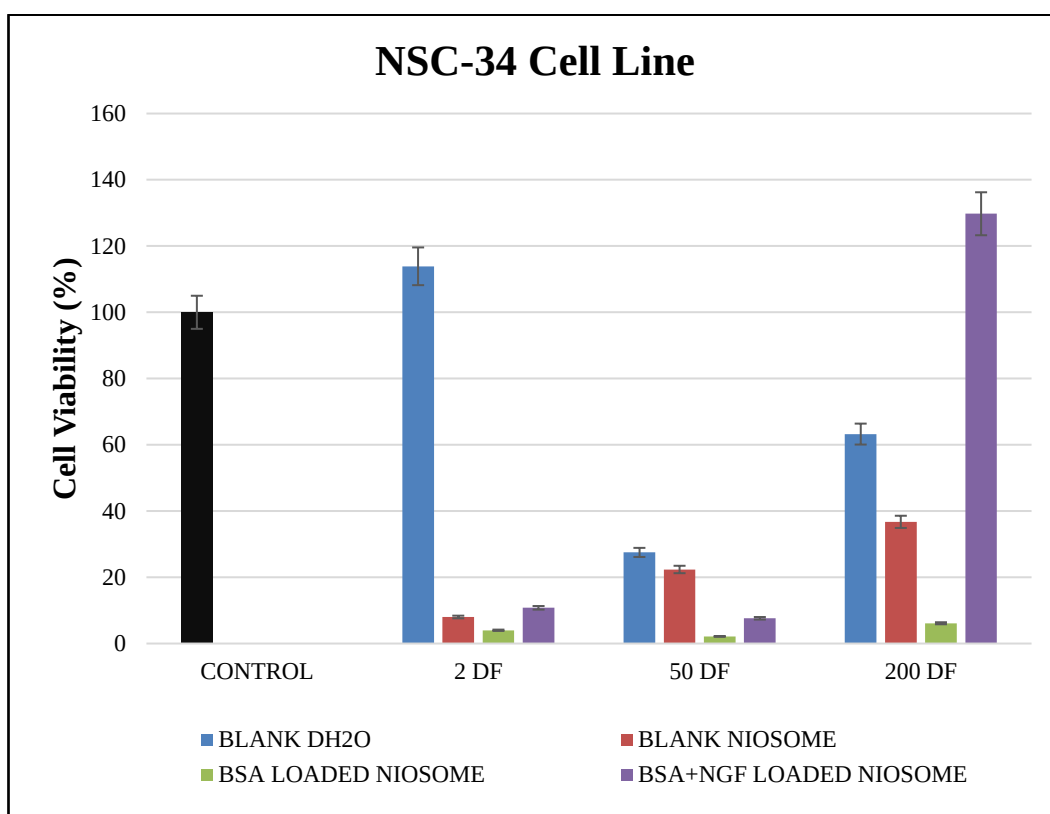


Figure 4.17: Cytotoxicity of BSA and NGF loaded niosome (Number of experiments=3, DF: Diluted factor, dH₂O: Distilled water).

For the prepared niosomes, three different dilutions were applied to the cells. Purpose of this process is observe the effects of different amounts of niosome, BSA and NGF on cell viability. The amount of ingredients of different concentration of niosomes were calculated and given in the Table 4.7.

Table 4.7: The amounts of ingredients in BSA and NGF loaded niosomes (DF: Diluted factor).

	Amount of NGF (ng/well)	Amount of BSA (µg/well)
2 DF Blank Niosome	-	-
50 DF Blank Niosome	-	-
200 DF Blank Niosome	-	-
2 DF BSA Loaded Niosome	-	50
50 DF BSA Loaded Niosome	-	2
200 DF BSA Loaded Niosome	-	0.5
2 DF BSA and NGF Loaded Niosome	5.35	42.8
50 DF BSA and NGF Loaded Niosome	0.214	1.714
200 DF BSA and NGF Loaded Niosome	0.055	0.43

5. DISCUSSION

In the present work preparation and optimization studies have been carried out in order to obtain the formation of niosome vesicles and to select the appropriate preparation parameters to be used in further formulations. In this study, niosome formulations were prepared by reverse phase evaporation method using 3 different surfactants Span 20, Span 60 and Span 80. Non-ionic surfactants were chosen in this study because of their low toxicity. Cholesterol has been used as a membrane stabilizing agent. The interaction between cholesterol and surfactant layer depends on the structure of the lipid chain and hydrophilic head group. The addition of cholesterol increases the loading efficiency of drugs into niosomes. It has been observed that BSA has been loaded successfully in all types of formulations. In particular, F1-S3 formulation, which has high entrapment efficiency, low particle size and also contains higher amount of BSA per unit weight, was chosen as the most suitable formulation for further in vitro tests (Table 4.5). Among the niosomes prepared with different surfactants, the encapsulation efficiency highest was with Span 60 which is 39.6% and size of 205.6 nm. According to the formulation prepared with Span 60, niosomes were prepared with different cholesterol ratios. The most suitable ratio for Span 60: Cholesterol was found as 6.25:6.25 mM. The encapsulation efficiency was obtained as 42.6 % and size of particle was determined as 205.6 nm.

Particle size distribution of the prepared niosome formulations is trimodal. Such distributions can also be observed in other studies on niosomes [46]. Polydispersity index values (PDI) are between 0.3 and 0.5 in all measurements. PDI is a parameter that indicates the width of the distribution and the uniformity of the size. If the PDI value is above 0.7, it indicates that the particle size distribution of the sample is very wide and it is not suitable for measuring with DLS. The results found in our study were found to be suitable for the DLS method.

A decrease in average particle size was observed with the increase of sonication duration. This result is consistent with the literature stating that the particle size of niosomes can be reduced by sonication [47]. However, when the sonication time was increased, disruption of niosomes was observed in the structure

of the niosomes. In terms of obtaining smaller particle sizes, the most suitable sonication method is water bath sonication and application duration was determined as 15 min. Niosomes obtained by this method are around 200 nm in size (Figure 4.4).

More detailed characterizations were done with the optimized final niosomal formulation. The total amount of BSA was calculated using the BCA assay kit. Encapsulation efficiency (EE %) ranges from 29-45%. The highest encapsulation efficiency was calculated as 45% for BSA loading. The encapsulation efficiency of BSA and NGF loaded niosomes calculated approximately $64\% \pm 3$. According to these results, BSA and NGF loaded niosome encapsulation efficiency was higher than the BSA loaded niosome and quite high encapsulation rate for NGF loading to niosomes.

In this study, a nanocarrier based on BSA loaded niosomes, capable of releasing BSA molecules slowly, has been developed (Figure 4.14). Span surfactants, which are more hydrophobic, form more compact niosomes in the presence of cholesterol [46]. Slow drug release from formulations will be beneficial in reducing the severity of toxic side effects that may be seen in healthy tissues in vivo. The release profile can be controlled by the CH percentage as one of the formulation parameters. According to release kinetic studies, BSA and NGF release from niosomes continued for up to 5 days. In addition, the high EE% and the rather slow release of BSA and NGF from niosomes make this system valuable for drug delivery and tissue engineering applications.

In order to characterize the thermal stability features of niosomes, their storage was carried out at $+4\text{ }^{\circ}\text{C}$, $+25\text{ }^{\circ}\text{C}$. The release result showed us that the increasing temperature accelerates BSA release from the niosome system (Table 4.6). Thus $+4\text{ }^{\circ}\text{C}$ is more convenient for the storage temperature of niosomes.

The mean particle sizes of blank niosome and BSA loaded niosome are 184.7 nm and 218.4 nm, respectively (Figure 4.7). The size of BSA and NGF loaded niosomes found approximately 185 ± 1.0 nm and the polydispersity index was 0.1.

Optical microscope images gave a qualitative idea of the size and shape of the niosome vesicles. It has been shown that vesicular structures can be obtained using the reverse phase evaporation method with Span 60 (Figure 4.5). The effect of the agitation method used on the shape of the vesicle was also clearly seen (Figure 4.6).

BSA loaded niosomes were dyed with FM4-64 which is a fluorescent probe as membrane marker. In this way, the spherical structure of the niosomes was observed by fluorescence invert microscope (Figure 4.12).

From the images obtained by SEM, it was observed that the spherical structures are visualized more clearly and the sonication process reduces the size of the niosome. Non-sonicated niosomes were visualized as spherical shapes with wide variation in sizes in the images obtained by SEM microscopy. We have thus shown that niosomes have been successfully created and that in spherical shapes.

Firstly, in order to examine the cytotoxicity of niosomes, a blank niosome solution was prepared and applied to the cells at different concentrations. Thus, in order to determine the optimum concentration for the cell viability. As a result of the cell viability assay in Figure 4.13, it was observed that there was no reduction in cell viability with 50, 200, and 500 times diluted niosomes. Later, BSA and NGF loaded niosomes were prepared and cell viability was evaluated on the NSC-34 cell line. In order to see the NGF effect on cell viability equal amount of blank niosome and BSA loaded niosome was used as control.

NGF and several other growth factors have promising therapeutic features on degenerative and traumatic central nervous system diseases. The results of experiments show that NGF delivery to brain is difficult because of Blood Brain Barrier (BBB). For effective passage from BBB, nanocarriers are the encouraging choice. In this study, we prepared and tested basic features of niosomes as a nanocarrier for NGF delivery. Although successful and consistent results were obtained in this study, more detailed in vitro and in vivo animal tests should be performed and further determination of safest NGF dosage and its release, should be investigated. Moreover, a reasonable study approach to achieve therapeutic targets can be rate-controlling process in the evolvement of complicated treatments goals in the future.

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BIOGRAPHY

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APPENDICES

Appendix A: Publications within the Scope of Thesis Study

Öztürk G., (2019), “The Effects of Growth Factor Loaded Niosomes on Neuronal Cell Survival”, GTU Graduate Studies Symposium and Introductory Days, 17-18 June.

