REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

FORMULATION AND EVALUATION OF NANOSTRUCTURED LIPID CARRIERS (NLC) FOR TOPICAL APPLICATION

Pharm. ISRA T. ANWAR

Cosmetology Program
M.Sc. THESIS

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SUPERVISOR
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Sağlık Bilimleri Enstitüsü Müdürlüğü'ne;

Bu çalışma jürimiz tarafından Kozmetoloji Programında Yüksek Lisans Tezi olarak kabul edilmiştir.

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Bu tez, Hacettepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği'nin ilgili maddeleri uyarınca yukarıdaki jüri üyeleri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu kararıyla kabul edilmiştir.

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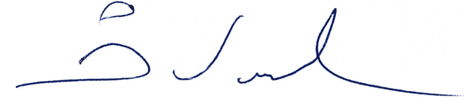

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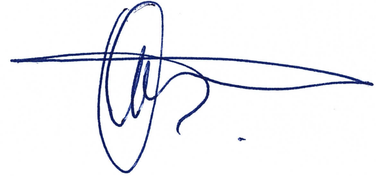

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ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor *Prof. Dr. Süeda Hekimoğlu* who has supported me throughout my thesis with her perceptive advice and extensive knowledge. I attribute the level of my Masters degree to her encouragement and effort, and without her this thesis would not have been completed or written. I also thank the members of my graduate committee for their guidance and suggestions, especially *Assoc. Prof. Dr. İmran Vural* for all her advice, encouragement and help in cell culture studies.

I would like to thank *Prof. Dr. Erhan Palaska* and *Dr. Keriman Özadalı* for their kind support and help during FTIR analysis and melting point evaluation, and *Prof. Dr. Sevim Dalkara* for her advice and guidance.

I would like to express my appreciation to *Assoc. Prof. Dr. R. Neslihan Gürsoy*, *Assist. Prof. Dr. Sibel Bozdağ Pehlivan, Assist. Prof. Dr. Hakan Eroğlu*, *Dr. Yasemin Çırpanlı*, *Pharm. Can Sarısözen (M.Sc.)* for their invaluable advice and guidance.

I would like to give my hearty thanks to all colleague and dear friends in the Department of Pharmaceutical Technology; Pharm. Süheyla Toprak (M.Sc.), Pharm. Özge Çevik, Chem. S. Burçin Hizmetli, Pharm. Gülnur Kübra Özeser, Pharm. Nazlı Erdoğar, Pharm. Z. Gülşen Öner, Bio. R. Eren Ayata (M.Sc.), Pharm.Tuğba Gülsün İnal (M.Sc.), Bio.Gözde Aygül, Pharm. Burçin Yavuz (M.Sc.), Pharm. Seçil Çaban (M.Sc.), Pharm. Kıvılcım Öztürk (M.Sc.), Pharm. Serhat Kozlu (M.Sc.), Pharm. Hasan Demir, Pharm. Harika Öcal (M.Sc.), Pharm. Elif Sarıgöl, Pharm.Yağmur Akdağ, Pharm. Merve Cansız and Pharm. Ertan Özdemir for providing me such a wonderful, cheerful environment and sharing with me happy, stressed and sad moments.

My endless love and gratitude is extended to my beloved family; my mother, my father, my brother and my sisters for their understanding, endless love, motivation, and encouragement.

ABSTRACT

Anwar, I. T., Formulation and evaluation of nanostructured lipid carriers (NLC) for topical application, Hacettepe University, Institute of Health Sciences, Master Thesis in Pharmaceutical Technology, Cosmetology Program, Ankara, 2011. Nanostructured lipid carriers (NLC) are smart delivery systems which are the second generation of solid lipid nanoparticles (SLN). In this thesis, blank and 0.5% and 1% salicylic acid loaded NLC and SLN formulations with various oil/fat ratio NLC(70:30), NLC(50:50), NLC(40:60) were developed for cosmeceutical application. Precirol ATO5 as solid lipid, Labrafac lipophile as liquid lipid, Tween 80 as hydrophilic and Lipoid S100 as lipophilic surfactants were used. Formulations were prepared by using high shear homogenization and ultrasonication technique. Then after they were characterized by evaluating particle size, polydispersity index, zeta potential, pH and viscosity measurements. The particle size of blank formulations were 138.74nm for NLC(70:30), 155.6nm for NLC(50:50), 168.6nm for NLC(40:60), and 235nm for SLN($d_{0.5}$). After loading SA into the formulations an increase was obtained in particle size of all formulations and this increase was higher for the SLN formulation. Although particle size of SA loaded NLC formulations increased, they indicated a narrow size distribution. However SLN formulation indicated a very broad size distribution value after SA loading. All blank formulations displayed zeta potential values ranging between -25 and -30mV, but after SA loading these values were showed a decrease due to the low pH value and acidic character of SA. The encapsulation efficiency of formulations were 43.24% for %0.5SA-NLC(50:50), 34.84% for %1SA-NLC(50:50), 36.94% for %0.5SA-NLC(40:60), 29.76% for %1SA-NLC(40:60), and 21.45% for %0.5SA-SLN. Because of the higher oil content in NLC(50:50) it indicated the highest encapsulation efficiency among other formulations. The in vitro release profile of formulations were also evaluated and at the first 30 min NLC formulations indicated a burst effect, followed by slower release profile and continued with a controlled release behavior after 8 h. SLN displayed a slower release behavior than NLC because of its the high viscosity and particle size value. Thus the increase in oil/fat ratio and the loaded amount of SA in NLC formulations caused an increase in the release rate in the following order 0.5% SA-SLN < 1% SA-NLC(40:60) < 0.5% SA-NLC(40:60) < 0.5% SA-NLC(50:50) < 1% SA-NLC(50:50). The in vitro cytotoxicity of the blank and 0.5% SA loaded NLC(50:50) were investigated during 12 h and 24 h by cell culture studies utilizing L929 mouse fibroblast cell line and results indicated that NLC with concentrations in range 0.125% - 0.5% were found to be non-cytotoxic. Stability studies of blank and SA loaded NLC(50:50), NLC(40:60) and SLN were performed at 25°C and 4°C during 90 days by means of particle size, zeta potential, viscosity, encapsulation efficiency and SA content in formulations. As a result blank and SA loaded NLC(50:50) and NLC(40:60) were found to be stable during storage at both temperatures.

Key Words: Nanostructured lipid carriers (NLC), salicylic acid, in vitro release, cytotoxicity, stability.

ÖZET

Anwar, I. T., Deriye uygulanan nanoyapılı yağ taşıyıcıların (NLC) formülasyonu ve değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Teknoloji, Kozmetoloji Programı Yüksek Lisans Tezi, Ankara, 2011. Nanoyapılı yağ taşıyıcılar (NLC) akıllı taşıyıcı sistemler olup katı yağ nanokürelerin (SLN) ikinci kuşak sistemleri olarak tanımlanmaktadır. Tez çalışması kapsamında kozmesötik etkisi olan %0.5 ve %1 salisilik asit ile yüklenmiş farklı sıvı yağ/katı yağ oranı ile NLC(70:30), NLC(50:50), NLC(40:60) ve SLN formülasyonları tasarlanmıştır. Formülasyonlarda, katı yağ olarak Precirol ATO 5, sıvı yağ Labrafac Lipophile, hidrofilik yüzey etkin madde Tween 80 ve lipofilik yüzey etkin madde olarak da Lipoid S100 kullanılmış ve yüksek hızda karıştırma ve ultrasonikasyon yöntemiyle formülasyonlar hazırlanmıştır. Karakterizasyon çalışmalarında partikül büyüklüğü, polidispersite indeksi, zeta potansiyeli, pH ve viskozite değerleri incelenmiştir. Boş NLC(70:30) 138.74nm, NLC(50:50) 155.6nm, NLC(40:60) 168.6nm, ve SLN($d_{0.5}$) 235 nm partikül büyüklüğü değerinde olup, salisilik asit yüklenmesiyle tüm formülasyonların partikül büyüklüğünde önemli bir artış gözlenmiştir. SA yüklenmiş NLC formülasyonları homojen ve küçük polidispersite indeksi değerlerine sahipken , SLN formülasyonunda mikron boyutlarda partikül boyutu değerleri ve geniş bir dağılım elde edilmiştir. Boş formülasyonlarda zeta potansiyeli -30 ve -25mV aralığında bir değer elde edilmiş, fakat SA yüklendikten sonra SA'in düşük pH değeri ve asidik özelliğinden dolayı NLC ve SLN'ye yüklendikten sonra zeta potansiyelinde düşüş gözlenmiştir. Salisilik asidin hapsedilme etkinliği, % 0.5SA-NLC(50:50) için % 43.24, %1SA-NLC(50:50) için % 34.84, % 0.5SA-NLC(40:60) için % 36.94, %1SA-NLC(40:60) için % 29.76, ve % 0.5SA-SLN için % 21.45 olarak bulunmuştur. NLC(50:50)`in sıvı yağ oranının diğer formülasyonlara göre daha yüksek olması, en yüksek hapsedilme etkinliği değerini vermiştir. NLC formülasyonlarının in vitro salım profilleri incelendiğinde ilk 30 dakikada hızlı bir salım ve sonra daha yavaş bir salım hızı gözlenmiş ve 8. saatten sonra salım kontrollü bir şekilde devam etmiştir, SLN'de ise yüksek viskozite ve partikül boyutu değerinden dolayı daha yavaş bir salım profili gözlenmiştir. NLC'lerde sıvı yağ oranı artışı ve yüklenen SA miktarı artışıyla salım hızında artış sırası % 0.5SA-SLN < %1 SA-NLC(40:60) < %0.5 SA-NLC(40:60) < %0.5 SA-NLC(50:50) < %1 SA-NLC(50:50) şeklinde bulunmuştur. Boş ve %0.5 SA içeren NLC(50:50) formülasyonları kullanılarak L929 fare fibroblast hücre hattı üzerinde yapılan sitotoksisite çalışmalarında 12 ve 24 saat sonunda NLC'in % 0.125 ve % 0.5 arasındaki konsantrasyonlarda sitotoksik olmadığı sonucuna varılmıştır. Boş ve SA yüklü NLC(50:50), NLC(40:60) ve SLN formülasyonları 25°C ve 4°C stabilite çalışmalarında 90 gün boyunca partikül büyüklüğü, zeta potansiyeli, viskozite, hapsedilme etkinliği ve SA'in formülasyon içindeki stabilitesi incelenmiş ve her iki sıcaklıkta boş ve SA yüklü NLC(50:50) ve NLC(40:60) stabil bulunmuştur.

Anahtar Kelime: Nanoyapılı yağ taşıyıcılar (NLC), salisilik asit, in vitro salım, sitotoksisite, stabilite.

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SYMBOLS AND ABBREVIATIONS

CV	Coefficient of Variance
DMEM	Dulbecco's Modified Eagle's Medium
EE	Encapsulation Efficiency
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectrum
h	Hour
LC	Loading Capacity
LOD	Limit of Detection
LOQ	Limit of Quantification
metOH	Methanol
NLC	Nanostructured Lipid Carriers
PBS	Phosphate Buffered Solution
PI	Polydispersity Index
PS	Particle Size
RE	Relative Error
SA	Salicylic Acid
SE	Standard Error
SLN	Solid Lipid Nanoparticles
UV	Ultraviolet
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
$\bar{X}$	Arithmetic Average
ZP	Zeta potential
λ_{max}	Wavelength of the maximum absorbance value

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1. INTRODUCTION

The lipid nanoparticles were getting an increasing attention from the pharmaceutical technology research groups from the beginning of the 1990s. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been investigated as carrier systems for various applications. In this thesis different NLC formulations containing salicylic acid were developed for topical application.

The advantages of NLC and the factors affecting NLC formulations were studied in detail. Different production techniques with respect to various solid lipids, liquid lipids, surfactants and co-surfactants have been used for production of NLC and SLN formulations in literatures (1-3). High shear homogenization and ultrasonication technique is used in this work to develop NLC and SLN using Precirol ATO 5 as solid lipid, Labrafac lipophile as liquid lipid, Lipoid S100 as lipophilic and Tween 80 as a hydrophilic surfactants.

Because of the cosmeceutical properties of salicylic acid (SA), it was chosen as a model drug to incorporate into the NLC and SLN. Salicylic acid is a beta hydroxy acid applied topically for the treatment of hyperkeratotic and scaling skin conditions such as dandruff and seborrhoeic dermatitis, psoriasis, and acne. Salicylic acid produces desquamation of hyperkeratotic epithelium by dissolving the intercellular cement, which causes cornified tissue to soften, macerate, and desquamate. It is used in cosmetics at concentrations less than 3%.

The aims of this thesis were to develope optimized NLC formulations after discussing pre-formulation studies with various oil/fat ratios and to evaluate the effect of increasing oil content in NLC and the difference between the blank and SA loaded NLC and SLN in terms of particle size and distribution, zeta potential, viscosity, encapsulation efficiency, loading capacity, and stability of blank and SA loaded NLC and SLN formulations in terms of particle size and distribution, zeta potential, encapsulation efficiency, loading capacity, viscosity, chemical stability of SA in NLC and SLN formulations at both 4°C and 25°C, and in vitro release behaviour of SA through these formulations.

Cytotoxicity studies of blank and SA loaded NLC were also performed in 12 and 24 hours in order to evaluate whether these formulations are cytotoxic or not before and after incorporation of SA into NLC. Therefore these studies have been evaluated and discussed according to the obtained results.

2. THEORETICAL PART

2.1. Nanostructured Lipid Carriers (NLC)

Nanostructured Lipid Carriers (NLC) are new generation of colloidal carrier systems which are produced using blends of solid lipids and liquid lipids. The blends for the particles matrix are produced by controlled mixing solid lipids with liquid lipids. Because of the liquid lipid presence in these mixtures, a melting point depression compared to the pure solid lipid is observed, and the obtained blends are solid at room and body temperature (1,4,5).

2.1.1. Historical Review From SLN to NLC

Solid lipid nanoparticles (SLN) are definable from nanostructured lipid carriers (NLC) by their composition of the solid particle matrix. SLNs were designed and developed as an alternative carrier system to emulsions, liposomes and other polymeric nanoparticles (1).

SLN are identical to an oil-in water emulsion, except that the liquid lipid (oil) part of the emulsion is replaced by a solid lipid having particle size ranging between 50 nm and 1000 nm (2). SLN are produced by replacing the liquid lipid (oil) of an o/w emulsion by a solid lipid or a blend of solid lipids, the lipid matrix being solid at both room and body temperature. NLC were developed to minimize or avoid some problems that are obtained from SLN as drug loading such as gelation problem, and leakage of drug during storage and NLC with higher lipid concentration can be prepared, in which simplifies the production of final product (1,2).

NLC consist of a mixture of various lipids, solid lipid(s) is blended with liquid lipid in different ratios (6,7). In comparison with SLN, NLC show a higher loading capacity and a controlled and faster release profile due to their liquid parts and so that NLC is considered being the smarter, new generation of lipid nanoparticles (4,8).

In case of SLN is almost perfect crystallized in structure with an identically shaped molecules similar to a brick and mortar with limited loading capacity for active compounds and because of the solid lipid matrix that crystallizes and causes

drud expulsion. However lipid matrix of the NLC shows imperfections similar to a building from very different shaped stones, the increased number of imperfections leads to an increase in the loading capacity for active compounds (Figure 2.1) (1).

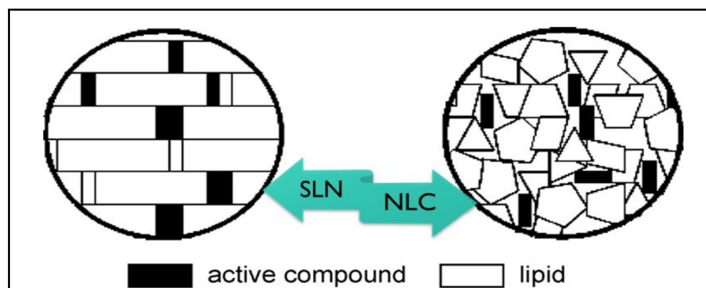


Figure 2.1. Structures of SLN and NLC (1) .

❖ **Advantages of NLC** (1,4,5,9-11)

- NLCs are new generation of lipid nanoparticles providing improved properties for drug loading of the active compound inside the particle matrix during storage.
- As the loading capacity is increased and expulsion of the drug during storage was avoided.
- Possibility to incorporate hydrophilic and lipophilic drugs.
- Increasing drug penetration into skin by creation of saturated system with NLC leading to an increased flux of the active compound.
- Improved physical stability are observed by changing the lipid concentration of NLC.
- Drugs showing higher solubility in oils than in solid lipids can be dissolved in the oil, and the chemical stability of sensitive active compounds are provided by surrounding lipids.
- Most of the lipids are GRAS "Generally Recognized as Safe" usually used in topical cosmetic or pharmaceutical preparations and the existing physiological and biodegradable lipids exhibiting low systemic toxicity and low cytotoxicity.

- The small size of the NLC/SLN provides close contact to stratum corneum and can cause an increase in the amount of drug penetrating into the mucosa or skin.
- Due to their lipid matrix, a controlled release from these carriers is possible. This becomes an important tool when it is necessary to develope a release for prolonged period of time, to reduce systemic absorption, and irritation in high concentrations.

❖ **Disadvantages of NLC**

Recently very few disadvantages for the NLC were reported in literatures (12-16) and they can be described as following:

- The non-spherical shape of particles in NLC and SLN may lead to higher polydispersity index compared to the nanoemulsions and because of the dilution process during size measurments of SLN and NLC differences were found between diluted and undiluted formulations (12).
- Although they are recognized as safe nanocarriers due to their composition, some studies reported cytotoxicity effect of these systems due to the combination of different solid lipids, liquid lipids and surfactants with various concentrations may lead to an increased cytotoxic effect after application with higher particle concentrations (13,14).
- Some recent studies demonstrated that NLC have no advantages over nanoemulsions (12,15).

2.1.2. Composition of NLC and SLN Formulations

NLC and SLN formulations are composed of lipid matrix, aqueous phase and surface active agents. There are various solid and liquid lipids used in preparation of SLN and NLC formulations. Apifil, Precirol ATO 5, Compritol 888 ATO, Daynasan 114, 116, 118 and stearic acid are the solid lipids frequently used in SLN and NLC formulations (17-22). Labrafac Lipophile, Labrafil, Oleic Acid, Labrasol, squalene are the liquid lipids used in combination with the solid lipids to prepare NLC (18,22-24). In addition different surfactants used in these systems lipophilc and/or hydrophilic character such as Tween 80, Tween 20, pluronic F68, sodium dodecyl

sulphate, sodium deoxycholate as hydrophilic surfactants, and types of soybeanlecithin (Lipoid S100, Lipoid S75), and myverol as lipophilic surfactants (25-28).

The lipid matrix used in this thesis is Precirol ATO 5 as solid lipid and Labrafac Lipophile as liquid lipid, surface active agents are Tween 80 as hydrophilic and Lipoid S100 as lipophilic surfactants. The dispersion phase is deionized water.

- **Precirol™ ATO 5 (Glyceryl Palmitostearate)**

Precirol™ ATO 5 is a market product of Gattefossé GmbH (Weil am Rhein, Germany). It`s synonyms are glycerin palmitostearate and glycerol palmitostearate. It has a melting point between 52°C and 56°C and HLB value of 2. Precirol ATO 5 forms lipid matrix for modified release tablets, provides lubrication for capsule filling, is used as taste-masking agent in coating technology (29,30) . It is used as a solid lipid in preparation of lipid particles, especially used in SLN and NLC formulations. In a study of Liu et al. (31) for topical application, isotretinoin loaded SLN formulation was evaluated using Precirol ATO 5 as solid lipid. Tween 80 and soybean lechitin were used as surfactants to stabilize SLN. Fang et al. (32) formulated SLN and NLC containing psoralen by using Precirol ATO 5 as the solid lipid for topical treatment of psoriasis. The characterization of physicochemical properties of SLN and NLC formulations and skin permeation of psoralen released from SLN, NLC were performed.

- **Labrafac™ Lipophile (Medium-chain triglycerides)**

Labrafac™ Lipophile is a market product of Gattefossé GmbH (Weil am Rhein, Germany). Synonyms of it are caprylate/caprate triglyceride, medium chain triglycerides. It is a liquid lipid with a HLB value of 2. In cosmetics and topical pharmaceutical preparations, medium-chain triglycerides are used as a component of ointments, creams, and liquid emulsions and have a number of advantages in pharmaceutical formulations, which include better spreading properties on the skin; good penetration properties; good emollient and cosmetic properties; good solvent properties; and good stability against oxidation (33-35).

- **Tween 80**

Tween 80 (polyoxyethylene 20 sorbitan monooleate) is a nonionic polysorbate derivative surface active agent with a high HLB value of 15. Tween 80 is widely used in topical formulations as SLN and NLC. Generally Tween 80 used combined with soybean lecithin in these formulations (32) . It is hydrophilic surfactant that is used widely as emulsifying agents in the preparation of stable oil-in-water pharmaceutical emulsions (34,36) . They may also be used as solubilizing agents for a variety of substances In a study of Chen et al. (37) was preparing podophyllotoxin loaded SLN formulation and stabilized by Tween 80 and soybean lecithin.

- **Soybean Lecithin (Lipoid S100)**

Lecithins are generally used in pharmaceutical products as dispersing, emulsifying, and stabilizing agents used in topical products such as creams and ointments. They differentiate in their physical form, from viscous semiliquids to powders, depending on the free fatty acid content. Lipoid S100 is light yellow in colour and is soluble in aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, mineral oil, and fatty acids. It is practically insoluble in cold vegetable and animal oils, polar solvents, and water (34,38).

It is frequently used in the formulation of NLC and SLN as lypophilic surfactant to improve stabilization (32). The main compound of lecithin is phosphatidylcholine. The composition of phospholipids in Lipoid S100 (w/w) is 94 % phosphatidylcholine, 0.5% N-Acyl-phosphatidylethanolamine, 0.1% phosphatidylethanolamine, 0.1% phosphatidylinositol, and 3% lysophosphatidylcholine (34,38).

Lecithin is a component of cell membranes and is therefore it has improved effect in skin permeation is related to both the solubilising effect of the lecithin matrix and the penetration enhancing effect of lecithin itself (34,39).

2.1.3. Types of NLC

Depending on the production method and composition, three types of NLC have been proposed (Figure 2.2) (1,4,9,40,41):

2.1.3.1. Imperfect Type (Type I)

This type of NLC generally has imperfections in the lipid matrix to fix and load the active compound in molecular form and amorphous clusters. By suitable used chemically different lipid molecules imperfect lipid crystals are formed to form the matrix by using solid lipids or liquid lipids. Because of the difference in their structure, they cannot fit together very well leading to more imperfections in the crystal structure leaving enough space to load the active compound, thus leading to improved encapsulation efficiency and higher drug loading capacity.

2.1.3.2. Amorphous Type (Type II)

The lipid matrix of this type of NLC is in amorphous state, solid, and with no crystallinity behaviour. They are produced by mixing solid lipid and liquid lipid in such a combination that the particles solidify after cooling but they do not recrystallize, thus remaining in the amorphous state. This type of NLC can be formed by the mixture of special lipids such as isopropylmyristate and hydroxyoctacosanylhydroxystearate, which do not recrystallize after homogenization and cooling. These lipids have ability to form solid particles of amorphous lipid structure and because the matrix maintained in the polymorphic α form, this can avoid the crystallization, and minimize drug expulsion.

2.1.3.3. Multiple Type (Type III)

This type of NLC is similar to multiple oil/fat/water (O/F/W) type of lipid nanoparticles characterized by the presence of tiny nanofractions of oil in the solid matrix. This controlled nanostructure results by mixing a solid lipid with a higher amount of liquid lipid. The liquid lipid needs to be chosen so that the melted solid lipid and liquid lipid are soluble at the production temperature. When the liquid is used in a high concentration that is above its solubility in the solid lipid at room

temperature, nanofractions are formed. This type of NLC enables high loading capacity due to the higher solubility of active compound in liquid lipid, which solubilises the active compound to a higher extent and control of drug release due to encapsulating lipid particle matrix.

Figure 2.2. Theoretical models for the structure of NLC (40,41).

2.1.4. Production Techniques of NLC

Production techniques of SLN are also applied for preparation NLC formulations (5,42):

- High Shear Homogenization and/or Ultrasonication Technique
- High Pressure Homogenization Technique
- Microemulsion Based Technique
- Double Emulsion Technique
- Solvent Emulsification-Evaporation Technique
- Solvent Emulsfication-Diffusion Technique
- Melting Dispersion Technique
- Phase Inversion Technique
- Lipid Nanopellets and Nanospheres

2.1.4.1. High Shear Homogenization and/or Ultrasonication Technique

High shear homogenization and ultrasonication are dispersing techniques which were initially used for the production of solid lipid nanodispersions. Both methods are widespread and easy to handle. However, dispersion quality is often compromised by the presence of microparticles. The first SLN was developed from lipid microparticles produced by spray congealing followed by lipid nanopellets produced by high speed stirring or sonication (43).

In this method the NLC/SLN prepared by dispersing the melted lipid phase in the aqueos surfactant solution at the same melting temperature using a high speed stirrer (Ultra-turrax) at different rpm for a certain time. The obtained pre-emulsion ultrasonified by a probe sonicator. In order to prevent recrystallization during homogenization, production temperature kept at least 5ºC above the lipid melting point. By cooling down the obained nanoemulsion in an ice bath NLC formulations can be obtained (44,45).

It is reported that the advantages of this technique are common and easy to handle. It is a simple, available and effective method to produce NLC without organic solvents; but it also having the limitation that, it may require an extra step of filtration of formed NLC emulsion in order to remove impurity materials produced during ultrasonication and is mostly understood by the presence of microparticles (45).

2.1.4.2. High Pressure Homogenization Technique

Two homogenization step, the hot and the cold homogenization techniques were developed by Muller et al. (2,46).

- **Hot Technique**

The hot technique which the formulations prepare at temperatures above the melting point of the used lipid and can therefore be regarded as the homogenization of an emulsion. A pre-emulsion of the drug loaded melted lipid phase and the hot aqueous surfactant phase is obtained by high-shear mixing device (Ultra-Turrax). This is followed by treating the mixture with a high pressure homogeniser by

applying different numbers of production cycles. Subsequently the dispersions allowed to recrystallise at room temperature forming NLC (2,47).

- **Cold Technique**

Cold homogenization is prepared with the lipids containing drug and therefore called as milling of a suspension. Cold homogenization has been developed to prevent temperature sensitive drugs from degradation, partitioning of hydrophilic drug from lipid phase to aqueous phase, and complexity of the crystallization step of the nanoemulsion leading to several modifications and/or supercooled melts. The first step of preparation is same as hot homogenization which can be performed by dispersion, dissolving or solubilisation of the drug in the melted lipid. Then the drug-lipid mixture is rapidly cooled either by liquid nitrogen or dry ice. The drug containing solid lipid is milled by a mortar or ball mill to micron size, and microparticles are dispersed in cooled emulsifier solution obtaining a pre-emulsion. Then this pre-emulsion is homogenized at high pressure homogenization at room or below room temperature, where the cavitation force is strong enough to break the microparticles to form SLNs/NLCs. This method minimizes the thermal exposure of drug, but it does not avoid it because of the melting of the drug-lipid mixture in the initial step (2,48).

2.1.4.3. Microemulsion Based Technique

Microemulsions are clear, thermodynamically stable system composed of a lipophilic phase, surfactant and co-surfactant and water. The concept of microemulsion technique for the production of these systems was developed and optimized by Gasco et al. (49,50) . The method based on dilution of microemulsions. They are made by stirring an optically transparent mixture at 65–70 ºC which is composed of a low melted fatty acid with the liquid lipid, a surfactant, co-surfactant and water. The hot microemulsion is dispersed in cold water (2–3 ºC) under mechanical stirring. The volume ratios of the hot microemulsion to cold water are in the range of 1:25 to 1:50 under mechanical stirring to form NLC/SLN dispersion. The dilution process is determined by the composition of the microemulsion (42,51).

2.1.4.4. Double Emulsion Technique

In double emulsion technique the drug (mainly hydrophilic drugs) was dissolved in aqueous solution, and then was mixed with sufficient lipid-surfactant mixtures which were dissolved in organic solvents forming a primaty emulsion. This primary emulsion was stabilized by adding a stabilizer and this stabilized primary emulsion was dispersed in aqueous phase containing hydrophilic surfactant forming w/o/w emulsion. NLC was formed by stirring double emulsion and evaporating organic solvent in evaporator or at room temperature for a certain time (52,53).

Double emulsion technique avoids the need to melt the lipid for the preparation of peptide-loaded lipid nanoparticles and the surface of the nanoparticles could be modified in order to sterically stabilize them by the incorporation of a lipid/PEG derivative. This technique is commonly used to encapsulate hydrophilic drugs. A major disadvantage of this technique is the formation of high percentage of microparticles (54,55).

2.1.4.5. Solvent Emulsification-Evaporation Technique

In solvent emulsification-evaporation method, the lipophilic material and hydrophobic drug were dissolved in a water immiscible organic solvent and then that is emulsified in an aqueous phase using high speed homogenizer Thenby evaporating the organic solvent by mechanical stirring at room temperature, the aqueos suspension were concentrated in a low pressure system forming NLC. However it is repoted that because of use of organic solvents in this method, it may cause an increase in toxicity effect for the obtained product (42,52,56-58).

2.1.4.6. Solvent Emulsification- Diffusion Technique

In solvent emulsification-diffusion technique, the solvent used must be partially soluble with water and this technique can be carried out either in aqueous phase or in oil (59). At the beginning, both the solvent and water were saturated together in order to produce the initial thermodynamic equilibrium of both liquid (60).

The saturation step was performed at that temperature when heating is required to solubilize the lipid, Then the lipid and drug were dissolved in water saturated solvent and this organic phase was emulsified with solvent saturated aqueous solution containing stabilizer using mechanical stirrer. Water used in ratio ranges from 1:5 to 1:10, were added to the system after the formation of O/W emulsion in order to allow solvent diffusion into the continuous phase, thus forming aggregation of the lipid in nanoparticles. Here both phases should be carried out at same temperature and the diffusion step was performed either at room temperature or at the temperature under which the lipid was dissolved (61). Throughout the process constant stirring was provided and the diffused solvent was eliminated by vacuum distillation or lyophilization (26,62-64).

2.1.4.7. Melting Dispersion Technique

In this technique the first step is melting the drug, solid lipid and liquid lipid in an organic solvent regarded as oil phase and at the same time water phase was also heated to same temperature as oil phase. Then in second step, the oil phase added in to a small volume of water phase and the resulting emulsion was stirred at higher rpm for few hours. At last it was cooled down to room temperature to give NLC. The last step was same as solvent emulsification-evaporation method except that in melting dispersion method no organic solvent had to be evaporated. It is reported that reproducibility was less than that of solvent emulsification-evaporation method but more than ultrasonication method (26,65,66).

2.1.4.8. Phase Inversion Technique

This technique is developed by Heurtault et al. (67,68) and consists of two steps. The first step to form particles by magnetic stirring of all the components while heating from ambient temperature to 85 ºC at a rate of 4 ºC/min. Three cycles of progressive heating and cooling in between 85 and 60 ºC at a rate of 4 ºC/min were realised to obtain the phase inversion. The second step was a fast cooling and dilution process with cold water (0 ± 1ºC) in order to divide the hot initial structure. Then, a slight magnetic stirring was applied to the nanosuspension during 5 min to form NLC.

2.1.4.9. Lipid Nanopellets and Nanospheres

In the 1989, Spesier et al. (43,69) were the first to report making solid lipid particles for drug delivery application. They created an initial nanoemulsion by dispersing melting lipid in a surfactant solution using high speed mixing or ultrasonication; the nanoemulsion was subsequently spray dried/ spray congealed to produce the lipid pellets so further called as lipid nanopellets.

The first lipospheres developed by Domb et al. (70) are solid, water insoluble microparticles that have a layer of a phospholipid embedded on their surface. Lipospheres comprise a core formed of a hydrophobic material solid at room temperature and a phospholipid coating surrounding the core. They are prepared by melting the core material, adding phospholipid along with an aqueous medium and dispersing the melted material at increased temperature by mixing techniques, such as mechanical stirring or sonication. Cooling leads to solid lipospheres.

2.1.5. Characterization of Nanostructured Lipid Carriers

Characterization processes of NLC are:

- Particle Size and Distribution Analysis
- Zeta Potential Analysis
- Crystallization and Lipid Modification Analysis

2.1.5.1. Particle Size and Distribution Analysis

It is reported that particle size and distribution of NLC dispersions can be determined by photon correlation spectroscopy (PCS) or laser diffractometry (LD) (2,24,42,71). PCS is common technique used to measure the size of particles in the submicron range. Dynamic light scattering (DLS) and quasi-electric light scattering are synonyms for PCS. PCS measurements are based on the random “Brownian” motion of particles. The scattered light intensity from the speed at which the particles are diffusing due to Brownian motion is measured (72) .

However in case of larger particle size measurements laser diffractometry (LD) is a technique for measuring the particles size of nanoparticles and

microparticles in the range of 10 nm to 2000 µm. It is based on the phenomenon that particles scatter light in all directions and diameters indicate the percentage of particle volume below a certain size (71,73) . Ricci et al. (44) determined the particle size of NLC after dilution with distilled water using photon correlation spectroscopy (PCS) to determine the mean particle size and population distribution of the bulk particle dispersion. Hentschel et al. (74) were investigated the particle size of β-carotene loaded NLC formulations using PCS to measure intensity weighed particle size, and also by using LD to indicate the percentage of particle volume weighed size of NLC.

2.1.5.2. Zeta Potential Analysis

Zeta potential is the electric potential of a particle in a suspension. It is a parameter which is very useful for the evaluation of the physical stability of colloidal dispersions. Zeta potential measurements allows predictions about the storage stability of colloidal dispersions. In general, particle aggregation is less likely to occur for charged particles with high zeta potential value due to electric repulsion (2,75,76) .

Freitas and Muller (75) investigated the effect of light and temperature on zeta potential of these nanocarrier systems and they studied the relation between these variables. They concluded that structural changes have been occured during storage the change in zeta potential proves the existance of such changes in the system and they are accelerarated by factors of light and temperature.

Besides Varshosaz et al. (77) were investigated particle size and zeta potential of valproic acid loaded NLC formulation using PCS after diluting NLC with deionized water and they evaluated influence of different factors on zeta potential of NLC. In the same manner, Hu et al. (78) evaluated the effect of storage time on zeta potential of dispersed NLC and SLN formulations in their stability studies.

2.1.5.3. Crystallization and Lipid Modification Analysis

Particle size analysis is a necessary but not the only method to characterize NLC. Degree of lipid crystallinity and modification of lipid are also important analysis because they are strongly related with the drug incorporation into NLC and release rate from NLC (42) .

It is supposed that, below the melting point of lipids the super-cooled melts are in form of lipid emulsions and no lipid crystallization occur in this state. However this is not desired form for the SLN/NLC, because the advantages of these systems are dependent on the solid or semisolid state. This cooling step may be affected by the size, surfactants and the loaded drugs.Thus, sometimes the recrystallization of the melted lipid may take longer time forming supercooled melt form. Where the lipid is in crystalline solid state it is existed in crystalline β-modification. This β-modification is the most stable case for these solid lipids. As the solid lipid melts and then solidifies and transition from β`-modification to β-modification occurs. Transition to β means a highly ordered crystalline packing, and hence a reduction in amorphous regions and crystal defects. However lipid dispersions recrystallize firstly forming α-modification and this by time display stable forms. Due to the type of lipid modification in formulations, the order of decrease in drug incorporation efficiency is as follows (42,79,80):

Super-cooled melt > α-modification > β`-modification > β-modification

The β-modification is a potential problem in SLN. By creating less ordered solid lipid matrix (α or β’) by blending solid lipid with liquid lipid a higher loading capacity can be achieved with avoiding drug expulsion in NLC. Surface morphology, lipid modification and thermal analysis of NLC dispersions can be characterized by the following techniques:

2.1.5.3.1. Thermal Analysis Techniques

- Differential Scanning Calorimetry (DSC) (8,24,25)
- Thermogravimetry Analysis (TGA) (8,28)

✓ **Differential Scanning Calorimetry (DSC)**

In general, the production technique of SLN and NLC effects the polymorphism of the lipid, which is frequently different in the formulation after solidification, in comparison to the bulk material (8). DSC is usually used to get information about both the physical and the energetic properties of a compound or formulation. DSC measures the heat loss or gain as a result of physical or chemical changes within a sample as a function of the temperature. DSC analysis has been used to determine the state and the degree of crystallinity of lipid dispersions. It allows the study of the melting and crystallization behavior of crystalline material like lipid nanoparticles and also it is useful to understand solid dispersions like solid solutions, simple mixtures or as in the case of NLC, drug and lipid interactions and the mixtures of solid lipids and liquid lipids (oils) (24,25).

✓ **Thermal Gravimetry Analysis (TGA)**

Beside characterization of particle size distribution and crystallinity of lipid nanoparticles, the chemical stability of the active ingredient could be determined in NLC by TGA . Souto and Muller (28) have reported that the chemical stability of the active ingredient loaded into NLC can also be evaluated by this method and they investigated the chemical stability of ketoconazole loaded into NLC at high temperature to support the chemical stability of the drug under production conditions.

2.1.5.3.2. Surface Morphology and Lipid Modification Analysis

- Light microscopy
- Scanning Electron Microscopy (SEM)
- Transmission Electron Microscopy (TEM)
- FTIR-Infrared and Raman spectrosopy
- Atomic force microscopy (AFM)
- X-Ray Scattering

✓ **Light Microscopy**

The characterization of these systems could be performed also by light microscopy. The particle size of colloidal carriers could be determined using Light microscopy with or without polarized light and also it is a method for detemination of the morphology and structure of NLC (71). Obeidat et al. (81) have reported that this method could be used in detection of larger particles which become visible and could be observed during stability studies as a result of the instability cases of NLC. In addition in product development, it is only important to screen for preservatives which do not influence the physical stability of the colloidal system.

✓ **Scanning electron microscopy (SEM)**

SEM is a type of electron microscopy that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. This method can be used to study surfaces of solid objects directly surface topography, composition and conductivity and also the surface morphology of NLC formulations can be visualised by SEM (7).

✓ **Transmission electron microscopy (TEM)**

Transmission electron microscopy (TEM) is a method of probing the microstructure of rather delicate systems such as micelles, liquid crystalline phases, vesicles, emulsions, and also nanoparticles (17,82). On the other side TEM is also used to study the surface morphology of NLC (82).

✓ **AFM (Atomic Force Microscopy)**

AFM is a key tool in the imaging of soft materials, biological and colloidal systems (83,84) in which utilizes the force acting between a surface and a probing tip resulting in a spatial resolution of up to 0.01 nm for imaging. The AFM investigations revealed the disk-like structure of the particles (42,85).

Shahgaldian et al. (58) studied the AFM imaging of calixarene based nanoparticles dispersed in various gels used in topical preparations and they concluded that nanoparticles which are not involved in surface features can be

observed due to their effects on local surface forces and the local mechanical properties of the gels.

- ✓ **FTIR-Infrared and Raman spectroscopy**

FTIR spectroscopy shows promise in its ability to detect the conformations of lipid molecules in matrices. Lin et al. (24) used FTIR to characterize the molecular dynamics of components of monocaprate loaded NLC and SLN.

- ✓ **X-Ray Scattering:**

The lipid crystalline structure, which is related to the chemical nature of the lipid, is a key factor in determining whether the liquid oil is expelled or incorporated into a carrier system (45). Lin et al. (24) used the x-ray diffraction to investigate changes of the microstructure in the lipid crystallization process of monocaprate loaded NLC using x-ray diffractometer.

2.1.6. Incorporation of Active Compounds into NLC

As mentioned in 2.1.3, depending on the production method and composition of lipid blend of NLC and types of NLC (Figure 2.2) factors determining the incorporation of drug into these carriers are (2):

- Drug solubility in melted lipid
- Miscibilty of the drug and lipid melt
- Chemical and physical structure of lipid matrix
- Polymorphic state of lipid material and production temperature

The loading capacity of active compound is increased and expulsion of the active compound during storage can be avoided by giving the lipid matrix a certain nanostructure. Drug molecules can be incorporated as amorphous clusters or in between imperfections of the lipid matrix created in the NLC structure, thus loading increases with increasing the number of imperfections within the lipid matrix. NLC are considered to be smart carriers also because their lipid modification and content

of liquid lipids could be controlled and designed by the solubility property desired for the incorporated drug (1,86).

Loading capacity and encapsulation efficiency are different from each other. The percentage of drug encapsulated inside the lipid nanoparticles relative to the total drug added specifies the encapsulation efficiency. It specifies how many percent of drug are included in the particles and how many percent of free drug are still present in the dispersion medium. Loading capacity refers to the percentage of drug incorporated into the lipid nanoparticles relative to the total weight of the lipid phase (87).

In the imperfect NLC, as solid lipids and liquid lipids are blended, the difference in the structure of the lipids and special requirements in crystallization process lead to a highly disordered, imperfect lipid matrix structure offering space for drug molecules and amorphous clusters of drugs to be incorporated. Compared to SLN, a potential problem was the formation of a perfect crystal which can assimilate to brick and mortar structure. Drug loading into SLN is limited due the formation of the lipid crystal and drug expulsion is occured by any crystallization process forming a perfect crystal. Thus by avoiding crystallization as in the amorphous NLC, the lipid matrix is solid but not crystalline. This can be achieved by mixing special lipids and the absence of crystallization avoids drug expulsion during crystallization (1,88).

The multiple type of NLC which is considered to be similar to w/o/w emulsions. The solid lipid matrix contains tiny oil nanofractions. Hereby when the solubility of drugs in oil is higher than their solubility in solid lipids leads to the formation of multiple type NLC (1,4). Hence, compared to SLN, the presence of liquid lipids in NLC are characterized by their higher drug incorporation rate and chemical stabilisation of the active compound which is released at a controlled rate (89).

Souto et al. (90) were developed clotrimazole loaded SLN and NLC and they studied the incorporation rate and percentage of the active compound in these systems. They considered NLC formulations have a liquid core and thus they obtained the imperfect NLC type that had resulted higher incorporation of the drug in NLC more than SLN. As a result the incorporation of active compounds into NLC

especially the imperfect NLC and amorphous NLC are though to provide more space and flexibility of incorporation to reach to the desired prolonged release profile (4,86).

2.1.7. Release of Active Compounds from NLC

Release of active compounds from NLC and release mechanism of these systems can be controlled by adjusting the content of liquid lipid depending on the incorporation model of NLC (4). Drug release from NLC can be realized by 2 different mechanisms:

- Burst Release
- Sustained Release

✓ **Burst Release**

Burst release as well as sustained release has been reported for both SLN and NLC. Burst release might improve the penetration of active compounds (2). Results indicate that in the initial releasing stage, the release rate mainly depends on some drugs which are free and adsorbed or deposited on the surface of lipid nanoparticles. They can be dissolved and spread rapidly. The reason for the rapid release rate can be attributed to two parts. First, it is possible due to the rapid diffusion of the free drug molecules. Second, it is because the amount of the drugs loaded in the inner part of nanoparticles is more than that of loaded in the surface layer, and the surface of nanoparticles combined with more surfactant molecules, which is good for dissolution from the surface layer. Therefore, the phenomenon of a burst release indicates the release mechanism is mainly as diffusion (86,91).

It is reported by Muller et al. (1,92) that the regulation of drug release into skin layers and drug penetration across skin membranes can be achieved by NLC. Due to the application of NLC formulation on skin with the increasing temperature and water evaporation, the lipid polymorphic structure of NLC transforms from the unordered form to the ordered form leading to a triggered release and thus creating a supersaturation effect of NLC leading to enhanced drug penetration into skin.

✓ **Sustained Release**

This release mechanism is important for active ingredients that are irritating at high concentrations, and for improving drug release to the skin over a prolonged period of time. Sometimes controlled fast release might be desired after diffusion and degradation. This release is thought that it should be triggered by an impulse when the particles are administered. By lipid particle degradation in the body fluid, drug release from lipid particles can occur (2,40).

Drug release from SLN and NLC has been evaluated using steroids as well as other agents as model drugs (46). It was concluded by the study of the release mechanism of valproic acid from NLC shows that drug release from NLC is more affected by the surfactant concentration than other factors. The type of lipid, surfactant and sonication time are also effective factors. Increasing surfactant concentration increases release rate percentage that drug release from NLC is more affected by the surfactant concentration than other factors. Increasing the sonication time decreased the drug release percentage that may be because of more interaction between particles, more aggregation and increasing the size as well as decreasing its surface area (77).

There are products which are desired to obtain a prolonged release after application onto skin as in case of UV blockers, perfumes and insect repellents in which they may cause skin irritation or allergic reactions (92,93). Their release can be controlled by incorporating into SLN and NLC systems. Prolonged release for SLN was observed for insect repellents such as DEET (94). SLN and NLC are colloidal carrier systems providing controlled release profiles for many substances (28) , such as ascorbyl palmitate (18), clotrimazole (90), ketoconazole (28) and suncreens (1,10,11).

Controlled drug release from NLC systems could be explained considering both drug partitioning between the oil nano compartments and the solid lipid and a successive partitioning of drug between solid lipid and water (44). Joshi et al. (95) investigated the drug release from NLC dispersion could show an initial burst release in the first hours and this is explained by fact the amount of drug encapsulated and the free drug remained in the outer shell of the NLC. Thus initial burst may be due to

release of this unencapsulated drug getting released first from the micelles then from the oil globules in the dissolution medium. Complete release occurred at the end of 24 h indicating that the drug encapsulated in the solid lipid core can also leach out even though at a slower rate.

2.1.8. Stability of NLC

Physical and chemical stability studies of NLC have been evaluated at different temperatures 4°C, 25°C, 40°C and at different time intervals by evaluating particle size and distribution, zeta potential and the lipid crystallinity and modification of NLC. Different factors such as type and concentration of used lipid blends, surfactants and production techniques are reported to be effective on NLC stability during storage at various temperatures (25,53,68,74) .

It is reported by Hu et al. (78) that the stability of NLC during prolonged storage can be assessed after storage a definite volume of dispersion into vials at 25°C and 4°C and monitoring changes in particle size, zeta potential, and drug content as a function of time. It was found that for the case of 25 °C, the particle sizes of both NLC and SLN were increased significantly. However, the particle growth was slower when nanoparticles were stored at 4 °C. This result is thought to be because of the increasing in the kinetic energy of system which coild accelerate collision of particles leading to aggregation during storage. Effect of storage time on the drug loading was also studied and found that NLC exhibited a good ability to reduce drug expulsion. Stability of NLC formulation and the active compounds loaded into NLC have been discussed in terms of type and concentrations of the used lipid matrix, surfactants, also chemical stability of drugs in NLC is an important factor in stability studies (4,18,77,96).

The protective effect of NLC against chemical degradation of incorporated drug was further demonstrated by Uner et al. (97) in which they investigated that Ascorbyl Palmitate loaded NLC had good chemical and physical stability at 4°C and 40°C for three months was observed. Besides, Han et al. (25) studied the effect of different surfactants and different blends of surfactants in NLC stored several weeks and 1 year at 4°C. As in these results it is concluded that surfactant type and blends of surfactants could effect the physical and chemical stability of the formulations.

Lipid nanoparticle dispersions with increasing lipid content were produced and it is found that the highly concentrated NLC dispersions form a pearl-like network (Figure 2.3). Muller et al. (1,4) investigated the particle size of these systems during 6 months storage and they obtained that no increase was observed in particle size and also the particles were in a fixed position and could not undergo collision and flocculation. The higher concentrated NLC were practically unchanged in size during storage avoiding the problems of lower concentrated SLN dispersions. It is reported that after administration of NLC and dilution with fluids, the network is destroyed releasing single and nonaggregated particles. However the concentrated solid lipid nanoparticle dispersion aggregate during storage time. Concluding that NLC are stable systems during storage time. Thus also because of the highly imperfect types of NLC, they could also improve stability during storage thus by avoiding burst effect and leakage of loaded active compounds.

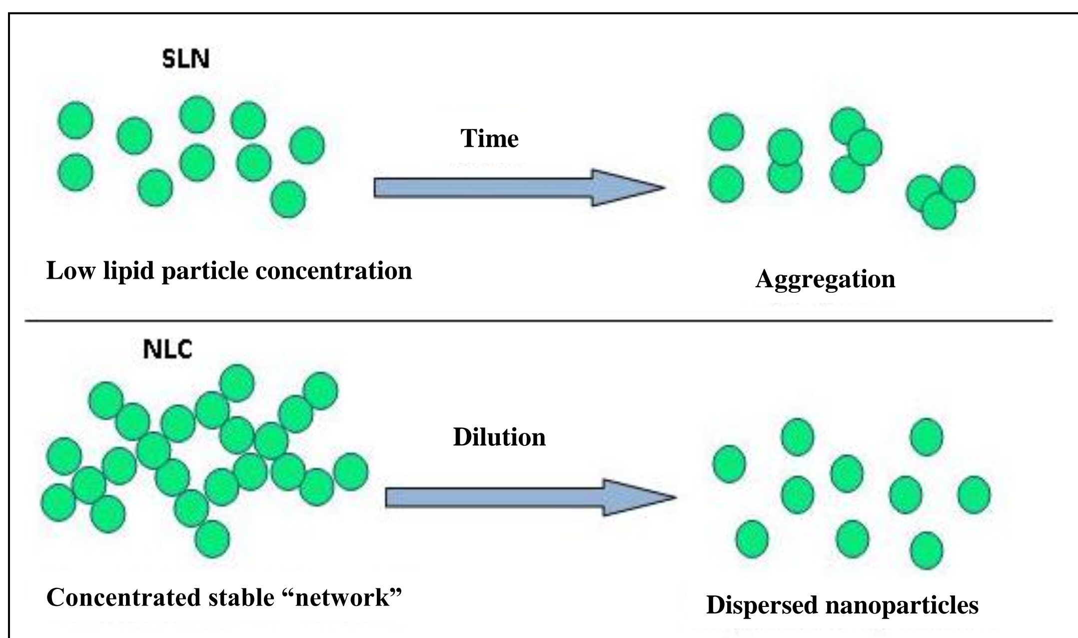


Figure 2.3. Aggregation phenomenon in low concentrated SLN dispersions (upper), and stability of high concentrated NLC (lower) (4).

2.1.9. Cytotoxicity of NLC

Different factors affecting the NLCs cytotoxicity were studied by evaluating the composition of NLC formulations containing various amounts and types of solid and liquid lipids, surfactants and as well as the effect of the formulations were evaluated before and after drug loading (14,98).

In general cytotoxicity studies are usually evaluated by applying the formulations on cells and then results are expressed in terms of cell viability and metabolical changes obtained by using colorometric MTT, XTT or WST-1 assays (14,99). Cytotoxicity studies of SLN and NLC were studied on different cell lines and evaluated by means of cell viability, proliferation, and biological activity in literatures (14,100). Scholer et al. (98) have reported in their study that the nature of the lipid matrix and concentration of nanoparticles influenced their cytotoxic effects on macrophages. A variety of different emulsifiers have been used for the preparation of SLN and NLC, including bile salts, poloxamers, and other ionic and nonionic surfactants which can induce irritative, hemolytic, or sensitizing action. Besides, Li et al. (101) investigated the efficasy and safety of bufandienolides loaded NLC on human astrocytoma cell line (U87-MG) and human gastric carcinoma (HGC-27) cell line. They found that entrapped drugs in NLC were able to reduce the cytotoxicity of bufadienolides. Hence cytotoxicity tests on cell lines showed that bufandienolide loaded NLC had a comparable cytotoxicity to the free drug by reducing the adverse effects of the bufandienolide solution. In addition, Muller et al. (14) investigated cytotoxicity of SLN formulations on Human Promelotic cells with regard to the used lipids and surfactants. They studied SLN composed of solid lipids Dynasan 114 or Compritol ATO 888 and stabilized with one of these surfactants: Lipoid S75, Lipoid KG, Poloxamers (184, 188, 235, 335, 407) or Tween 80. They were found that the nature of lipid had no effect on cell viability results. However cytotoxicity was increased with increased nanoparticle concentration and this is concluded to be due to the used surfactant type and concentrations but no cytotoxic effect observed for SLN stabilized with Lipoid S75 on Human Promelotic (HL60) cells among the other surfactants.

2.2. Pharmaceutical and Cosmetic Applications of NLC

The investigated applications of these carriers are :

- Parenteral Application
- Oral Application
- Ocular Application
- Pulmonar Application
- Carriers for Vaccines and For Brain Targetting
- Dermal and Cosmetic Application

2.2.1. Parenteral Application

NLC can be injected either intravenously, intramuscularly, subcutaneously or to the target organ, because of their small size. SLN and NLC provide a sustained release depot of the drug when administered subcutaneously or intramuscularly. The drug can be incorporated between fatty acid chains, lipid layers or imperfections in the lipid nanoparticles. Depending on the drug/lipid ratio and solubility, the drug is located mainly in the core of the particles, in the shell or molecularly dispersed throughout the matrix (102). The in vivo study of antimalarial activity of nanoject-NLC formulations was investigated by Joshi et al. (95) and they found that nanoject-NLC were significantly more effective as compared to the conventional enjectable formulation.

Besides coating of NLC with PEG are thought to increase stability and plasma half life of NLC/SLN in order to decrease phagocytic uptake, and therefore improves the biovailability of drugs. The inability of potent drugs to pass blood brain barrier (BBB), which is formed by the endothelium of brain vessels, basal membrane and neurological cells causes a limited treatment of central nervous system diseases such as brain tumors, AIDS, neurological and psychiatric disorders. By hydrophilic coating of colloidal carriers they could improve the transport of these drugs through BBB (103,104).

2.2.2. Oral Application

The three different types of lipid nanoparticles SLN, NLC, and LDC (Lipid Drug Conjugate) represent promising carrier systems for the oral delivery of lipophilic and also hydrophilic drugs, especially for hydrophilic drugs showing a reduced stability and a limited bioavailability. Lipid nanoparticles utilizes the absorption enhancing properties of lipids, which are used for new improved delivery systems and oral dosage forms (87). Lipid-based drug delivery systems are expected as promising oral carriers because of their potential to increase the solubility and improve oral bioavailability of poorly water-soluble and/or lipophilic drugs (105).

Zhuang et al. (63) investigated that NLC could improve the gastrointestinal absorption and offer a potential approach to enhance oral bioavailibility of poorly water soluble drugs with low dissolution rate in intestinal tract . The aim of the study was to evaluate the in vivo and in vitro efficacy of vinpocetine loaded NLC formulation. As a result it was concluded that in vitro release study indicated that vinpocetine–NLC showed a sustained release profile of vinpocetine without obvious burst release effect. In vivo pharmacokinetic study showed that the relative bioavailability of vinpocetine–NLC formulation was much higher as compared with vinpocetine suspension in wistar rats after oral administration.

2.2.3. Ocular Application

Bioavailability of traditional ocular drug delivery systems such as eye drops is very poor because eye is protected by a series of complex defense mechanisms that make it difficult to achieve an effective drug concentration within the target area of the eye. Many approaches have been developed to solve the problem in recent years, of which colloidal drug delivery system has been showed more attention (106).

Ophthalmic drug delivery with long pre-corneal retention time and high penetration into aqueous humor and intraocular tissues is a limiting factor for the treatment of ocular diseases and disorders. Within the study of cyclosporin loaded NLC, the conjugate of Cysteine-Polyethylene Glycol monostearate (Cys-PEG-SA) was synthesized and was used to compose the thiolated NLC (Cys-NLC) as a potential nanocarrier for the topical ocular administration of Cyclosporine A (CyA).

The rapid cross-linking process of Cys-PEG-SA in vitro was found in simulated physiological environment. The in vitro CyA release from Cys-NLC was slower than that of non-thiolated NLC due to the cross-linking of thiomers on the surface of nanocarriers. After topical ocular administration in rabbits, the in vivo ocular distribution of CyA was investigated in comparison of Cys-NLC with non-thiolated NLCs and oil solution. The results showed that CyA concentration in systemic blood was very low and close to the detection limit. These results demonstrated that the thiolated NLC could deliver high level of CyA into intraocular tissues due to its bioadhesive property and sustained release characteristics (107).

2.2.4. Pulmonar Application

Recently biodegradable nanoparticles have indicated many advantages among inhalation drug delivery systems in terms of protecting the active compound from degradation and releasing the drug in a controlled way for prolonged periods of time. Because SLN and NLC have good tolerability, biodegradability and better stability during shear forces arised during nebulization, they are found to be good carrier systems for pulmonar drug delivery (108).

Patlolla et al. (109) explored the possibility of NLC as a novel carrier system for inhalation drug delivery of celecoxib. They studied the in vitro release effect of celecoxib-NLC, aerodynamic properties and in-vitro cytotoxicity. The investigation was also aimed to evaluate the in-vivo pulmonary deposition and systemic availability of aerosolized celecoxib loaded NLC. As a result they concluded that the celecoxib-NLC formulation was able to release the celecoxib in a controlled way for a prolonged period of time and the aerodynamic diameter was within the nebulization limits. The plasma samples analysis indicated the controlled release of celecoxib from the celecoxib-NLC formulation, where the celecoxib plasma levels were maintained at constant levels for 6 h after nebulization and in the case of the celecoxib-Solution the plasma levels fell rapidly below detection limits after 6 h (109).

2.2.5. NLC as Carriers for Vaccines and for Brain Targeting

NLC and SLN were used as carriers of different drugs for targetting because of their loading and release modifications and the carrier lipids are biodegredable and safe.

Adjuvants are used in vaccination to enhance the immune response (110). The side effects of Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) are too strong to be employed. The lipid components of SLN are degraded more slowly than emulsions, providing a longer lasting exposure to the immune system. Degradation can be slowed down even more when using sterically stabilizing surfactants that hinder the anchoring of enzyme complexes. In a first study SLN have been tested as adjuvant in comparison to FIA in sheep. The two SLN formulations indicated 43 and 73% of the immune response of FIA investigated as standard (2).

Hsu et al. (111) were prepared an anti-parkinson drug, apomorphine loaded NLC with a cationic charge to investigate whether the duration of brain targetting and accumulation of drugs in brain can be improved. They concluded that as compared to the aqueous solution, the brain targeting efficiency of NLC was greatly improved according to in vivo bioluminescence monitoring. NLC successfully targeted the midbrain and may greatly improve the ability of drugs to treat Parkinson’s disease.

2.2.6. Dermal and Cosmetic Applications of NLC

Topical treatment of skin disorders has the advantage that more effective drug concentrations can be obtained at the site of disease, and systemic side effects can be reduced compared to oral or parenteral drug administration. Topical drug administration is still a problem in pharmaceutics due to the difficulties in controlling and determining the exact amount of drug that reaches to different skin layers. The physicochemical properties of drugs and the vehicles are considered to be the main properties responsible for the drug distribution in the skin. Lipid nanoparticles have been investigated to improve the treatment of various skin diseases. Apart from the treatment of skin diseases by topical application, side effects of certain drugs can

also be decreased by topical treatment. The possibility to enhance the percutaneous absorption with lipid nanoparticles was observed and these carriers may even allow drug targeting to the skin or even to its layers. Thus, they are thought to have the potential to improve the benefit/risk ratio of topical drug therapy (1,89,92).

SLN and NLC are attractive colloidal carrier systems for skin applications due to their different desirable effects on skin. They are based on non-irritant and non-toxic lipids and thus they are considered to be suitable carriers that can be used on damaged or inflamed skin (112). Many studies have been reported on the topical application of NLC and during the last few years, SLN and NLC have been studied with active compounds such as vitamin K and insect repellents (DEET) (94,113), vitamin E, α-tocopherol (112), retinol (114), ascorbyl palmitate (18), clotrimazole (90), valdecoxib (51) , α-lipoic acid , and Co-enzyme Q10 (96,115).

Joshi et al. (6) compared a NLC-based gel of celecoxib with a micellar gel with the same composition regarding the *in vitro* skin penetration using rat skin and the pharmacodynamic efficiency by aerosil induced rat paw edema The in vitro permeation of celecoxib from NLC gelwas less than the permetration fromthe micellar based gel, which confirms findings about nanoparticles leading to a drug deposit in the skin resulting in sustained release. The in vivo comparison of the percentage edema inhibition produced by NLC and micellar gel showed a significant higher inhibition after application of the NLC based gel up to 24 h.

Ketoprofen and naproxen are non-steroidal anti-inflammatory drugs used for the treatment of musculoskeletal disorders, rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. Puglia et al. (116) prepared ketoprofen and naproxen loaded NLC which were incorporated into gels and compared to reference gels containing ketoprofen or naproxen solution, respectively. The in vitro percutaneous absorption, the in vivo active localization in the stratum corneum and the anti-inflammatory effect were studied. NLC were able to reduce the drug penetration through excised human skin while it was found by tape-striping test that the drug permeation and drug accumulation in the horny layer was increased (116).

Certain dermal and cosmetic benefits of NLC formulations were mentioned in published literatures (5,117-120):

- Providing chemical stability of active compounds
- Adhesiveness ,occlusion and skin hydration
- Lubrication, smoothness and emolliency

2.2.6.1. Providing chemical stability of active compounds

Liposomes and emulsions have a limited extent to protect chemically instable active compounds against degradation. They belong to the most frequently used carriers for the delivery of cosmetic active compounds. Fast partitioning of lipophilic active compounds between oil phase and water occurs because of the liquid character of oil droplets in O/W emulsions. Similarly for liposomes lipophilic active compounds take place in the lipid blayer and the same partitioninf of phenomena occurs. Degradation of the active compound occurs in the water phase. Hydrophilic active compounds dissolve in the water phase of liposome and exchange occurs with the external water phase. This exchange could be decreased by increasing the viscosity of liposome blayers. In case of a solid particle matrix these exchange and degradation are avoided or at least slowed down. The exchange between the inner and outer phase of these systems does not occur or it is slower.

2.2.6.2. Adhesiveness, occlusion and skin hydration

Adhesive effect increases with the decrease of particle size of nanoparticles. The adhesiveness is indicated for polymeric nanoparticles and for liposomes and nanoparticles form cause a film formation on the skin after application and thus producing a hydration effect on skin. The dermis loses its elasticity during getting aged. Because of the hydration properties of these systems, it can be assumed that lipid nanoparticles may enhance skin elasticity that is an important step to prevent or delay skin aging. It is considered that SLN and NLC may also increase penetration of active compound into skin. Thus occlusion and consquently skin hydration can be enhanced.

2.2.6.3. Lubrication, smoothness and emolliency

Reducing skin damage was one of the objectives of the cosmetic and dermal products. The lubricating effect and mechanical barrier property of lipid nanoparticles protect and support the skin, which is especially useful in skin irritation and allergic reactions cases. The regulation of emolliency of cosmetic formulations based on lipid nanoparticles is accomplished by the degree of hydration obtained with such systems. It is reported that the lubrication effect of cosmetics are thought to be important to reduce scrub effect and scratch of skin. Thus lipid nanoparticle should have spherical-like shape to improve the lubrication action.

2.3. Cosmeceuticals

Drugs have been defined as compounds used in the treatment and prevention of disease, or are intended to affect a physiologic function or structure of the body, and according to EU cosmetics are defined as the substance or preparation intended to be placed in contact with the various external parts of the human body epidermis, hair system, nails, lips and external genital organs or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, correcting body odours, protecting them or keeping them in good condition (121).

There is a border line between these two fields, for which the term cosmeceuticals has been defined. Cosmeceuticals represent combination between cosmetics and pharmaceuticals and are intended to enhance both the health and beauty of the skin by external application and they are defined as cosmetical effective products that enchance and change the condition and functions of skin and skin layers with a certain physiological effect (122,123).

The skin is the largest organ of the body and it has several functions, the most important is being the barrier to the environment, controlling the inward and outward transport of water, electrolytes and various substances while providing protection against micro-organisms, ultraviolet radiation, and toxic agents. It is consisted of three layers: epidermis, dermis and subcutaneous layer (124).

The epidermis is the outer layer of skin and the main cells of the epidermis are the keratinocytes, which synthesise the protein keratin. Protein bridges called desmosomes connect the keratinocytes, which are in a constant transition case from the deeper layers to the superficial. The five separate layers of the epidermis are formed by varying levels of differentiation. Moving from the lower layers to the upper, the five layers of the epidermis are: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (124,125).

The stratum corneum is the outermost layer of the epidermis, is approximately 10–20 μm thick and is responsible from the barrier function of skin. The stratum corneum is also known as nonviable epidermis and consists of large, flat dead cells that have migrated up from the stratum granulosum and it has lipophilic character due to the epidermal lipids oriented between the keratin filled corneocytes. The structure of the stratum corneum can be described by a “brick and mortar” model. The corneocytes comprise the bricks and the epidermal lipids between these cells are mortar (124-127).

Throughout the stratum basale layer of the epidermis there are keratinosocyte, melanocyte, and Langerhans cells. Melanocytes, which produce the pigment melanin, one of the main contributors to skin colur and also, hair and eyes colour. The epidermis also contains Langerhans' cells, which are part of the skin's immune system. These cells help detect foreign substances and defend the body against infection, they also play a role in the development of skin allergies. Moreover keratinocytes are the most important epidermal cells in which they are undergo the morphological changes within the layers of epidermis, in which are called as corneocytes at the surface of epidermis. These epidermal cells turn over time from stratum basale to stratum corneum is occurring every 21 days (124,128) .

The dermis is below the epidermis layer and is composed of fibroblasts, which produce collagen, elastin and structural proteoglycans, together with the mast cells and macrophages. 70 % of the dermis is consisted from collagen fibres, giving it strength and toughness. Elastin can maintain normal elasticity and flexibility of the skin, while proteoglycans have ability to maintain viscosity and hydration of skin (124,128).

It is accepted that cosmeceutical products must be as safe as a cosmetic and should not act as a drug. They have performance characteristics that suggest pharmaceutical action, but when necessary they are registered and sold as a cosmetic (129,130). Their sites of effect are intended to be on the epidermis and dermis. Thus two different functions are achieved when applying cosmeceutical active compound on skin. Firstly, it may be required to have an effect on skin surface, these are epidermal cosmeceuticals. The second function is that they effect the viable epidermis and dermis when applied on skin (125,131,132).

Cosmeceuticals used for their certain properties and effects on skin such as hydration, protecting, radical scavenging, and antiaging. Application of lipids to the skin can enhance skin hydration by several mechanisms. The most common one is occlusion, which causes a reduction in the transepidermal water loss (TEWL) of skin. Common occlusive substances in moisturizers are petrolatum, beeswax, lanolin, and various oils. Hence, moisturizers and moisture retaining subtances such as urea, occlusive agents like lanolin and propylene glycol protect skin from dryness, smooth a rough skin surface and fine wrinkles. Chemical peeling agents AHA and BHA effect the epidermis by improving corneocyte activity by reducing cohesion between corneocytes, causing exfoliation of stratum corneum layer and thus stimulate epidermal turn over effect. Besides, cosmeceuticals like vitamin C has effects viable epidermis by inhibition of tirozinase enzyme and thus melanin synthesis can be reduced in which is responsible from skin pigmentation (133,134).

Skin cell proliferation, and differentiation can be enhanced by stimulating collagen synthesis in dermis. Cosmeceuticals like peptides, proteins, AHA, BHA and vitamis A and E can increase thickness of dermis as well as increasing both epidermal and dermal glycosaminoglycan (GAG) deposition. The deposition of epidermal and dermal GAG may affect collagen and elastic fiber formation in which acts to improves signs of skin aging and also they bind a large amount of water, which helps to regulate skin hydration and enable cell mobility within skin (134,135). Vitamin E regenerates hair follicles and it has free radical scavenging effect, thus antioxidant effect can be performed and they can effect periferic blood circulation of skin. Another example for cosmeceuticals is coenzyme Q10 which has

antioxidant effect that can lead to a significant improvement of mitochondrial functions in the skin and reduction of wrinkles (126,134,136-141).

2.4. Cosmeceutical Ingredients

Cosmeceutical active ingredients can be classified to four main titles:

- Vitamins
- Peptides and Proteins
- Botanicals
- Hydroxy Acids

2.4.1. Vitamins

Vitamins are essential compounds for the human organism. Some vitamins can be synthesized, but some need to be obtained by an sufficient diet. The most important vitamins are A, B, C, D, E, and K, as well as folic acid. In addition to their certain specific functions, vitamins are useful for prevention, as well as for topical and systemic treatment of photoaging and chronologic skin aging. They are also effective in the management of acne vulgaris. Some vitamins are used as drugs or some as cosmeceutical compounds are also of great interest for topical treatment (135).

Their mechanism of effect on skin is wide. Vitamin A has the ability to stimulate epidermal proliferation and differentiation leading to thicker skin, vitamin B is precursor of endogenous enzym cofactors to obtain antioxidant effect, vitamin C has an antioxidant activity and tyrosinase inhibition effect leading to skin lightening effect and also has potential to increase collagen production to reduce wrinkle appearance, vitamin E is an oil soluble antioxidant and has free radical scavenger effect that caused by UV exposure, and vitamin K has the effect of stabilizing the surface capillary system of the skin and therefore it has the ability to reduce erythema as well as dark eye contours (134,138) .

2.4.2. Peptides and Proteins

Peptide cosmeceuticals are one of the new, popular options for the skin aging treatment. Peptides are short amino acid sequences that are components of larger proteins, such as collagen. The theoretical benefits of applying peptides to the skin were discovered during wound healing research (142,143) .

Peptides have the ability to increase fibroblast production of collagen or inhibit collagenase reduction of existing collagen and should improve the clinical appearance of the fine and wrinkles visible in both chronological and photoaged skin. Some bioactive amino acid chains have been discovered, as a result of wound healing research and genomic studies on the growth and stimulation of human skin fibroblasts, in which stimulate human skin dermal fibroblast growth and decrease the depth and length of wrinkles (139,143). There are three main categories of cosmeceutical peptides described in the literature (143):

- Signal peptide: They have the ability to increase dermal remodeling by stimulating human dermal skin fibroblasts, inhibiting collagenases and increasing ground substance production.
- Neurotransmitter-affecting peptide: They decrease muscle contraction through its interactions at the neuromuscular junction.
- Carrier peptide: They relieve in the delivery of a cofactor required for wound healing and various other enzymatic processes necessary for renewing the dermis.

2.4.3. Botanicals

Nowadays most of the interested topic is to replace synthetic products to use natural active compounds for cosmetic applications. Because of the safety of botanical cosmetic active compounds, they are widely used due to their many important and useful properties. Thus they are considered to be used for healing many skin disorders, for skin protection, hydration, and also for massaging. These active compounds are extracted from roots, barks, rhizoms, seeds, leaves, flowers, and also dried fruits of plants. Fatty acids, saponins, flavonoides, tanens,

phytosterols, vitamins, proteins, and peptides are also the components that are extracted from the plants (144,145). There are numerous botanical extracts that are used as a cosmeceutical. They can be classified by their functions and effect on skin for the miscellanous skin care, anti-inflammatory, antioxidant and antiaging functions (146,147).

- **Skin Care Agents**

Common cosmeceutical use of botanical extracts is to provide treatment, soothing, regenerate, protect, or relax the skin. Mucilages have a soothing, cooling effect on the skin. Some plants such as yeast, wheat, potatoes, Morus alba, or white mulberry are rich in ceramides and glycosylceramides and could be used for their effect on skin or hair to provide hydration or reconstitute epidermal barrier function (144,146,148).

Arbutin which is extracted from bearberry leaves has an inhibitory effect on the synthesis of melanin thus showing a bleaching effect on skin. Chamomile is one of the main botanicals used in skin care products for its soothing, antiseptic, anti-inflammatory effects on epidermis as well as it is used for lightenning of hair colour (144). Squalene, linaloic acid and oleic acid are plant butters and oils found in many plants such as coffee, mango, soya, and corn that have role to maintain skin and reduce water loss and hence provide skin hydration effect (147).

Recently, certain botanical extracts used in cosmeceutical creams combination for anticellulite functions. Tea, coffee, Guarana, and cocoa extracts are rich in methylxanthines (caffeine, theobromin). They accelerate lipid degradation by inhibiting cAMP-phosphodiesterase enzyme. Flavonoids are inhibitor of this enzyme, and phytsterols have effects on fat storage or degradation, as well as on adipocyte differentation. Additionally methylxanthins effect the lipoprotein lipase enzyme by reducing transition of fatty acids to adipocytes (144,147) .

- **Anti-inflammatories**

Flavonoids are botanical extracts have anti-inflammatory effect on skin and are found in certain botanicals such as Ginkgo biloba, Saccharomyces cerevisiae, green tea, echinacea, thuja, and sarsaparilla. Glycyrrhiza glabra, Ginkgo biloba and

green tea leaves contain flavonoids, and flavonol glycosides that have anti-inflammatory effects. These flavonoids have also inducing effects human skin fibroblast proliferation and increasing collagen and extracellular fibronectin were also reported (144,146,149).

Sarsaparilla possess topical benefits due the presence of plant sterols and saponins. It has both antiseptic and antipruritic properties. Thus, it is incorporated into topical formulations to treat both eczema and psoriasis (146).

- **Antioxidant and Antiaging Agents**

Free radicals play important role on skin in sun damage and also in aging process. They cause degradation of collagen, elastin, and cell membranes. Hence these negative effects of free radicals can be avoided by some botanical extracts used as cosmeceutical active ingredient (147).

Flavonoids, phytosterols and polyphenols are known by their antioxidant effect to be against antiaging process induced by UV damage and free radicals. The degradation of glycosaminoglycans and collagen, with a consequent reduction in skin thickness are main changes appeared during skin aging (144,146,147).

Besides, ascorbic acid is an antioxidant and a key element in collagen synthesis extracted from many plants. It stimulates the production of RNA coding for collagen. It is also an anti-inflammatory agent that degrades and eliminates histamine and it protects the skin against free radical damage (147).

Saccharomyces cerevisiae (yeast extract) includes vitamin B complex in it and is used for treatment of oily skin as well as its alcohol extract enhances oxygen consumption and metabolism of skin tissue. In addition antiwrinkle effect, antipigment, and collagen synthesis effects are also included to the cosmetic effects of this extract (144,147).

2.4.4. Hydroxy Acids

Dermatologists and consumers have become interested with group of hydroxy acids, thoughted to provide antiaging and skin smoothing effects. Hydroxy acids, which are incorporated into a wide group of topical formulations (133,141,150):

- Alpha Hydroxy Acids (AHA)
- Beta Hydroxy Acids (BHA)
- Combination Hydroxy Acids (CHA)
- Trihydroxy Acids (THA)
- Polyhydroxy Acids (PHA)

2.4.4.1. Alpha Hydroxy Acid (AHA)

This group has hydroxyl group in the alpha position. The linear, aliphatic nature of the AHA structure is water soluble,or can be said they have hydrophilic properties. The AHAs include monocarboxylic acids (glycolic acid, lactic acid, mandelic acid) with one hydroxy group, dicarboxylic acids (malic acid, tartaric acid) with two hydroxy groups, and tricarboxylic acids (citric acid). AHAs have effect on the stratum corneum by peeling the dead cells exist on the outer part and thus their effect differ according to the concentrations used either for cosmeceutical or dermatologic purposes. At low concentrations they reduce the intracelluar corneocyte cohesion by effecting ionic bonds of corneocytes. A superficial peel and desmolytic effect occurs at low concentration, and at high concentrations the stratum corneum suddenly becomes detached at its lower-most levels, and desquamates as large parts or pieces. So these actions cause an improving in the hyperkeratotic disorders and increasing plasticization and flexibility of the stratum corneum. In dermis they cause epidermolysis and increases water content, thus stimulates collagen synthesis (133,134,141).

2.4.4.2. Beta Hydroxy Acid (BHA)

BHA, is an organic aromatic carboxylic acid with a hydroxy group in the beta position. Salicylic acid is the beta hydroxy acid, has effect in inducing exfoliation in

the oily areas of the skin, it can enter the medium of the sebaceous unit and also it has a keratolytic activity. Becasue of the solubilization effect of SA in intercellular cement, it is thought to have reducing effect of corneocyte cohesion (133,148,151).

BHA is approved as an exfoliant in the treatment of acne in over-the-counter products at a concentration of 3% or less. Recently BHA has been rediscovered for its ability to improve the appearance of aged skin through exfoliation of the skin surface and within pores (133,152).

Several studies suggest that AHA can increase the sensitivity of skin to ultraviolet radiation. Recently, β-hydroxy acids or combinations of AHA and BHA have also been incorporated into antiaging skin care products (134,153).

2.4.4.3. Combination Hydroxy Acids (CHA)

The free acid component of the hydroxy acids can provide antiaging effect on the skin. Combination of AHA and salicylic acid formulations can be performed. However the difference in their pKa value may cause some difficulties to formulate them optimally.

The pKa for the AHAs is 3.83, while the pKa for BHA is 2.98. Products are preffered to be formulated at a pH close to the pKa for optimal free acid concentration at optimal efficacy (133).

2.4.4.4. Trihydroxy Acids (THA)

Trihydroxy acids (THAs) formulations represent combination of three alpha hydroxy acids. An example given in the literature for this, the glycolic acid is combined with the malic acid and the citric acid (133).

2.4.4.5. Polyhydroxy Acids (PHA)

Polyhydroxy acids are reported to be chemically similar to AHAs, but they are larger than hydroxy acids in molecular weight. They are able to exfoliate only the skin surface with limited dermal penetration and means that there will be less stinging, burning, and irritation on skin through this decreased penetration (133,142).

2.4.5. Uses and Therapeutical Effects of Salicylic acid on Skin

Salicylic acid is a β-hydroxy acid applied topically for the treatment of hyperkeratotic and scaling skin conditions such as dandruff and seborrhoeic dermatitis, ichthyosis, psoriasis, and acne. Salicylic acid produces desquamation of hyperkeratotic epithelium by dissolving the intercellular cement, which causes softenning, hydration, and desquamation. Salicylic acid is used as a keratolytic at concentrations of 3% to 6%. It is destructive to tissue at concentrations above 6%. Concentrations 6% to 60% are used to remove corns and warts, in the treatment of psoriasis and other hyperkeratotic disorders. It has been previously reported the effectiveness of this agent for acne and mild photoaging. Salicylic acid peels offered a clinically significant advantage for treatment of acne vulgaris, oily skin, textural changes, melasma, and postinflammatory hyperpigmentation (141,152,154,155).

Salicylic Acid is used in cosmetic formulations as a denaturant, anti-dandruff agent, and skin conditioning agent. It is used in cosmetic products at concentration ranges from 0.0008% to 3%. It is well known to have keratolytic effect on normal human skin. Because of its use as exfoliating agent, and thought that repeated use may effectively increase exposure of the dermis and epidermis to UV radiation (156,157).

2.5. Salicylic Acid

2.5.1. Chemical Structure

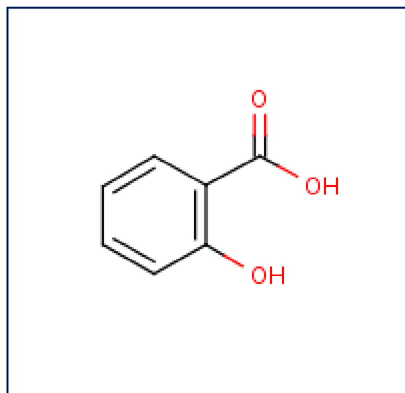


Figure 2.4. Chemical structure of salicylic acid.

- **CAS number**: 69-72-7
- **Synonyms**: Orthohydroxybenzoic acid; Acido Orthoxibenzoico; Acidium Salicylicum; Salizylsaure (158).
- **Chemical name**: 2-hydroxybenzoic acid (158).

2.5.2. Physicochemical Properties

- **Molecular Formula**: $C_7H_6O_3$ (159).
- **Molecular Weight**: 138.12 (159).
- **Colour**: Colourless or white solid crystals (158).
- **Description**: Salicylic acid is a white crystalline powder with a sweetish acrid taste. If prepared from natural methyl salicylate, it may have a faint mint like odour. It is available in forms of ointments, cream, gel, transdermal patches, liquids and plaster (158).
- **Solubility**: Salicylic acid is soluble 1 in 460 to 550 of water, 1 in 15 of boiling water, 1 in 3 to 4 in alcohol, 1 in 3 in ether and 1 in 45 in chloroform (156).
- **Melting Range**: 158°-161°C (159).
- **LogP** : 2.011 ± 0.247 (at 25°C) (156).
- **Refractive index** : 1.565 (156).

- **Stability and storage**: Discolours in sunlight and when heated to decomposition. Decomposes into phenol and CO_2 when rapidly heated at atmospheric pressure. Should be stored in well closed containers and protect from light (156).

2.5.3. Mechanism of Action of Salicylic Acid

Hydroxy acids have a common mechanism of action on skin surface as it is described that these acids involve exfoliation of stratum corneum. This accelerated exfoliation then results in improvement effects to skin surface texture and colour appearance. Salicylic acid is a good keratolytic agent. It encourages the conditions of dead skin cells by dissolving the intercellular cement that binds the epithelial cells together leading to the reduction in the cell cohesion. Salicylic acid also has a lipophilic character that facilitates the peeling of the top layer of skin and the opening of plugged follicles, which leads to a renewal of the skin cell replacement cycle. Thus, salicylic acid has a strong comedolytic effect. Salicylic acid affects the arachidonic acid cascade and has anti inflammatory properties also. Hence, it is useful in reducing both non-inflammatory as well as inflammatory acne lesions (141,157).

2.5.4. Contraindications and Side Effect of Salicylic Acid

It is reported that Salicylic acid is easily absorbed from the skin and at high concentrations it may cause toxicity, itching sense through exfoliation of skin with discomfort when used topically and may also cause an allergic contact rash in some people. When applied to large areas of skin, it may be absorbed into the blood circulation and induce salicylism, also it should not be applied to irritated or inflamed skin. A common side effect is dryness of skin which is usually mild. Other side effects are heavy breathing, dizziness, hearing impairment, digestive disorders, and nausea (141,150,156,157,160,161).

3. EXPERIMENTAL PART

3.1. Materials and Equipment

3.1.1. Chemicals

Apifil (PEG-8 Beeswax)	Gattefosse, France
Ca^{++} Mg^{++} free PBS	PBS BULBECCO (L1825), Biochrom AG, Germany
Chloroform	Merck, Germany
Compritol 888 ATO (Glyceryl Behenate)	Gattefosse, France
DMEM (Dulbecco's Modified Eagle's Medium)	Biochrom, Germany
Fetal Bovine Serum (FBS-S0113)	Biochrom AG, Germany
Labrafac Hydrophile (Caprylic/Capric Triglyceride PEG-4 Esters)	Gattefosse, France
Labrafac Lipophile WL 1349 (Caprylic/Capric Triglyceride)	Gattefosse, France
Labrafil M 1944 cs (Oleoyl Macrogolglycerides)	Gattefosse, France
L-Gultamine	Biochrom AG, Germany
Methanol	Merck, Germany
Mouse Fibroblast Cells (L929 Cell Line)	American Type Culture Collection (ATCC), USA
Oleic Acid	Sigma-Aldrich, Germany
Penicillin/ Streptomycin (10000U/ 10000 $\mu g.mL^{-1}$)	Biochrom AG, Germany
Pluronic F68	Gattefosse, France
Potassium dihydrogen phosphate	Carlo-Erba, Italy
Precirol ATO 5 (Glyceryl Palmitostearate)	Gattefosse, France
Salicylic Acid	Carlo Erba, Italy
Sodium Hydroxide	Riedel de Haen, Germany
Soybean Lecithin (Lipoid-S100)	Lipoid-GMBH, Germany

Tween 80 (Polyoxyethylene (20) sorbitan monooleate)	Sigma-Aldrich, Germany
Trypan Blue	Sigma-Aldrich, Germany
Trypsin-EDTA (0.05% / 0.02% w/v, L2143)	Biochrom AG, Germany
WST-1 Premixed Cell Proliferation Reagent [(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)]	Clontech,CA-USA

3.1.2. Equipment

Equipment	Supplier
Cellophane Membrane (MWCO 12400 Da)	Sigma-Aldrich, Germany
Centrifuge	HERME Z383 K, Hermle Laboritechnik GmbH, Germany
Chiba Needles	Bacton-Bickinson, Norway
Cone-Plate Viscometer	Brookfield- DVII, USA
Cronus syringe filter (PTFE,0.45µm)	Cronus Filter, UK
Electronic Balance Ax200	Shimadzu, Japan
Franz Diffusion Cells	Ildam, Turkey
FTIR	Perkin Elmer, England
Horizontal agitator with thermostated water bath	Memmert, Germany
Immersible magnetic stirrer	Variomag, Germany
Incubator	SANYOMCO-18AIC, SANYO Electric Biomedical Co.Ltd., Japan
Insulin Syringe (1mL)	DOPA, Turkey
Inverted Microscope Leica DMIL, DFC 320	Leica, Germany
Magnetic stirrer	Heidolph, Germany
Mastersizer 2000	Malvern Instruments Ltd., UK
Melting Point Apparatus	Thomas Hoover, USA
MFS-25 Syringe Filter (0.45µm)	Advantec MFS, USA
Micropipette	Eppendorf, Germany
Microplate Reader	VERSAmax, Molecular Device Corporation, CA, USA
Plate for cell culture (96 wells)	Cellstar, Greiner bio-one,Germany
pH-Meter	Sartorius, Germany
Polarized Light Microscope DMEP, (DFC 320 with camera)	Leica, Germany
Spectrophotometric Quartz Cell	Hellma, Germany

Syringe (5 mL)	AYSET, Turkey
Thermostated Heater	Haake, Germany
Ultrafiltration pomp	Watson-Marlow, England
Ultrasonic Bath	Branson, USA
Ultrasonical Probe	Bandelin- Sonopuls, Germany
Ultraturax T25	IKA-WERKE, Germany
UV-Spectrophotometer (UV-1800)	Shimadzu, Japan
Vertical Laminar Airflow Cabinate	BHG 2000 S/D, Faster, D Group Company, Italy
Viva Flow 50 (30 kDa)	Sartorius, Germany
Vortex	MS1 Minishaker IKA
Water Bath	Edmund Buhler-Tubingen, Germany
Water Bath for Cell Culture	SBD 300, Şimşek Laboriteknik, Turkey
Zetasizer Nanoseries Nano ZS	Malvern, England

3.2. EXPERIMENTS AND METHODS

In this section the physicochemical properties of salicylic acid, formulation characterization and evaluation with in vitro release studies, cell culture and stability studies were performed.

3.2.1. Physicochemical Properties of Salicylic Acid

In order to determine the physicochemical properties of salicylic acid (SA), the FTIR spectrum, melting point, and the UV spectrum in two different solvents were determined. Two different calibration curves were evaluated and validated in the two different solvents and the solubility of SA was determined.

3.2.1.1. FTIR Spectrum

The FTIR spectrum of SA in crystal solid state was recorded in the region between 600-4000 cm^{-1} on a Perkin Elmer FT-IR Spectrometer.

3.2.1.2. Melting Point of SA

Metling point of SA was determined using Thomas Hoover melting point apparatus. SA in crystal-solid form was placed in a capillary tube and when the temperature of the apparatus was reached to 10°C below the melting point of SA, the tube was placed in the apparatus. The melting point was recorded as SA started to melt and this process is repeated six times.

3.2.1.3. UV Spectrum

The UV spectrum of SA with concentration 25 µg/mL in PBS (pH 7.4) and 20 µg/mL SA concentration in chloroform:methanol (1:1, v/v) solution was recorded between λ= 200-500 nm.

3.2.2. Calibration curves

In order to determine the encapsulation efficiency, loading capacity, in vitro release of SA from formulations, stability and the amount of SA in the formulations,

two different calibration curves one in phosphate buffer solution pH 7.4 and the other in chloroform:methanol (1:1, v/v) solvent system were established.

Solubility, in vitro release, encapsulation efficiency and loading capacity studies were performed by the established calibration curve equation of SA in PBS pH 7.4. The calibration line of PBS pH 7.4 is established starting from stock solution of 200 µg/mL concentration and then diluting it to different concentrations to prepare 6 calibration standards of 6 series. All standards were prepared in volumetric flasks and were protected from light to prevent discolour of the SA. Concentrations of the standards: were 3, 5, 10, 15, 20, and 30 µg/mL and they were measured spectrophotometrically at λ_{max}= 296 nm using the UV-spectrophotometer.

On the other hand for the spectrophotometric assay to study the amount and stability of SA in the formulations, calibration line in the organic solvent that consists of chloroform: metOH (1:1, v/v) was established too. The stock of 200 µg/mL was prepared and 6 calibration standards were prepared of 6 series from the stock solution. Standards were prepared in volumetric flasks and protected from light. Concentrations of the standards in the organic solvent were: 3, 5, 10, 15, 20 and 25 µg/mL and they were measured spectrophotometrically at λ_{max}= 304 nm using UV-spectrophotometer.

3.2.3. Spectrophotometric Analysis of SA

As described in 3.2.2. calibration curve which was established in PBS pH 7.4 which is used to determine SA concentration in formulations during the in vitro release study, encapsulation efficiency, and loading capacity studies. Besides, chloroform:metOH (1:1, v/v) was used to determine the SA concentration during stability studies in the formulations spectrophotometrically. Analytical method validation studies were performed for both calibration curves obtained from two systems.

3.2.4. Analytical method validation

Analytical method validation is used to show the reliability of the chosen analytical method for analysis of the active ingredient (162-166). The parameters studied for this purpose:

- Linearity
- Accuracy
- Precision
    - Repeatability
    - Reproducibility
- Sensitivity
    - Limit of Detection (LOD)
    - Limit of Quantification (LOQ)
- Specificity (selectivity)
- Stability during the analysis

Linearity

The linearity is the ability of analytical procedure to produce test results which are propotional to the concentration or amount of analyte in samples within a given concentration range, either directly or by means of a mathematical transformation (163,164).

Calibration curves for the two systems [PBS pH 7.4 and chloroform:metOH (1:1, v/v)] were established as mentioned in 3.2.2. and each 6 series of 6 calibration points are prepared from 200 µg/mL stock solution. The absorbance for each concentration of the solutions were measured to establish the calibration line. The equation, intercept, and the correlation coefficient of the calibration line were calculated in order to demonstrate a clear correlation between response and analyte concentrations (test of departure from linearity) by using the test of significance of departure from linear regression.

Accuracy

The accuracy of an analytical method is defined as the degree in which the determined value of analyte in a sample corresponds to the true value. Accuracy may be measured in different ways and the method should be appropriate to the matrix. This is sometime termed trueness (163).

Accuracy can be determined by one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value with the known value. The second is to compare test results from the new method with results from an existing alternate procedure that is known to be accurate. The third approach, based on the recovery of known concentrations of analyte, is performed by spiking analyte in blank matrices. For assay methods, spiked samples can be prepared in triplicate. For impurity methods, spiked samples can be prepared in triplicate at three levels over a range that covers the expected impurity content of the sample. The percent recovery should be calculated. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. Accuracy criteria for an assay method according to FDA is that the mean recovery will be 100 ± 2 % at each concentration over the range of 80–120 % of the target concentration (164).

In this study to demonstrate the accuracy of the used method; two standards at low (5µg/mL) and the other at high (20 µg/mL) concentrations were chosen and prepared in volumetric flasks wrapped with aluminum foil. Standards (n=6) were prepared from 200 µg/mL stock solution. In order to measure the inter day and the intraday assay, solutions were prepared in consecutive 3 days and the absorbance of the known concentrations were measured by UV-spectrophotometer. The obtained absorbance values were replaced in the calibration curve equations and the relative error (%) were calculated for the expression of the recovery (%). The relative error was measured by the following equation:

$$\textbf{RE (\%)} = [(C_1 – C_2)/C_1] \text{ x}100 \qquad (3.1)$$

RE(%): Relative Error, C_1 : Known concentration, C_2 : Calculated concentration

Precision

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision is a measure of random errors, and may be expressed as repeatability and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation (SD) or coefficient of variation (CV %) of a series of measurements. A minimum of 5 replicate sample determinations should be prepared together with a simple statistical assessment of the results (163,164).

- **Repeatability** is the closeness of agreement between independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short time intervals (163).

For this purpose two standards at low (5 µg/mL) and high (20 µg/mL) concentrations were prepared from a stock solution at 200 µg/mL from both systems [PBS pH 7.4 and chloroform:metOH (1:1, v/v)] and prepared in volmetric flasks wrapped with aliminum foil. These two standards were measured six times for the intra-day assay and were prepared consecutively 3 days for inter day assay and also measured in the same manner at λ_{max}=296 nm (PBS pH 7.4) and λ_{max}=304 nm (chloroform:metOH (1:1,v/v)) by UV-spectrophotometer.

The measured absorbances were replaced in the calibration equation and arithmetic average ($\bar{X}$), standard error (SE) and the coefficient of variation (CV %) were calculated. In order to prove the repeatability of this method, the coefficient of variation should show a value less than 2%.

- **Reproducibility** is the closeness of agreement between test results obtained with the same method on identical test material in different laboratories with different operators using different equipment (163). For the intra day assays two standard solutions at low (5 µg/mL) and high (20 µg/mL) concentrations were prepared from a stock solution of 200 µg/mL from both systems and prepared in volmetric flasks wrapped with aliminum foil. Each standard was prepared 6 times and all were measured by UV-spectrophotometer. The obtained absorbance values were replaced

in the calibration line equation and the arithmetic average ($\bar{X}$), standard error (SE) and the coefficient of variation (CV%) were calculated. In order to show that the intra day assay is reproducible for this method, the coefficient of variation should show a value less than 2%.

On the other hand for the inter day assay also two standards at low (5 µg/mL) and high (20 µg/mL) concentrations were prepared from a stock solution of 200 µg/mL and prepared in volmetric flasks wrapped with aliminum foil. These samples were prepared consecutive 3 days, each one in different 6 volumetric flasks. Then they measured by UV-spectrophotometer the arithmetic average ($\bar{X}$), standard error (SE) and the coefficient of variation (CV%) were calculated. In order to conclude that the inter-day assay is reproducible for this method, the coefficient of variation should show a value less than 2%.

Sensitivity

- **Limit of Detection (LOD)**

The detection limit of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated as an exact value. It is expressed as a concentration at a specified signal-to-noise ratio, usually 3:1 (163,164). The LOD was calculated based on the standard deviation (SD) of the response or of y-intercepts of regression lines, and the slope (S) of the calibration curve at levels approximating the LOD according to the equation 3.2.

$$LOD = 3.3 \, x \, SD/S \tag{3.2}$$

- **Limit of Quatitation (LOQ)**

The limit of quantitation is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions. The limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for

the determination of impurities and/or degradation products or low levels of active constituent in a product (164) . The calculation method is based on the calculation of SD of the response or SD of y-intercepts of regression lines, and the slope (S) of the calibration curve according to the equation 3.3.

$$LOQ = 10\, x\, SD/S \quad (3.3)$$

Specificity (Selectivity)

Selectivity of a method refers to the extent to which it can determine analyte(s) in a complex mixture without interference from other components in the mixture (163,164). The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective.

Because the NLC did not dissolve in the phosphate buffer solution pH 7.4, the specificity process was evaluated by an in vitro release study by modified Franz Diffusion Cell using cellophane membrane (MWCO 12400 Da). For this purpose, 1 g from the each blank and SA loaded formulations were placed on the donor compartment and samples were taken from the diffusion media after 1 hour. Phosphate buffer solution versus the dialyzate of blank NLC, and phosphate buffer solution versus dialyzate of NLC containing SA were measured spectrophotometrically and the spectrums were obtained after 1:10 dilution process for each dialyzate.

In addition in order to determine the specificity of the calibration curve and validation of the method established by chloroform:metOH (1:1, v/v) system for SA, blank NLC and SA loaded NLC were dissolved in this system. After 1:5 dilution and filteration of the dissolved formulations through 0.45µm PTFE syring filter, the spectrums of chloroform:methanol (1:1,v/v) system versus the dissolved blank and SA loaded NLC were obtained.

Stability During Analysis

The stability of SA during analysis in each system PBS pH 7.4 and in chloroform:methanol (1:1,v/v) was studied. By preparing two standard solutions from the stock solution of 200 µg/mL. Two standards one at low (5 µg/mL) and the other at high (20 µg/mL) concentrations were prepared each in 6 different volumetric flasks wrapped by aluminum foil. The absorbance of the solutions were measured at the prepared moment, 1st, 3rd, 6th and 12th hours at λ_{max}=296 nm in PBS and λ_{max}=304 nm in the organic solvent system. All the solutions were kept at 25 °C and the covers of the volumetric flasks were wrapped with parafilm.

3.2.5. Solubility Test

The PBS pH 7.4 was selected as the release medium for the formulations (167,168). Thus to evaluate whethere this medium ensures the sink condition, the solubility of SA in PBS pH 7.4 was determined. According to USP, sink condition is defined as the volume of medium at least greater three times than that required to form a saturated solution of a drug substance (169).

For this purpose excess amounts of SA was weighed and placed in 50 mL vials and 25 mL of the PBS pH 7.4 were added. The siliconized vials were sealed with aluminum lids. Then the vials were wrapped with aluminum foil and placed in horizontal agitator of a water bath at 37°C and shaked at a 100 rpm. Sampling times were in the 1, 2, 3, 5 and 8th hours. In each measurement the samples were taken by injectors and filtered through 0.45 µm cellulose syringe filters then their absorbance were measured spectrophotometrically at λ_{max}=296 nm.

3.3. Formulation Studies

Different NLC formulations were prepared by high shear homognization-ultrasonication technique as described in 2.1.4.1. Formulations with different lipids and various oil/fat ratios were studied. The oil/fat phase concentration was 12% (w/w) and two different surfactants, one hydrophilic surfactant[Tween 80 (2.4 %,w/w)] and the second lypophilic surfactant [Lipoid S100 (0.2 %, w/w)] were used.

As reference formulation, SLN was also prepared in the same way and these formulations were compared with the NLCs in terms of particle size and distribution, zeta potential, viscosity, encapsulation efficiency, and loading capacity.

3.3.1. Pre-formulation Studies

Pre-formulation studies NLC with various oil/fat ratios were studied [10:90, 30:70, 40:60, 50:50, 70:30 (w/w, %)] using different mixtures of solid and liquid lipids, with Lipoid S100 as a lipophilic (0.2%, w/w), and Tween 80 (2.4%, w/w) , Pluronic F68 (2.4%, w/w) as hydrophilic surfactants. SA was used to load into the formulations at 0.5%, 1% and 2% (w/w) concentrations. Then the optimum formulations with suitable characteristics in particle size, polydispersity index and zeta potential were chosen for further studies.

3.3.1.1. Solid lipids, Liquid lipids and Surfactants Used in Formulations

For the developement of NLC formulations, different solid and liquid lipid blends could be used with different surfactant mixtures according to purpose of study (25,170) (Table 3.1.).

Table 3.1. Properties of solid/liquid lipids and surfactants used in pre-formulation.

Solid Lipids	Melting Point	HLB Value
Precirol ATO 5	52-56°C	2
Apifil	59-70°C	9
Comritol 888 ATO	70°C	2
Liquid Lipids		
Labrafac Lipophile	-	2
Labrafac Hydrophile	-	4 - 5
Labrafil M1944	-	3 - 4
Oleic Acid	-	1
Surfactants	-	
Tween 80	-	15
Pluronic F68	-	29
Lipoid S100	-	3

3.3.1.2. Lipid Screening

Before preparing NLC formulation, a lipid screening process was performed to determine the most suitable lipid for the formulation and the active compound to be incorporated into NLC. This is performed by dissolving increasing amounts of the active compound (SA) in various melted solid and liquid lipids, and determining the maximum amount of the active that could be dissolved in each lipid in two different temperatures (25ºC and 80ºC). SA was dissolved in various solid and liquid lipids in 5 different concentrations [1%, 2%, 3%, 5% and 10% (w/w)]. Solid lipids used were Precirol ATO 5, Apifil, Compritol 888 ATO, and liquid lipids were Labrafac Lipophile, Labrafac Hydrophile, Oleic Acid, and Labrafil. After dissolution process, the SA/lipid mixtures were investigated to observe the presence or absence of SA crystals visually at both temperatures (25ºC and 80ºC) and by using polarized light microscope at 25 ºC.

3.3.1.3. NLC Formulation Studies with Different Lipid Matrix and Different Oil/Fat Ratios

The influence of two hydrophilic (Tween 80 and Pluronic F68) with one lipophilic (Lipoid S100) surfactants were evaluated as first step during NLC pre-formulation studies. In this step, NLC formulations prepared using Precirol ATO 5 and Labrafac Lipophile lipid matrix [50:50 (%, w/w)] with the each hydrophilic surfactant separately. One with Pluronic F68 and the other with Tween 80 in the water phase, and each in combination with Lipoid S100 in the lipid phase. The influence of surfactant combinations were studied on NLC formulation in terms of obtained particle size and distribution results.

The second step for pre-formulation studies was preparing NLC using Precirol ATO 5 and Apifil as solid lipid and each blended with Labrafac Lipophile, Labrafac Hydrophile, Labrafil and Oleic Acid which were used as liquid lipids. All Formulations were compared with one another in terms of particle size, PI and zeta potential. Then the most suitable solid and liquid lipid matrix was chosen for further studies. The oil/fat ratio used to prepare NLC for this step was 50:50 (%, w/w) (Table 3.2).

Table 3.2. NLC(50:50) formulations with different solid and liquid lipid matrix.

NLC Formulations	Lipid Matrix
1	Precirol ATO 5 + Labrafac Lipophile
2	Precirol ATO 5 + Labrafac Hydro
3	Precirol ATO 5 + Labrafil
4	Precirol ATO 5+ Oleic Acid
5	Apifil + Labrafac Lipophile
6	Apifil + Labrafac Hydro
7	Apifil + Labrafil
8	Apifil + Oleic Acid

In the third step, in pre-formulation studies of NLCs using Precirol ATO 5 and Labrafac lipophileas lipid matrix were prepared with different oil/fat ratios [10:90, 30:70, 40:60, 50:50, 70:30 (%, w/w)]. The influence of oil/fat ratio on formulations was evaluated according to the particle size, polydispersity index, and zeta potential results.

3.3.1.4. Salicylic Acid Concentrations in Formulations

The NLC(50:50) formulations with Precirol ATO 5 and Labrafac lipophile lipid matrix (Table 3.2) were also prepared with loading three different SA concentrations [0.5, 1, and 2 (%, w/w)] and were studied in terms of PS, PI, and ZP.

On the other hand the two concentrations of SA [0.5 and 1 (%, w/w)] were used in the evaluation of NLCs with various oil/fat ratios [10:90, 30:70, 40:60, 50:50, 70:30 (%, w/w)].

3.3.2. NLC Formulations

After pre-formulation evaluation of the NLC and SLN formulations, Precirol ATO 5 and Labrafac Lipophile was selected as a the lipid matrix, Lipoid S100 and Tween 80 as lipophilic and hydrophilic surfactants respectively. In order to ensure

the cosmetic SA concentration acceptibility in the formulations, the most suitable SA loaded formulations that are selected for this study were 0.5 % and 1 % (w/w) SA .

On the other hand amoung the NLC formulations prepared with various oil/fat ratios, the selected NLC and SLN (Table 3.3) in range of particle size, polydispersity index, zeta potential and viscosity were NLC(70:30), NLC(50:50) and NLC(40:60). 0.5% and 1% SA loaded NLC(40:60), NLC(50:50) and 0.5% SA loaded SLN were selected for the encapsulation efficiency, loading capacity, in vitro release studies. The blank and 0.5% SA loaded NLC(40:60), NLC(50:50) and SLN were selected for stability studies.

Table 3.3. Composition of NLC with different oil/fat ratios and SLN (%, w/w).

Formulation Code	*Precirol ATO 5 (%)*	*Labrafac Lipophile (%)*	*Lipoid S100 (%)*	*Tween 80 (%)*	*Water (q.s)*
NLC (70:30)*	3.6	8.4	0.2	2.4	100
0.5% SA-NLC (70:30)**	3.6	8.4	0.2	2.4	100
1% SA-NLC (70:30)***	3.6	8.4	0.2	2.4	100
NLC (50:50)*	6	6	0.2	2.4	100
0.5% SA-NLC (50:50)**	6	6	0.2	2.4	100
1% SA-NLC (50:50)***	6	6	0.2	2.4	100
NLC (40:60)*	7.2	4.8	0.2	2.4	100
0.5% SA-NLC (40:60)**	7.2	4.8	0.2	2.4	100
1% SA-NLC (40:60)***	7.2	4.8	0.2	2.4	100
SLN*	12	-	0.2	2.4	100
0.5% SA-SLN**	12	-	0.2	2.4	100

* Blank formulations, ** Formulation loaded with 0.5% SA, *** Formulation loaded with 1% SA.

The NLC formulations were prepared by hot high-shear homogenization and ultrasonication technique. The lipid phase (Precirol ATO 5 and Labrafac Lipophile) was melted at 80 °C with Lipoid S100 to obtain a clear lipid mixture. At the same time, aqueous surfactant solution with Tween 80 has been heated at the same temperature.

The hot surfactant solution was then dispersed in the hot lipid phase using an mechanical agitate at 600 rpm for 1 min. After this immediatly hot lipid and aqueous phase mixture was stirred at 11000 rpm for 5 min using a high speed stirrer (Ultraturax T25). Then the obtained pre-nanoemulsion was homogenized with strength of 10 % for 15 min using the ultrasonical probe. In order to prevent recrystallization during homogenization, production temperature was kept at least 5 °C above the lipid melting point. The obtained nanoemulsion (O/W) was cooled down at the last 5 min during ultrasonication process in an ice bath to form NLC (Figure 3.1). SLN formulations were prepared in the same way.

Salicylic acid loaded formulations were prepared in the same manner. Incorporation of SA into the formulations was realized by adding SA to the melted oil/fat phase at 80°C and stirred with magnetic stirrer until dissolved. Then the remained steps of preparation were performed in the same way as for the preparation of blank formulations. The siliconized glass vials were immediately sealed after it was filled with the obtained formulation.

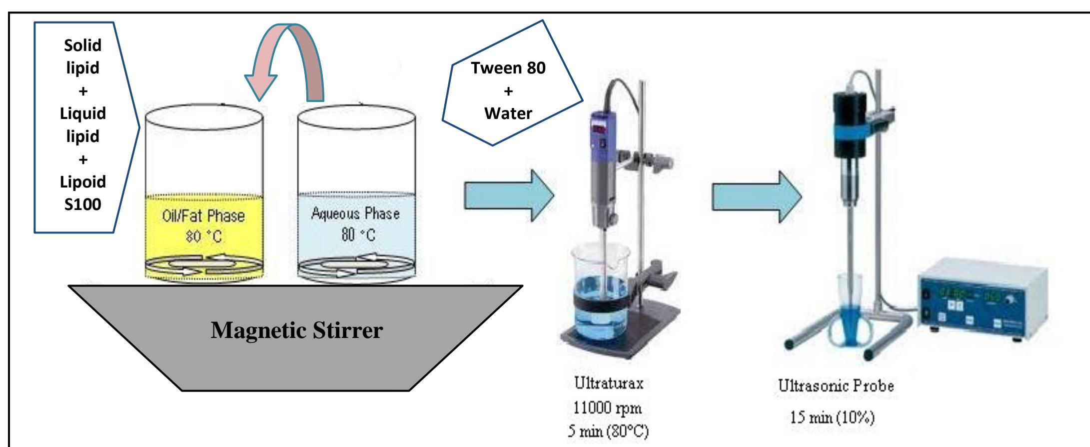


Figure 3.1. Scheme of preparation of NLC formulations.

3.3.2.1. Characterization of Formulations

For the characterization process the organoleptic and physical appearance, particle size, size distribution, zeta potential, viscosity and pH of the formulations were examined.

3.3.2.1.1. Macroscopical Evaluation

The formulation appearance were examined in terms of colour, viscosity and if there were any phase seperation or a sedimentation during and after production.

3.3.2.1.2. Particle Size Distribution and Zeta Potential

The particle size, polydispersity index (PI) and zeta potential of the blank and SA loaded NLCs were determined using a Malvern Zetasizer Nano ZS, Malvern Instruments (Malvern, UK). Before the measurements all the samples were diluted with deionized water (1:100) and analysed by Malvern Zetasizer Nano (n=5, 25°C). On the other hand, particle size of blank and SA loaded SLN were measured using Mastersizer 2000 Malvern Instruments (Malvern,UK). Besides, zeta potential of SLN formulations were measured using Malvern Zetasizer Nano ZS.

3.3.2.1.3. Viscosity

The viscosity of the blank and SA loaded formulations were analysed by Brookfield DV-II cone/plate viscometer using two different cone spindles (CPE). Viscosity of blank NLCs, 0.5% SA loaded NLC(70:30), NLC(50:50), NLC(40:60), 1% SA loaded NLC(70:30) and blank SLN were measured using CPE-40 cone. The viscosity of 1% SA loaded NLC(50:50) and NLC(40:60) and 0.5% SA loaded SLN were measured using CPE-52 cone. All measurements were performed in triplicate at 25 °C.

3.3.2.1.4. pH Value

The pH value of blank and SA loaded formulations were measured by direct immersion of electrode of pH-meter in the formulations using Sartorius pH-meter. All measurements were performed in triplicate.

3.3.3. Encapsulation Efficiency and Loading Capacity

The encapsulation efficiency (E.E.) and loading capacity (L.C.) of the NLC (50:50), NLC(40:60) loaded with 0.5% and 1% SA and SLN loaded with 0.5% SA were studied by ultrafiltration system using Vivaflow 50 Sartorius (MWCO 30 kDa) filter. For this process, 1 g of each formulation was weighed and diluted (1:100) with PBS (pH 7.4). The dispersed solution is then filtered with the ultrafiltration system. The clear fitrate that contained free SA was measured by UV spectrophotometer at λ_{max}= 296 nm. The concentration of the free SA was calculated by the calibration curve equation then the encapsulation and loading effeciencies of formulations were obtained.

The amount of encapsulated and the loaded SA was calculated by the difference between the total amount used to prepare the systems and the amount of SA that remained in the aqueous phase after isolation of the systems (Equations 3.1. and 3.2).

$$\text{E. E.} = \frac{\text{Total amount of SA} - \text{free amount of SA}}{\text{Total amount of SA}} \text{ x } 100 \qquad (3.4)$$

$$\text{L. C.} = \frac{\text{Total amount of SA} - \text{free amount of SA}}{\text{Total amount of the lipids in the system}} \text{ x } 100 \qquad (3.5)$$

3.3.4. In Vitro Release Studies

Drug release studies from topical formulations is not only an important step during the developing stages of new formulations but also a routine quality control test for assuring uniformity of the finished product.

3.3.4.1. Stability of SA During The In Vitro Release Study

In order to determine the stability of SA in the diffusion medium during the in vitro release studies, 20 µg/mL of SA solutions in PBS (pH 7.4) were prepared. These solutions were placed in the receptor compartment of the diffusion cell. The

upper part of the receptor compartment was closed with stretch film and the system was covered with aluminum foil to protect from light. The diffusion cells containing SA solutions were placed in the same manner in a thermostated water bath at 37 °C and samples were measured at the 0, 1, 3, 6, 10, 12 and 24 hours using UV spectrophotometer at λ_{max}=296 nm. Thus concentration of samples were calculated from the calibration curve of SA in PBS (pH 7.4) regression equation.

3.3.4.2. Formulations Used For In Vitro Release Studies

In vitro release studies were performed with SA loaded and blank two different NLCs with various oil/fat ratios. The 0.5% SA and 1% SA loaded NLC(40:60), NLC(50:50) formulations were chosen to perform release studies, and 0.5% SA loaded SLN was used for comparison.

3.3.4.3. Diffusion Apparatus

Modified Franz diffusion cells were used to evaluate the amount of drug release from the developed formulations. The Franz diffusion cell made from an inert glass that consisted of an receptor compartment and a donor compartment. The surface area of the release membrane was 2.5 cm^2 and the volume of the receptor phase was 10 mL with a side arm as sampling port. The donor compartment that is made from a socket glass was placed after the cellophane membrane (MWCO 12400 Da) is mounted between the two compartments and a metalic spring was used to fix the membrane and to position compartments tightly.

The temperature of the assay was accurately controlled at 37 °C to mimic human skin conditions. The receptor medium was composed of phosphate buffer solution (pH 7.4) and which was continuously stirred by magnetic bar at 600 rpm to avoid different concentrations within the receptor medium and to minimize stationary layers.

Samples of receptor solution, 1 mL aliquots, were withdrawn through the sampling port of the receptor compartment at various time intervals. The cells were refilled with fresh receptor solution to keep the volume of receptor phase constant during the experiment. The experiments were run for 24 hours.

3.3.4.4. Preparation of Diffusion Medium

Release studies of SA from formulations using PBS 7.4 is available in literatures and it is reported that PBS 7.4 could perform a suitable release medium for SA (167). Also this pH value was believed to be suitable for release studies of salicylic acid from formulations (168,171,172).

Thus fresh PBS with pH 7.4 was selected as diffusion medium after the solubility test of SA in this medium and it was concluded that this medium could supply the sink condition for SA. The PBS solution pH 7.4 was prepared according to USP 31-NF26 (173) and is soaked in the ultrasonic bath for 1 hour for degasing process.

3.3.4.5. In Vitro Release Studies

In the in vitro release studies the receptor compartment is filled with the diffusion medium and after placing the magnetic stirring rod, the upper part of the receptor compartment was covered attentively with the cellophane membrane (MWCO 12400 Da) which wetted overnight in deionized water. The donor compartment is placed on the top of receptor compartment with a spring system to provide a strained and fixed placement of the cellophane membrane. For sampling the Chiba needle is placed in the sampling port. In this way the the diffusion cells were placed with plexiglass sockets and dipped in a thermostated water bath at 37 °C and stirred at 600 rpm over a multi-magnetic stirrer. Because of the low viscosity of NLC (50:50) formulations it could cause a leakage between the receptor and donor compartments. For this reason the frame of the openning part in the bottom of donor compartment and of the upper part of receptor compartment were sticked together using silicone gum. Before this process the diffusion medium and the magnetic stirring rods were placed in the cell. The pre-wetted cellphane membrane were dried and placed tightly on the receptor compartment. Finally the donor compartment was placed on the receptor compartment and at the end some drops of distilled water was dropped on the membrane to prevent drying. Thenby the fixed cells were kept overnight. The next day the distilled water was removed away and the cells were

placed with plexiglass sockets in thermostated water bath at 37 °C over a multi-magnetic stirrer at 600 rpm.

1 g from the formulation was placed on the donor compartment by an injector. The blank formulations were used as blank. In order to protect the system from light and provide a dark condition for the all release system was completely covered with aluminum foil. Selected sampling times were at the 0.25, 0.5, 1, 2, 3, 4, 6, 8,10, 12, and 24 hours. 1 mL samples were taken from the sampling port by using Chiba needless and insulin injectors and the cells were refilled with the same amount of fresh diffusion medium at the same temperature to keep the volume of receptor compartment constant during the experiment.

The samples then were analysed by UV spectrophotometer at λ_{max}=296 nm. Samples were diluted with diffusion medium as required. The obtained absorbance were replaced in the calibration line equation to calculate the concentrations.

3.3.4.6. Spectrophotometric Analysis of SA

The amount of SA released from the samples was detemined using UV spectorphotometer at λ_{max}= 296 nm and replacing the absorbance value in the calibration equation of SA in PBS pH 7.4. To describe the kinetics of the SA release from the NLC and SLN, mathematical models such as zero-order, and Higuchi's model were used and discussed.

3.3.5. In Vitro Cell Culture Studies

In vitro cytotoxicity studies were performed using L929 mouse fibroblast cell line. Because of the higher encapsulation efficiency of NLC(50:50); blank and 0.5% SA loaded NLC(50:50) formulations were used in this study.

3.3.5.1. Preparation of Complete Cell Culture Medium

The complete cell culture medium for L929 cells was prepared by adding fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine to the Dulbecco`s Modified Eagle's Medium (DMEM) to have the final concentration of 10% v/v, 50 Unit/mL, 50µg/mL and 0.002 mM/mL respectively.

3.3.5.2. Preparation of L929 Cells for Cytotoxicity Studies

L929 cells were brought out from -180 ºC nitrogen tank and placed in laminar air flow cabin. Then, they were added to 10 mL medium in 15 mL sterile tubes and centrifuged at 1000 rpm for 3 minutes, supernatant was discarded and cells were resuspended in fresh medium and transferred to 25 cm^2 cell culture flask. The flask was incubated in 5% CO_2, at 37 ºC and humid incubator. The medium of the flask was changed with fresh medium every 3-4 days and trypsinized when the cells approached to approximately 75 % of confluency. For trypsinization, 1 mL trypsin-EDTA solution (trypsin 0.005%, EDTA 0.002% w/v) was added to the flask and kept in the incubator for 2 minute. After that, the process was repeated through centrifugation, resuspension and incubation until introduced into 96 well microplate for cytotoxicity studies.

3.3.5.3. Determination of LD_{50} Value for SA

For the determination of LD_{50} value of SA; SA was dissolved in DMSO solution and dilutions from this solution were prepared in the complete cell culture medium. The prepared SA solution concentrations were 0.000625% , 0.00125%, 0.0025%, 0.005%, 0.01%, 0.04%, 0.16%, 0.32%, and 0.5%, and these solutions were applied to L929 cells in order to evaluate the LD_{50} value and cell viability (%) results (n=3).

3.3.5.4. Cytotoxicity Studies

To investigate the cytoxicity effect of SA, SA-loaded NLC and the blank NLC on L929 cell, the WST-1 assay was performed . WST-1 is a colorimetric assay that is based on the cleavage of the tetrazolium salt reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. The WST-1 reagent produces a water soluble formazan dye which can be detected by absorbance at 420nm-480nm (99,174). The WST-1 reaction product can be quantified in 0.5 to 4 hours without an additional solubilization step (99).

In order to obtain a monolayer cell population, cells were introduced into 96-well, flat-bottomed plates at 5 x 10^3 cells/100 µL/well. They were kept in the

incubator overnight to allow them to attach to the wells in 5% CO_2 at 37°C. Following day, the medium was removed and then triplicate wells were treated with 100µL for each blank NLC(50:50), 0.5% SA-NLC(50:50), and SA solution for 12 and 24 hours. The applied NLC concentrations were 0.125, 0.25, 0.5, 1 and 2 % (v/v). The equivalent SA concentrations in SA loaded NLC(50:50) formulation were 0.000625, 0.00125, 0.0025, 0.005 and 0.01% (w/v). After treating, the plates were incubated for 12 h and 24 h at 37°C in 5 % CO_2 incubator. According to WST-1 protocol, 10 µL of WST-1 was added to each well and the plates were again incubated for 2 h and the absorbance was measured at 440 nm. Cells grown in culture medium was used as a common control for all other samples.

3.3.6. Stability Studies

NLCs are described as stable systems for preivous stability studies and the structural specialities described and discussed physical and chemical long-term stability in literatures (1,13,25). By selecting suitable surfactants, lipids and storage conditions the physical and chemical stability of NLC can be improved too (18).

The stability study of blank and 0.5% SA loaded NLC formulations were performed with two different oil/fat ratios: NLC(50:50) and NLC(40:60). Stability study for blank and 0.5% SA from SLN was also performed for comparison.

The NLC and SLN formulation were stored in glass vials during 90 days at 25°C and 4°C. During storage period, the changes in physical appearance, colour, particle size, zeta potential, pH, viscosity, encapsulation efficiency, loading capacity of the formulations, and SA content in formulations were evaluated. The vials were covered and wrapped with aluminum foils and stored in dark to protect from light during storage.

3.3.6.1. Macroscopical Evaluation

The appearence of all formulations were evaluated during stability studies if there were aggregation, phase seperation, colour change or sedimentation.

3.3.6.2. Particle Size and Zeta Potential

Particle size and zeta potential of NLC and SLN formulations were measured as described in 3.3.2.1.2. Particle size and distribution of all formulations were measured in the 0, 7, 15, 30, 60 and 90 days.

3.3.6.3. Viscosity

The viscosity of all formulations were determined as described in 3.3.2.1.3. During stability studies, the viscosity measurements were performed in the 1, 7, 15, 30, 60 and 90 days.

3.3.6.4. pH value

pH of all the blank and SA loaded NLC and SLN formulations were measured as mentioned in section 3.3.2.1.4. pH values of all formulations were measured in the 0, 7, 15, 30, 60 and 90 days.

3.3.6.5. Encapsulation Efficiency and Loading Capacity

Encapsulation efficiency and loading capasity of the SA loaded formulations were determined as described in 3.3.3. For stability studies the measurements were performed in the 1, 7, 15, 30, 60 and 90 days.

3.3.6.6. Salicylic Acid Stability in Formulations

Salicylic acid content in the formulations were determined by using the calibration curve equation which was established from chloroform:metOH (1:1, v/v) system as described in 3.2.2. and 3.2.3. A 0.5 g of the loaded formulation was dissolved and diluted (1:100) with the organic solvent in a volumetric flask covered with aluminum foil. Then, this dilured sample is kept in ultrasonic bath for 30 min and filtered through 0.45μm PTFE syringe filters. The SA content was calculated from the obtained absorbance value measured by UV spectrophotometer at λ_{max}= 304 nm using the calibration equation.

3.3.7. Statistical Analysis

Results of the experiments were given by the average and the standard error values. Other statistical analysis were done by using SPSS 16 program. Calibrations and validation of the methods were evaluated by calculating their average, standard error and coefficient of variation or the relative standard error values. The stability results of validation and release studies were evaluated by using One-Way ANOVA for repeated measures.

The difference between more than 3 groups were evaluated statistically by using non-parametric test of Kruskal Wallis- one way analysis for variance test. In vitro release study results in terms of released amount and release rate were compared using non-parametric ANOVA-Kruskal Wallis one way analysis for variance test. The difference between 2 groups were compared by using Mann-Whitney U Test. Statistical significance was considered at $p<0.05$.

4. RESULTS

4.1. Physicochemical Properties of Salicylic Acid

Physicochemical properties of salicylic acid was determined by investigation of the following parameters:

4.1.1. FTIR Spectrum

The FTIR Spectrum of SA is given in Figure 4.1. The spectrum of SA revealed several absorption bands in the region of stretching vibrations of O-H and the aromatic C-H bonds. Thus the absorption bands at 3233 cm^{-1} was assigned to O-H. The absorption band of the hydroxyl group is most probably overlapped with the broadened band spreading from 3550 to 3200 cm^{-1}. While the absorption at bands at 2976-2161 cm^{-1} were assigned to the aromatic C-H bonds. The stretching vibrations of aromatic C=C bonds were assigned at 1441.91- 1481.68 cm^{-1}, while the carboxylic acid vibration band was assigned at 1652.15 cm^{-1}. The stretching vibration at 1236.2 cm^{-1} was assigned to the aromatic C-O bond and the abdorption band at 1292.2 cm^{-1} was assigned to the carboxylic C-O bond. The obtained FTIR Spectrum of SA (a) was found to be identical to the reference FTIR Spectrum (b) (175).

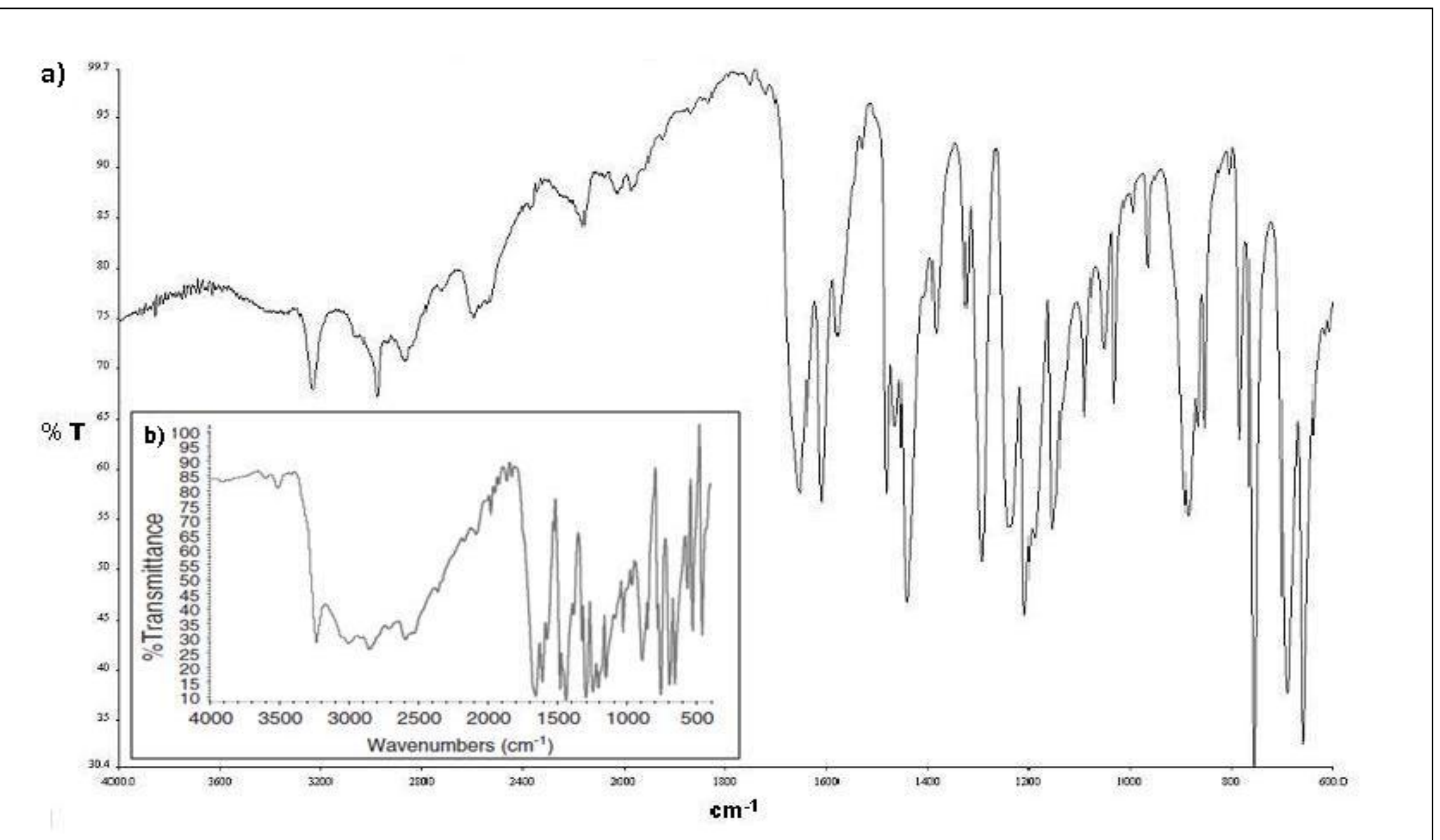


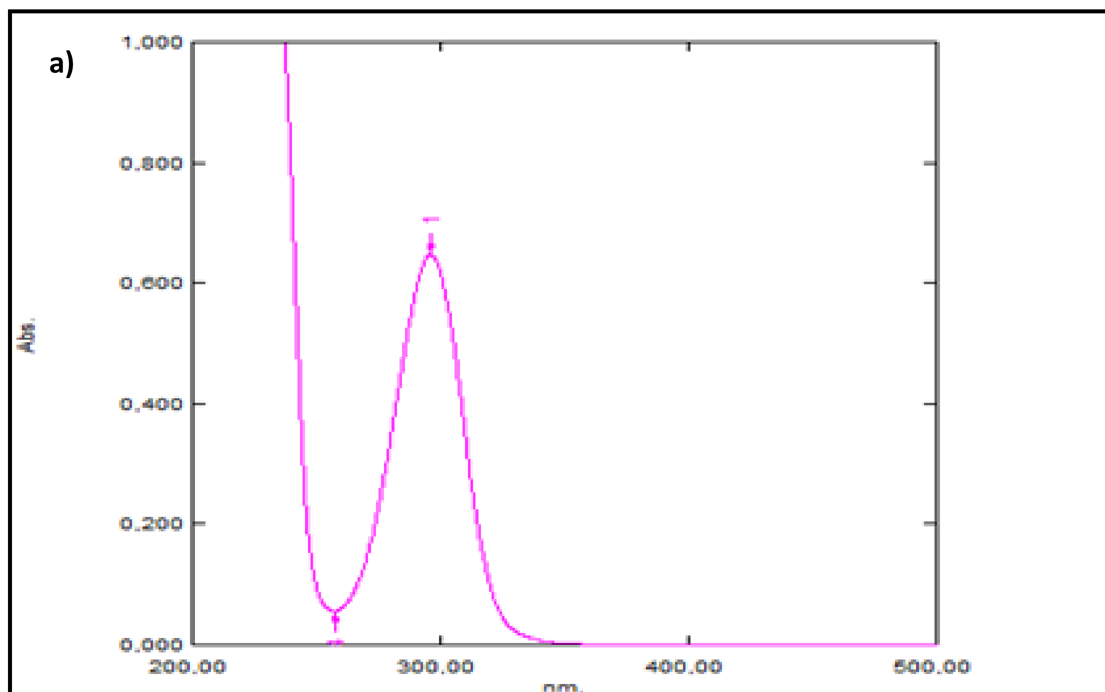
Figure 4.1. FTIR Spectrum of salicylic acid (a), and reference SA FTIR Spectrum (b).

4.1.2. Melting Point of Salicylic Acid

Melting point of SA was determined as mentioned in the section 3.2.1.2. and the result was found in the range of 158–161°C (n=6). This result was in accordance with the reference values (159).

4.1.3. UV Spectrum

As described in 3.2.1.3. the UV Spectrum of SA was established. The UV spectrum of 25 µg/mL SA in PBS pH 7.4 is given in Figure 4.2 (A) and the λ_{max} was found to be 296 nm. The UV spectrum of 20 µg/mL SA in chloroform:metOH (1:1, v/v) system is given in Figure 4.2 (B) and the λ_{max} was found to be 304 nm. The UV spectrum of SA in PBS pH 7.4 and in the chloroform:methanol (1:1, v/v) system were compatible with the references (176,177).



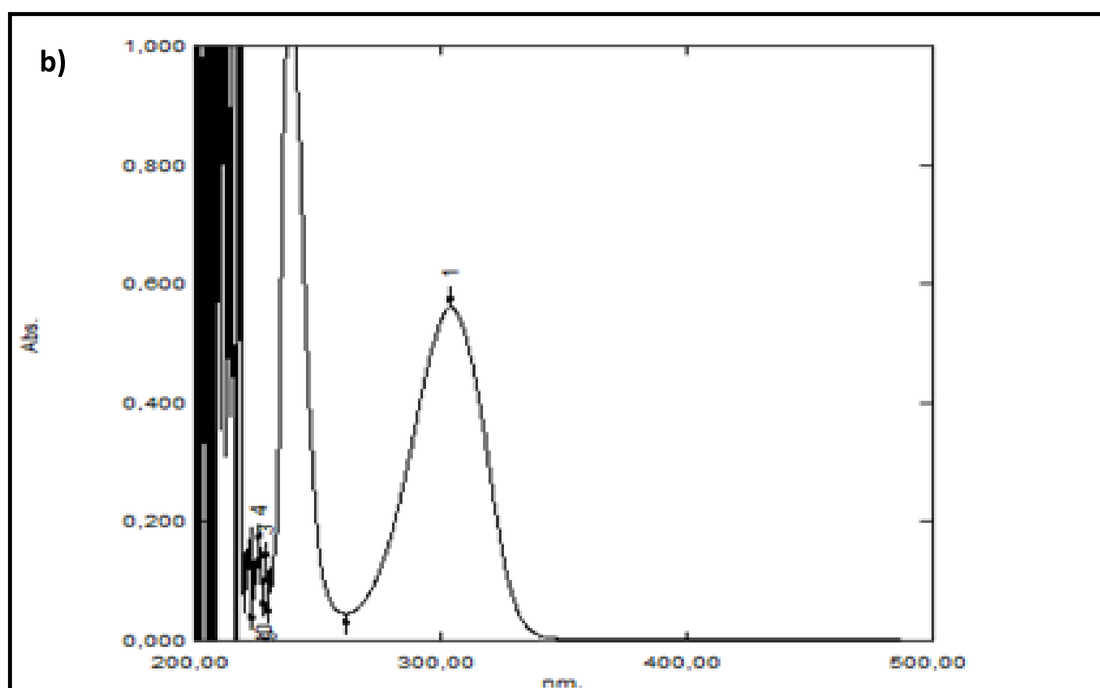


Figure 4.2. **(a)** UV Spectrum of SA in PBS pH 7.4 (λ_{max} =296nm), **(b)** UV Spectrum of SA in chloroform:metOH (λ_{max} =304nm).

4.1.4. Calibration Curves

The calibration curves of SA in PBS pH 7.4 and in chloroform:metOH (1:1, v/v) were established according to the method described in the section 3.2.2. The calibration curve of SA in PBS pH 7.4 is given in Figure 4.3. The slopes of different 6 series were calculated and the variation coefficients of these 6 slopes were found to be 0.248. The average values of the slopes and intercepts, along with other analytical data, are given in the Table 4.1.

The quatitative analysis of salicylic acid was determined by using the regression equation of the calibration curve. The calibration curves of SA in chloroform:metOH (1:1, v/v) was established according to the method described in the section 3.2.2. The calibration curve of SA in chloroform:metOH (1:1, v/v) is given in Figure 4.4. The slope of different 6 series were calculated and the variation coefficients of these 6 slopes were found to be 0.268. The average values of the slopes and intercepts, along with other analytical data, are given in the Table 4.2.

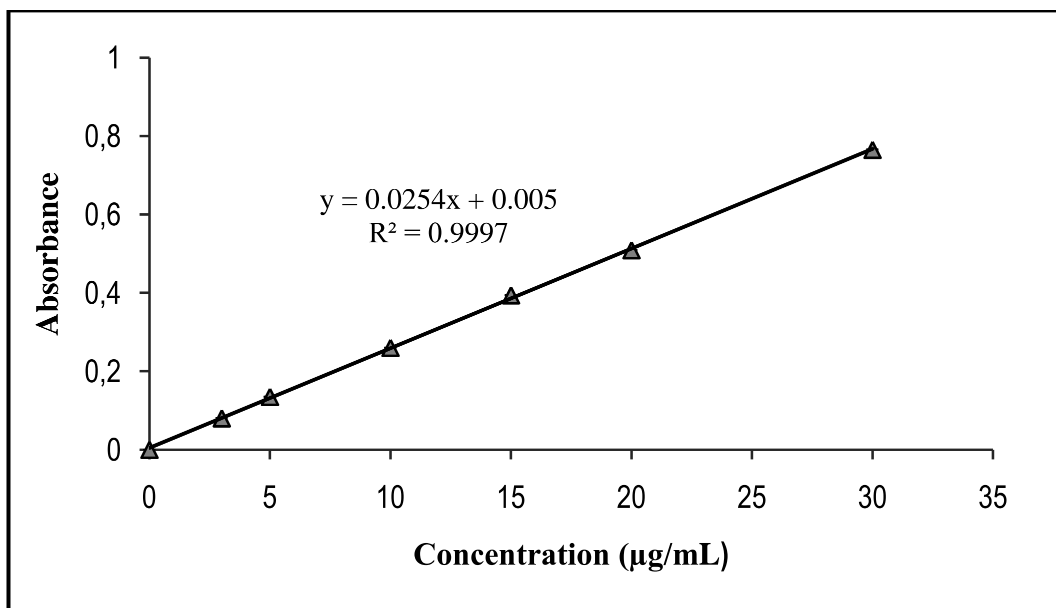


Figure 4.3. Calibration curve of SA in PBS pH 7.4 (n=6).

Table 4.1. Linear regression data (n=6) of SA in PBS pH 7.4.

Parameter	**Results**
Concentration Range	3 µg/mL - 30 µg/mL
Slope ($\bar{X}$ ± SE)	$0.025 \pm 2.6 \times 10^{-5}$
Intercept ($\bar{X}$ ± SE)	$0.005 \pm 1.4 \times 10^{-4}$
Correlation Coefficient (R^2)	0.9997

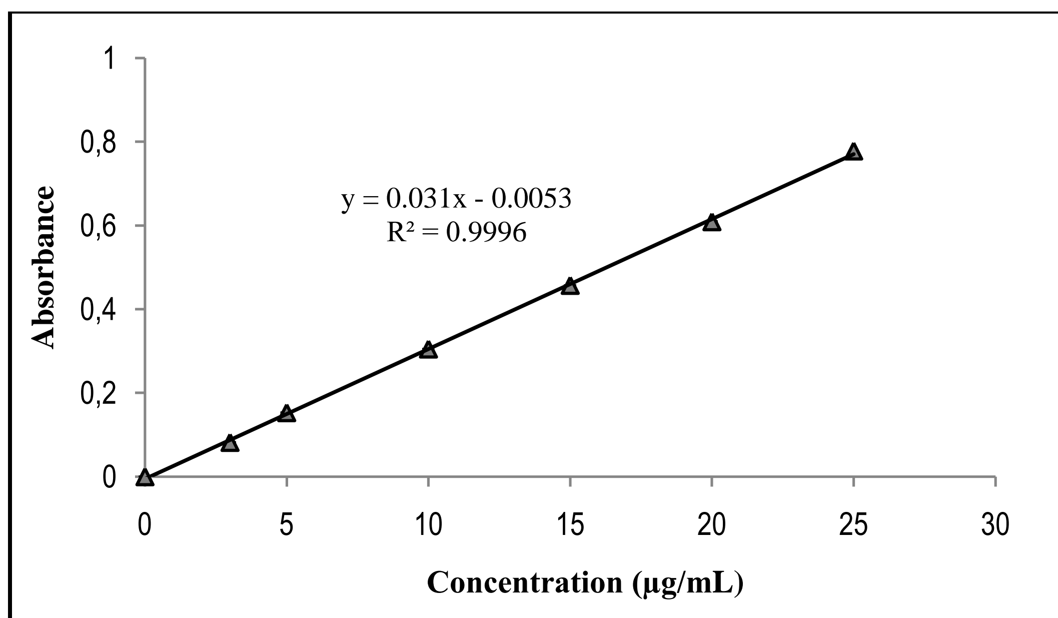


Figure 4.4. Calibration curve of SA in chloroform:metOH (1:1, v/v) (n=6).

Table 4.2. Linear regression data (n=6) of SA in chloroform:metOH (1:1, v/v) system.

Parameter	**Results**
Concentration Range	3 µg/mL - 25 µg/mL
Slope ($\bar{X}$ ± SE)	$0.031 \pm 3.4 \times 10^{-5}$
Intercept ($\bar{X}$ ± SE)	$0.0053 \pm 3.5 \times 10^{-4}$
Correlation Coefficient (R^2)	0.9996

4.1.5. Spectrophotometric Analysis of SA

Spectrophotometric analysis of SA in both systems were peformed by using the regression equations of calibration curves given in Figures 4.2 and 4.3.

4.1.6. Analytical Method Validation

Analytical method validation of both calibration curves shown in Figure 4.1 and 4.1 were investigated in terms of linearity, accuracy, precision, specificity and sensitivity. Analytical method validation of SA in both solvent systems were determined.

Linearity

Linearity of the calibration curves of SA in both PBS pH 7.4 and in chloroform:metOH (1:1, v/v) systems were determined statistically by using the test of departure from linearity as mentioned in 3.2.4. Salicylic acid concentration vs absorbance value graphics show a linear curve over the range of 3 – 30 µg/mL in PBS pH 7.4 and 3 – 25 µg/mL in chloroform:metOH (1:1, v/v) system. Regression analysis and correlation coefficients of both systems also proves the linearity.

Accuracy

As described in section 3.2.4. , two standard concentrations (5 µg/mL and 20 µg/mL) of salicylic acid on the calibration curve were prepared in both PBS pH 7.4 and chloroform:metOH (1:1, v/v) systems and analysed with six replicants (n=6) for each of these two concetrations for assessing intra-day and inter-day (3 consecutive days) for accuracy. Accuracy results are given in Tables 4.3 and 4.4. The intra-day and inter-day accuracy values for both standard concentrations in both systems were calculated from the measured absorbance values and the average concentration values ($\bar{X}$) with standard error (SE) and the relative error (RE) values were calculated.

Table 4.3. Intra-day and inter-day accuracy results of SA in PBS pH 7.4 (n=6).

	Concentration (µg/mL)	**Absorbance λ_{max}=296 nm**	**Calculated Concentration (µg/mL)**	**$\bar{X}$ (µg/mL) ± SE**	**RE (%)**
Intra-day[a]	**5**	0.135	5.118	5.066 ± 0.019	1.320
		0.133	5.039		
		0.133	5.039		
		0.134	5.079		
		0.134	5.079		
		0.133	5.039		
	20	0.508	19.803	19.843 ± 0.012	- 0.785
		0.510	19.882		
		0.510	19.882		
		0.511	19.921		
		0.507	19.764		
		0.508	19.803		
Inter-day[b]	**5**	0.136	5.157	5.085 ± 0.022	1.700
		0.133	5.052		
		0.133	5.052		
		0.134	5.066		
		0.135	5.105		
		0.134	5.079		
	20	0.508	19.816	19.843 ± 0.018	- 0.775
		0.509	19.856		
		0.509	19.843		
		0.510	19.882		
		0.509	19.829		
		0.509	19.843		

[a] intra-day (n=6) ; [b] inter-day (n=6, 3 days).

Table 4.4. Intra-day and inter-day accuracy results of SA in chloroform:metOH (1:1, v/v) system (n=6).

	Concentration (µg/mL)	Absorbance λ_{max}=304nm	Calculated Concentration (µg/mL)	$\overline{X}$ (µg/mL) ± SE	RE (%)
Intra-day[a]	**5**	0.152	5.074	5.09 ± 0.014	1.800
		0.153	5.106		
		0.153	5.106		
		0.152	5.074		
		0.154	5.139		
		0.151	5.042		
	20	0.610	19.848	19.80 ± 0.018	- 1.000
		0.607	19.752		
		0.610	19.848		
		0.609	19.816		
		0.607	19.752		
		0.608	19.784		
Inter-day[b]	**5**	0.153	5.106	5.097 ± 0.010	1.940
		0.153	5.106		
		0.152	5.085		
		0.153	5.106		
		0.154	5.128		
		0.151	5.053		
	20	0.610	19.838	19.805 ± 0.001	- 0.975
		0.608	19.795		
		0.609	19.816		
		0.608	19.784		
		0.608	19.784		
		0.609	19.805		

[a] intra-day (n=6) ; [b] inter-day (n=6, 3 days).

Precision

Precision is determined in terms of reproducibility and repeatability in both PBS pH 7.4 and chloroform:metOH (1:1, v/v) systems.

- **Repeatability**

Repeatability of of the analytical method in both systems were deternined as explained in section 3.2.4. Two standard concentrations, 5 µg/mL and 20 µg/mL of salicylic acid on the calibration curve were prepared in both PBS pH 7.4 and chloroform:metOH (1:1, v/v) systems and six replicants of the same standard for each concentration was measured consecutively. As a result the coefficient of variation results were found to be less than 2% and this proves the method`s repeatability.

Table 4.5. Repeatability test of SA in PBS pH 7.4 for precision (n=6).

Concentration (µg/mL)	Absorbance λ_{max}=296nm	Calculated Concentration (µg/mL)	$\overline{X}$ (µg/mL) ±SE	CV (%)
5	0.134	5.079	**5.059 ± 0.010**	**0.426**
	0.133	5.039		
	0.133	5.039		
	0.134	5.079		
	0.134	5.079		
	0.133	5.039		
20	0.507	19.763	**19.756 ± 0.010**	**0.150**
	0.506	19.724		
	0.507	19.763		
	0.507	19.763		
	0.508	19.803		
	0.506	19.724		

Table 4.6. Repeatability test of SA in chloroform:metOH (1:1, v/v) for precision (n=6).

Concentration (µg/mL)	Absorbance λ_{max}=304nm	Calculated Concentration (µg/mL)	$\bar{X}$ (µg/mL) ±SE	CV (%)
5	0.152	5.074	**5.074 ± 0.0003**	**0.400**
	0.153	5.106		
	0.152	5.074		
	0.152	5.074		
	0.152	5.074		
	0.151	5.042		
20	0.608	19.784	**19.811 ± 0.010**	**0.120**
	0.609	19.816		
	0.608	19.784		
	0.610	19.848		
	0.609	19.816		
	0.609	19.816		

- **Reproducibility**

Reproducibility of the analytical method in both systems were deternined as explained in section 3.2.4. Two standard concentrations (5 µg/mL and 20 µg/mL) of salicylic acid on the calibration curve were prepared in both PBS pH 7.4 and chloroform:metOH (1:1, v/v) systems and analysed with six replicants (n=6) for each of these two concentrations for assessing intra-day and inter-day (3 consecutive days) for precision.

Precision-reproducibility results are given in Tables 4.7 and 4.8. The intra-day and inter-day precision values for both standard concentrations in both systems were calculated from the measured absorbance values and the average concentartion values ($\bar{X}$) with standard error (SE) and the coefficient of variation (CV) values were calculated for the description of precision. As a result the coefficient of variation results were found to be less than 2% and this proves the method`s reproducibility for intra-day and inter-day assays.

Table 4.7. Reproducibility test of SA in PBS pH 7.4 for precision (n=6).

	Concentration (µg/mL)	Absorbance λ_{max}=296nm	Calculated Concentration (µg/mL)	$\overline{X}$ (µg/mL)±SE	CV (%)
Intra-day[a]	**5**	0.135	5.118	**5.066 ± 0.013**	**0.632**
		0.133	5.039		
		0.133	5.039		
		0.134	5.079		
		0.134	5.079		
		0.133	5.039		
	20	0.508	19.803	**19.843 ± 0.025**	**0.307**
		0.510	19.882		
		0.510	19.882		
		0.511	19.921		
		0.507	19.764		
		0.508	19.803		
Inter-day[b]	**5**	0.136	5.157	**5.085 ± 0.016**	**0.787**
		0.133	5.052		
		0.133	5.052		
		0.134	5.066		
		0.135	5.105		
		0.134	5.079		
	20	0.508	19.816	**19.845 ± 0.010**	**0.114**
		0.509	19.856		
		0.509	19.843		
		0.510	19.882		
		0.509	19.829		
		0.509	19.843		

[a] intra-day (n=6) ; [b] inter-day (n=6, 3 days).

Table 4.8. Reproducibility test of SA in chloroform:metOH (1:1, v/v) for precision (n=6).

	Concentrartion (µg/mL)	Absorbance λ_{max}=304nm	Calculated Concentration (µg/mL)	$\bar{X}$ (µg/mL)±SE	CV (%)
Intra-day[a]	**5**	0.152	5.074	**5.09 ± 0.014**	**0.665**
		0.153	5.106		
		0.153	5.106		
		0.152	5.074		
		0.154	5.139		
		0.151	5.042		
	20	0.610	19.848	**19.8 ± 0.018**	**0.225**
		0.607	19.752		
		0.610	19.848		
		0.609	19.816		
		0.607	19.752		
		0.608	19.784		
Inter-day[b]	**5**	0.153	5.106	**5.097 ± 0.031**	**0.507**
		0.153	5.106		
		0.152	5.085		
		0.153	5.106		
		0.154	5.128		
		0.151	5.053		
	20	0.610	19.838	**19.805 ± 0.035**	**0.097**
		0.608	19.795		
		0.609	19.816		
		0.608	19.784		
		0.608	19.795		
		0.609	19.805		

[a] intra-day (n=6) ; [b] inter-day (n=6, 3 days).

Sensitivity (LOD and LOQ)

The detection and the quantitation limit of the analytical method were analysed in both systems as described in section 3.2.4.

The obtained sensitivity results for the calibration curve in PBS pH 7.4 were found to be for LOD (µg/mL) = 0.04 and LOQ (µg/mL) = 0.133 and for the calibration curve in chloroform:methanol (1:1, v/v) were found to be LOD (µg/mL) = 0.082 and for LOQ (µg/mL) = 0.275.

Specificity (Selectivity)

Specificity of the analytical method was proven by the method described in section 3.2.4. The specificity of the method in both systems PBS pH 7.4 and chloroform:metOH (1:1, v/v) was tested.

For the specificity of the method in PBS pH 7.4 an in-vitro release was performed and the UV spectrum of dialyzate blank NLC and the SA loaded NLC were established after taking the samples from the released media in the 1st hour. Samples were analysed by UV spectrophotometer after dilution (1:10) There was found no interference to salicylic acid peak by the other components in the formulation (Figure 4.5).

On the other hand specificity of the method in chloroform:methanol (1:1, v/v) system was proven by dissolving 0.5 g of each formulation in this system and then topping up to 100 mL volume in a volumetric flask. After soaking the flask in a ultrasonic bath for 30 min, samples were analysed by UV spectrophotometer after dilution (1:5) and there were found no interference between SA peak and the other formulation components (Figure 4.6).

Spectrum results in each system were proven that there was no interference between salicylic acid and the other components in NLC at λ_{max}=296 nm in PBS pH 7.4 and at λ_{max}=304 nm in chloroform:methanol (1:1, v/v) systems.

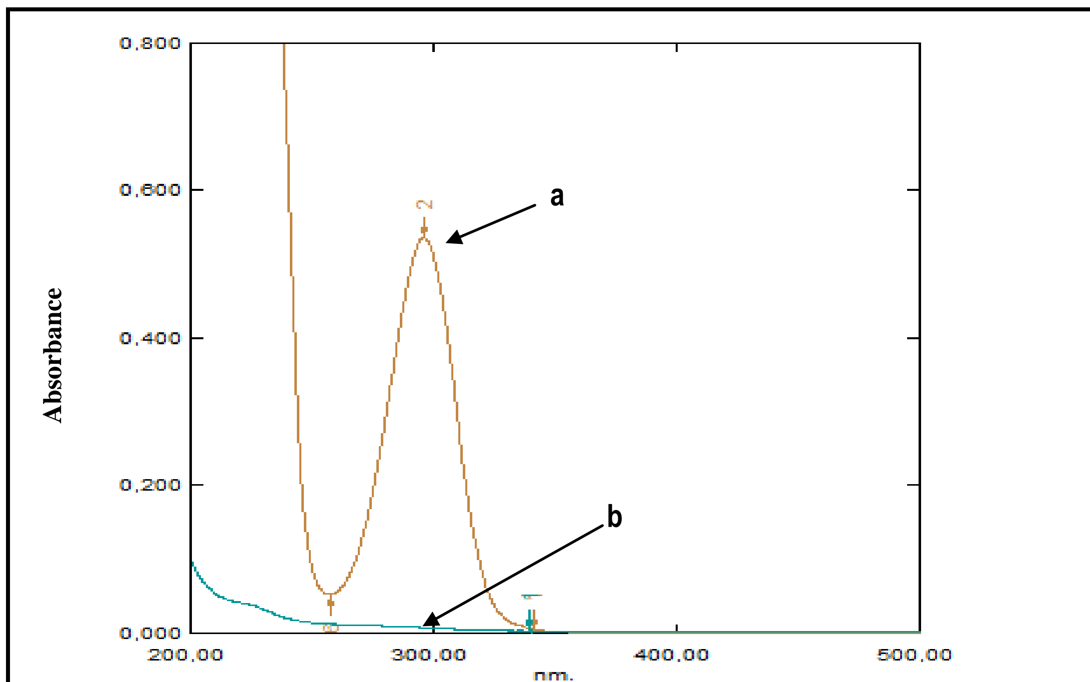


Figure 4.5. UV spectrum of the blank NLC and 1% SA-NLC formulations in PBS pH 7.4. (a) 1% SA-NLC (50:50), and (b) blank NLC (50:50).

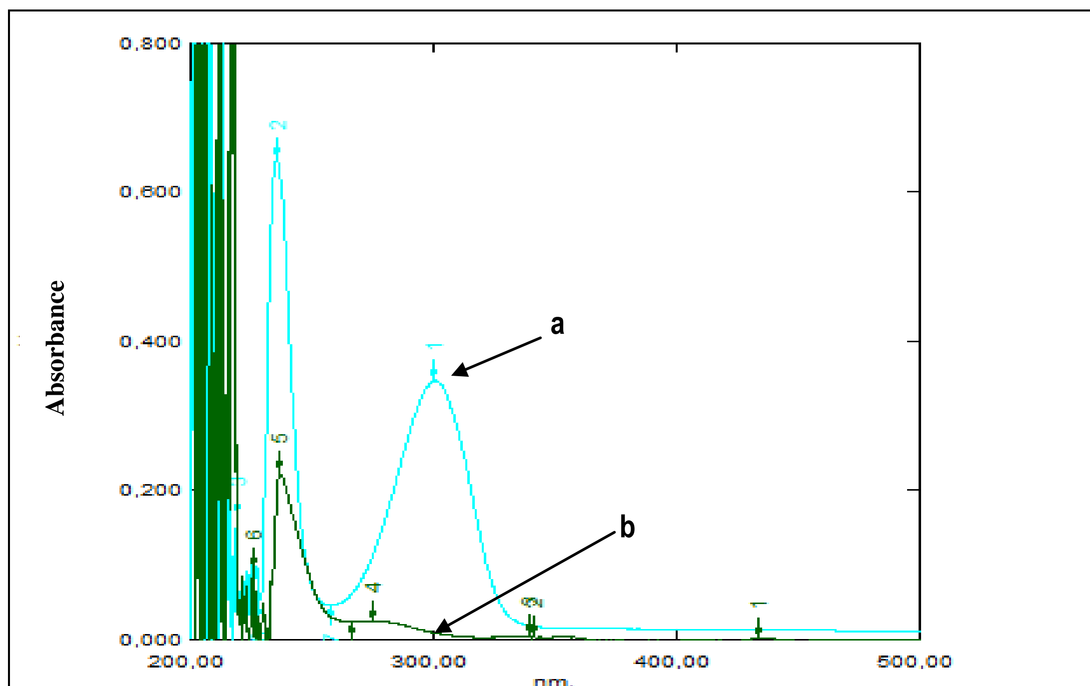


Figure 4.6. UV spectrum of the blank NLC and 1% SA-NLC formulations in chloroform:metOH (1:1, v/v). (a) 1% SA-NLC (50:50), and (b) blank NLC (50:50).

Stability During The Analysis

During the analysis samples are exposed 12 hours at 25°C for both systems. Therefore it was necessary to show that salicylic acid does not degrade during this period. The stability of SA during analysis in each system PBS pH 7.4 and in chloroform:methanol (1:1,v/v) was studied by preparing two standard solutions from the stock solution of 200 µg/mL. Two standards one of low (5 µg/mL) and the other high (20 µg/mL) concentrations.

The absorbance of the solutions were measured at the 0, 1, 3, 6 and 12 hours at λ_{max}=296 nm in PBS pH 7.4 and at λ_{max}=304 nm in the chloroform:methanol (1:1, v/v) system. Concentration of SA in each system was calculated using the regression equation of the calibration curves. The results obtained from both systems were statistically evaluated and there were found no statistical difference between the SA concentration results obtained in each hour ($p > 0.05$). This proves that salicylic acid is stable in both systems (PBS pH 7.4 and chloroform:metOH (1:1, v/v) during the analysis by UV spectrophotometer (Table 4.9 and 4.10).

Table 4.9. Stability test results of SA in PBS (pH 7.4), (n=6).

Time (h)	Concentration (µg/mL)	Absorbance λ_{max}=296nm	Calculated Concentrations $\overline{X}$ (µg/mL) ± SE	CV (%)
0	**5**	**0.134**	5.065 ± 0.019	0.940
1		**0.133**	5.058 ± 0.017	0.810
3		**0.134**	5.079 ± 0.010	0.490
6		**0.134**	5.079 ± 0.010	0.490
12		**0.133**	5.078 ± 0.010	0.480
0	**20**	**0.509**	19.830 ± 0.012	0.150
1		**0.508**	19.830 ± 0.017	0.210
3		**0.507**	19.760 ± 0.020	0.250
6		**0.507**	19.780 ± 0.020	0.240
12		**0.509**	19.850 ± 0.019	0.230

Table 4.10. Stability test results of SA in chloroform:metOH (1:1,v/v) system (n=6).

Time (h)	Concentration (μg/mL)	Absorbance λ_{max}=304nm	Calculated Concentrations $\overline{X}$ (μg/mL) ± SE	CV (%)
0	**5**	**0.153**	5.101 ± 0.010	0.476
1		**0.153**	5.101 ± 0.010	0.476
3		**0.153**	5.106 ± 0.008	0.400
6		**0.153**	5.096 ± 0.016	0.767
12		**0.153**	5.096 ± 0.011	0.517
0	**20**	**0.609**	19.805 ± 0.016	0.197
1		**0.609**	19.816 ± 0.012	0.146
3		**0.609**	19.811 ± 0.013	0.160
6		**0.609**	19.805 ± 0.016	0.197
12		**0.610**	19.832 ± 0.020	0.247

4.1.7. Solubility Test

As described in section 3.2.5. solubility test of SA in PBS pH 7.4 was performed. Results of the solubility test was shown in Table 4.11. Sampling times were in the 1, 2, 3, 5 and 8 hours and at the end of this period the difference between the amounts dissolved SA in PBS were compared statistically. There were found no difference between the amount SA dissolved in the 3rd, 5th and 8th hours ($p>0.05$). So it is concluded that in solubility test result, the saturation concentration of SA in PBS pH 7.4 was found to be 11.955mg/mL ± 0.103 and this concentration is concluded to be suitable and higher than the volume that required to provide sink condition for SA in diffusion medium. This result proves that PBS pH 7.4 provides the sink condition for salicylic acid to obtain release studies in this medium.

Table 4.11. Solubility test results of SA in PBS pH 7.4 (n=3).

Time (h)	Absorbance λ_{max}=296nm	Calculated Concentrations (µg/mL)	$\overline{X}$±SE (µg/mL)	Solubility $\overline{X}$ ± SE (mg/mL)	Solubility (%)
1	0.593 0.594 0.601	23.150 23.190 (dil.1/500) 23.464	23.268	**11.634 ± 0.100**	**1.163**
2	0.600 0.596 0.604	23.425 23.268 (dil.1/500) 23.583	23.425	**11.713 ± 0.090**	**1.171**
3	0.603 0.619 0.610	23.543 24.173 (dil.1/500) 23.820	23.845	**11.923 ± 0.180**	**1.192**
5	0.605 0.608 0.614	23.622 23.740 (dil.1/500) 23.976	23.790	**11.895 ± 0.104**	**1.190**
8	0.608 0.617 0.612	23.740 24.090 (dil.1/500) 23.900	23.910	**11.955 ± 0.103**	**1.196**

4.2. Formulation Studies

Formulations were prepared by high shear homogenization-ultrasonication technique as described in section 3.3.2. As a result of pre-formulation studies, blank NLC with NLC containing 0.5% and 1% salicylic acid with various ratios of liquid lipid/solid lipid (50:50, 40:60 and 70:30) and blank SLN with 0.5% SA loaded SLN were studied in terms of particle size and distribution, zeta potential and viscosity. Thus among formulations, the selected formulations for this study had small particle size with a narrow size distribution, and viscosity that is thought to be suitable for topical and cosmeceutical applications.

4.2.1. Pre-formulation Studies

NLC formulations were investigated by preparing with various solid and liquid lipid blends with various ratios as described in section 3.3.1. Then by analysing particle size and zeta potential the most suitable formulations were selected

for this study. As a result of pre-formulation studies, the optimized NLC and SLN formulations were developed and chosed for further studies according to their particle size, polydispersity index and zeta potential values.

4.2.2. Lipid Screening

As described in section 3.3.1.2. before the production of NLC formulation and in order to consider the solubility of SA in lipids, a lipid screening should be performed to determine the most suitable lipid for the active ingredient to be incorporated in the NLC.

Visual and microscopical evaluations for the lipid screening of the components were done whether salicylic acid could be dissolved in these liquid and solid lipids. Generally lipids could dissolve SA but crystals of SA were observed visually and microscopically in some of them. Visually there were found that SA dissolved compeletely at 1%, 2%, 3% and 5% SA concentrations in the solid and liquid lipids at both temperatures. However it is observed 10% SA was dissolved slower in both liquid and solid lipid melt at both temperatures. The screening test result is shown in Table 4.12. After 1 and 24 hour of this process the homogeneity of the matrix were investigated microscopically.

Table 4.12. Solubility results of SA in different solid and liquid lipids (25°C).

	Salicylic Acid % (w/w)									
	1%		2%		3%		5%		10%	
Time (h)	**1**	**24**	**1**	**24**	**1**	**24**	**1**	**24**	**1**	**24**
Precirol ATO 5	+	+	+	+	+	+	+	+	±	±
Apifil	+	+	+	+	+	+	+	+	±	±
Compritol 888 ATO	+	+	+	+	+	±	+	±	±	±
Oleic Acid	+	+	+	+	+	+	+	+	+	±
Labrafac Lipophile	+	+	+	+	+	+	+	+	+	+
Labrafac Hydro	+	+	+	+	+	+	+	+	+	+
Labrafil M1944	+	+	+	+	+	+	+	+	+	+

(+): SA dissolved completely (No crystals of SA observed), (±): SA dissolved but crystals of SA observed microscopically.

4.2.3. NLC Formulation Studies with Different Lipid Matrix and DifferentOil/Fat Ratios

Two different hydrophilic surfactants were seperately used during preparation of NLC(50:50) formulations. Results obtained that formulations with Lipoid S100 as lipohilic surfactant and Tween 80 as a hydrophilic surfactant showed the smaller polydispersity index in comparison to formulations prepared with Lipoid S100 and Pluronic-F68. Since NLCs containinig Lipoid S100 and Tween 80 had a narrower size distribution with good zeta potential values, they were selected as stabilizers in NLC and SLN.

After lipid screening results in order to select the best lipid matrix that incorporates SA homogeously in it, blends of Precirol ATO 5 with liquid lipids and Apifil with liquid lipids used in equal ratios [50:50, (% w/w)] to formulate NLC. The blank and SA loaded NLC formulations prepared with Apifil used as solid lipid blended with four different liquid lipids (Labrafac lipophile, Labrafac hydrophile, Labrafil and Oleic acid) indicated significantly larger particle size and distribution results in comparison with the formulations prepared with blends of Precirol ATO 5 as solid lipid and the four different liquid lipids. The increased order of particle size obtained from the blank formulations with various lipid matrix was Ap+LL < Pr+LL < Pr+LB < Pr+OA < Pr+LH < Ap+LB < Ap+LH < Ap+OA. Although the blank formulation with Ap+LL indicated small particle size results, however after SA loading Ap+LL indicated larger particle size and broad size distribution results than Pr+LL ($p<0.05$). The formulations prepared with Precirol ATO 5 and different liquid lipids were found that Precirol ATO 5 with Labrafac lipophile indicated the best results in terms of particle size and narrow size distribution before and after SA loading. So as lipid matrix Precirol ATO 5 as solid lipid with Labrafac lipophile as liquid lipid were selected to formulate NLC (Table 4.13).

Table 4.13. Particle size, PI and ZP of blank and SA loaded Pre-formulations with different lipid matrix (n=5).

	Sample Name	**Z-Avr** $\bar{X}$ **±SE**	**PI** $\bar{X}$**±SE**	**Pk 1** $\bar{X}$**±SE**	**Pk 1**	**ZP** $\bar{X}$**±SE**
		nm		**nm**	**%**	**mV**
Pr±LL	NLC(50:50)	155.60±0.61	0.141±0.01	187.22±1.57	100	-30.90±0.50
	0.5%SA-NLC(50:50)	168.54±0.40	0.168±0.01	205.5±4.35	100	-8.78±0.54
	1%SA-NLC(50:50)	190.94±3.50	0.182±0.004	220.98±4.45	100	-8.50±2.72
Pr±LH	NLC(50:50)	201.30±2.20	0.20±0.03	245.30±0.70	100	-25.50±1.20
	0.5%SA-NLC(50:50)	421.80±11.80	0.50±0.20	442.50±11.10	89.5	-17.20±0.20
	1%SA-NLC(50:50)	647.50±34.00	0.60±0.02	442.50±11.10	60	-19.20±0.30
Pr±LB	NLC(50:50)	178.60±3.90	0.22±0.01	225.10±8.10	100	-26.80±0.30
	0.5%SA-NLC(50:50)	225.60±5.10	0.225±0.02	287.30±15.10	100	-21.10±0.40
	1%SA-NLC(50:50)	274.00±4.2.00	0.385±0.01	425.20±25.10	100	-17.10±0.10
Pr±OA	NLC(50:50)	188.80 ± 1.32	0.22±0.03	210.40 ± 3.30	96	-22.20±0.20
	0.5%SA-NLC(50:50)	302.20 ± 2.31	0.35±0.03	388.40± 5.62	87	-18.10±0.10
	1%SA-NLC(50:50)	515.94±13.29	0.60±0.01	582.20±32.43	88	-12.60±0.40
Ap±LL	NLC(50:50)	145.54± 1.21	0.19±0.01	179.50 ±2.21	99	-39.50±0.22
	0.5%SA-NLC(50:50)	185.48 ±1.53	0.34±0.02	269.98±12.23	98	-17.90±0.31
	1%SA-NLC(50:50)	252.84 ± 3.76	0.36±0.03	332.38±13.35	99	-20.20±0.50
Ap±LH	NLC(50:50)	265.40±1.40	0.32±0.10	310.60±0.90	82.4	-42.20±0.30
	0.5%SA-NLC(50:50)	198.20±3.60	0.21±0.01	210.20±3.40	88.7	-18.80±0.21
	1%SA-NLC(50:50)	622.00±45.10	0.70±0.40	1092.20±13.40	67.3	-16.20±0.22
Ap±LB	NLC(50:50)	256.40±3.20	0.30±0.06	298.60±0.08	100	-34.30±0.12
	0.5%SA-NLC(50:50)	425.20±6.50	0.34±0.10	654.10±11.70	86.2	-20.10±0.41
	1%SA-NLC(50:50)	780.80±12.20	0.40±0.05	1100.10±54.30	76.5	-22.10±0.10
Ap±OA	NLC(50:50)	345.60±1.20	0.26±0.02	367.10±11.70	90	-36.40±0.17
	0.5%SA-NLC(50:50)	590.10±12.30	0.45±0.30	890.10±67.20	65.7	-20.20±0.27
	1%SA-NLC(50:50)	654.32±32.80	0.62±0.20	877.70±56.20	89.5	-18.70±0.30

**Ap:Apifil, Pr:Precirol, LL:Labrafac Lipophile, LH:Labrafac Hydrophile, LB:Labrafil, OA:Oleic acid.

After selecting Precirol ATO 5 and Labrafac lipophile as the lipid matrix in NLC, NLC formulations with various oil/fat ratios 10:90, 30:70, 40:60, 50:50 and 70:30 (%,w/w) were prepared. Among the studied NLC with various oil/fat ratios, studies with NLC(10:90) and NLC(30:70) were discontinued because of the obtained higher particle size and broad size distribution results. The obtained high particle size for the NLC(10:90) and NLC(30:70) are thought to be because of the low liquid lipid

content compared with the NLCs with the order of increasing liquid lipid contents NLC(40:60), NLC(50:50) and NLC(70:30). Thereby, particle size results for blank NLC (10:90) were found to be 222.4 nm±2.26, and for NLC(30:70) 482.5 nm± 28.1. For the SA loaded formulation results, 0.5%SA-NLC(10:90): 239.5nm±4.31, 1%SA-NLC(10:90): 2.22 µm±32.4, 0.5%SA-NLC(30:70): 215.2 nm±0.51, 1%SA-NLC(30:70): 230.5nm ± 0.1 and for 1% SA-SLN: 2.4µm±82 . The particle size and distribution results of the selected NLC formulations will be given in the characterization studies section 4.2.5.

4.2.4. Salicylic Acid Concentration in Formulations

In cosmetic formulations SA should be used in concentrations below 3% (156) . As described in section 3.3.1.4., three different concentrations 0.5%, 1% and 2% of SA were incorporated into NLC(50:50) and results obtained that 2% SA loading into NLC(50:50) formulation were not succeeded and indicated a very broad size distribution with a very large particle size value out of zetasizer measurement range and thus it was measured by mastersizer and the particle size ($d_{0.5}$) was found 18.7µm±0.7. The same result was obtained for the 1% SA loaded SLN in which indicated particle size ($d_{0.5}$) was 21.6µm±0.6. Therefore studies with 2% SA loaded NLC and 1% SA loaded SLN were discontinued.

As a result, due to the suitable particle size and distribution results obtained from 0.5% and 1% SA loaded NLC(70:30), NLC(50:50), NLC(40:60) and 0.5% SA loaded SLN, they were chosen for further studies. These results will be given in the further characterization section 4.2.5.2. The composition of optimum NLC formulations with various oil/fat ratio and with incorporated SA concentrations were given in Table 4.14.

Table 4.14. Composition of selected formulations (%, w/w).

Formulation Code	SA (%)	Precirol ATO 5 (%)	Labrafac Lipophile (%)	Lipoid S100 (%)	Tween 80 (%)	Water q.s.
NLC(70:30)	0.5 /1	3.6	8.4	0.2	2.4	100
NLC(50:50)	0.5 /1	6	6	0.2	2.4	100
NLC(40:60)	0.5 /1	7.2	4.8	0.2	2.4	100
SLN	0.5	12	-	0.2	2.4	100

4.2.5. Characterization of Formulations

Characterization of the 3 different NLC formulations with various oil/fat ratios [NLC (70:30), NLC (50:50), NLC (40:60)] and SLN were selected for these studies. For this purpose blank NLCs, 0.5% and 1% SA loaded NLC, blank and 0.5% SA loaded SLN were investigated in this study.

As mentioned in 3.3.2.1. for the characterization process the organoleptic and physical appearance, particle size distribution, zeta potential, viscosity and pH of the formulations were studied.

4.2.5.1. Macroscopical Evaluation

All blank and SA loaded NLC and SLN formulations were homogenious in appearance, milky coloured, and with different viscosities depending on the difference between oil/fat ratios of NLC. By increasing the amount of the liquid lipid in NLC, the viscosities of the formulations were found to be lower and thus SLN was the most viscose and cream like among them. The higher liquid lipid content in NLC the lower the viscosity of the formulation was observed. Also it is observed that salicylic acid content in the formulations increased the viscosity of the formulations except for the NLC(70:30).

4.2.5.2. Particle Size, Size Distribution and Zeta Potential

The Z-average (PS), peak 1 intensity (Pk 1), polydispersity index (PI) and zeta potential (ZP) of the blank and SA loaded NLC formulations were evaluated (Table 4.15), and the zeta potential of blank and SA loaded SLN were determined using Malvern zetasizer Nano ZS. On the other hand particle size of blank and SA loaded SLN formulation were characterized by Malvern Mastersizer (Table 4.16).

The particle size of blank NLC(70:30), NLC(50:50) and NLC(40:60) were found to be statisticaly significant from one another ($p<0.05$). So that by increasing the oil content in NLC a significant decrease in particle size of blank fomulations was obtained ($p<0.05$). Blank NLC(40:60) showed higher particle size, and this particle size value decreased with the increase of oil content as NLC(70:30) indicated the smallest particle size. Polydispersity results for NLC showed that the particle

distribution was monomodal with narrow size distribution results (PI<0.2) (Table 4.15).

Zeta potential values for the blank NLC formulations were varied in range -25.68 and -30.9 mV and there were no statistical significance between the zeta potential values of blank NLCs (p>0.05). However after SA loading a significant dercrease in ZP value was observed for the both SA concentrations. Thus, the zeta potential of blank and SA loaded NLCs were statistically significant (p<0.05) and no statistical difference were found between 0.5% and 1% SA loaded NLCs (p>0.05).

Table 4.15. Z-average (PS), PI, peak intensity (Pk 1) and ZP of the NLC formulations (n=5).

Formulation Code	Z-Avr (PS) $\bar{X} \pm SE$	PI $\bar{X} \pm SE$	Pk 1 $\bar{X} \pm SE$	Pk 1 Area Int.	ZP $\bar{X} \pm SE$
	nm		nm	%	mV
NLC (70:30)	138.64±0.36	0.134±0.001	159.66 ± 3.31	100	-25.70 ± 0.51
0.5% SA-NLC (70:30)	146.00±0.80	0.152±0.001	174.00 ± 1.43	100	-10.70 ± 0.21
1% SA-NLC (70:30)	157.00±1.60	0.157±0.002	185.40 ± 3.60	100	-9.80 ± 1.45
NLC (50:50)	155.60±0.61	0.141±0.001	187.22 ± 1.57	100	-30.90 ± 0.50
0.5% SA-NLC (50:50)	168.54±0.40	0.168±0.001	205.50 ± 4.35	100	-8.78 ± 0.54
1% SA-NLC (50:50)	190.94±3.50	0.182±0.004	220.98 ± 4.45	100	-8.50 ± 2.72
NLC (40:60)	168.30±1.30	0.164±0.001	213.42 ± 1.15	100	-27.80 ± 0.37
0.5% SA-NLC (40:60)	183.60±2.10	0.172±0.001	222.90 ± 0.03	100	-8.89 ± 1.15
1% SA-NLC (40:60)	201.78±2.14	0.185±0.005	228.48 ± 3.60	100	-8.80 ± 2.63

Particle size results of SA loaded NLC formulations were found to be higher than the blank ones. Loading amount of SA into the formulations increased the particle size and 0.5% SA loaded NLC formulations were statistically significant from 1% SA loaded NLC formulations (p<0.05) and the increasing order of particle size from the smallest one was NLC(70:30) < 0.5%SA-NLC(70:30) < 1% SA-NLC(70:30). Similar results were obtained for NLC(50:50) and NLC(40:60). By increasing the SA concentration, particle size of NLC formulations increased significantly (p<0.05). NLC(50:50) < 0.5%SA-

NLC(50:50) < 1%SA-NLC(50:50), and hence for NLC(40:60) was NLC(40:60) < 0.5% SA-NLC(40:60) < 1% SA-NLC(40:60) (p<0.05) (Figure 4.7).

The particle size of 0.5% SA loaded NLC formulations were evaluated and as a result it was observed that NLC(70:30) which include the higest oil/fat ratio indicated lower particle size than NLC(50:50) and followed by NLC(40:60) (p<0.05). 1% SA loaded formulations indicated similar results. The increasing order from smallest particle size was 1%SA-NLC(70:30) < 1%SA-NLC(50:50) < 1%SA-NLC(40:60) and they al were statistically significant from one another (p<0.05).

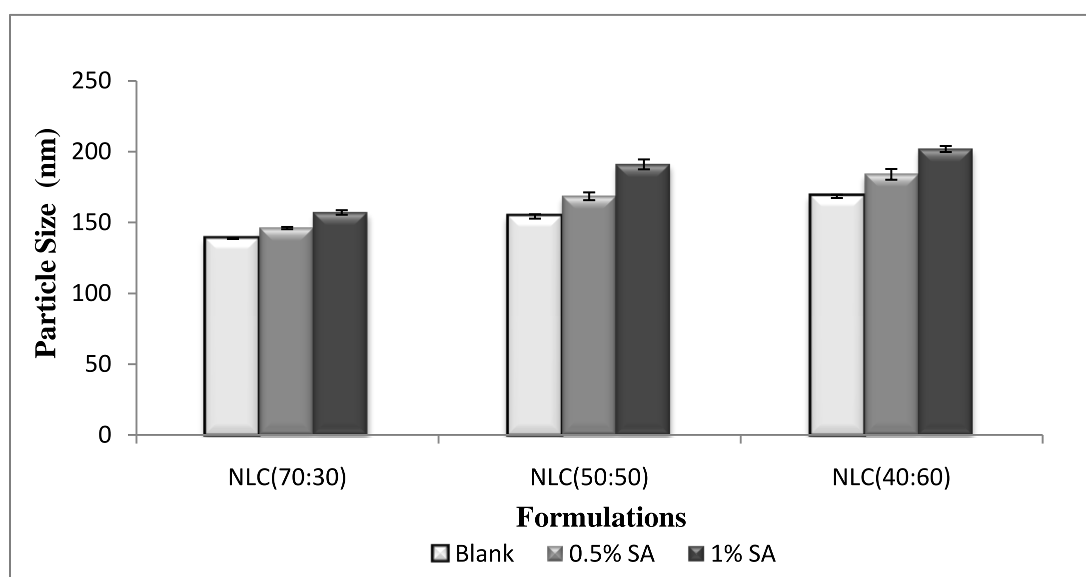


Figure 4.7. The particle size (Z-avr) of blank and SA loaded NLC formulations (n=5).

Salicylic acid loaded formulations caused significantly increase in the particle size (p<0.05) and hence a decrease in the zeta potential value (p<0.05) was observed for all formulations. While blank NLC formulations indicated zeta potential value in range -25.68 and -30.9 mV, after SA loading this value decreased significantly at 0.5% SA and also 1%SA loaded NLC formulations (p<0.05). However there were no statistical difference in zeta potential values between 0.5% and 1% SA loaded NLC formulations (p>0.05) and between 0.5% SA-NLC(70:30), 0.5% SA-NLC(50:50), 0.5% SA-NLC(40:60) (p>0.05).

Blank and SA loaded SLN formulations were characterized by Mastersizer 2000 and 0.5% SA loaded SLN formulation indicated a broad size distribution reached to micron-range. The particle size ($d_{0.5}$) of 0.5%SA-SLN was significantly higher than blank SLN ($p<0.05$). Two batchs of 0.5% SA-SLN were prepared and it is observed that the obtained results for SA loaded SLN formulation were not repeatable (Table 4.16).

Table 4.16. Particle size (volume weighed), size distribution and ZP of blank and 0.5% SA-SLN (µm) (n=5).

		$\bar{X}$ (µm) ± SE			Particle size distribution (SPAN) $\bar{X}$ ±SE	ZP (mV) $\bar{X}$ ±SE
		$d_{(0.1)}$	$d_{(0.5)}$	$d_{(0.9)}$		
Blank SLN		0.155 ± 0.01	0.235±0.002	0.571 ± 0.01	1.77 ± 0.03	-26.20 ± 1.35
0.5% SA-SLN	**Batch 1**	0.273 ± 0.04	0.783 ± 0.08	2.910 ± 0.33	3.40 ± 0.46	-8.20 ± 0.50
	Batch 2	0.300 ± 0.05	1.050 ± 0.25	21.830 ±18.86	15.2 ± 9.15	-8.56 ± 0.50

The obtained particle size and distribution (SPAN) results showed a wide size distribution for 0.5% SA-SLN in the first batch. The results in the first batch indicated smaller particle size than the second batch and was statistically significant ($p<0.05$). The SPAN value of 0.5% SA-SLN in batch 2 was significantly higher than batch 1 ($p<0.05$). The obtained SPAN value shows that blank SLN had significantly lower SPAN value than both batchs of SA loaded SLN ($p<0.05$) (Table 4.16 and Figure 4.8).

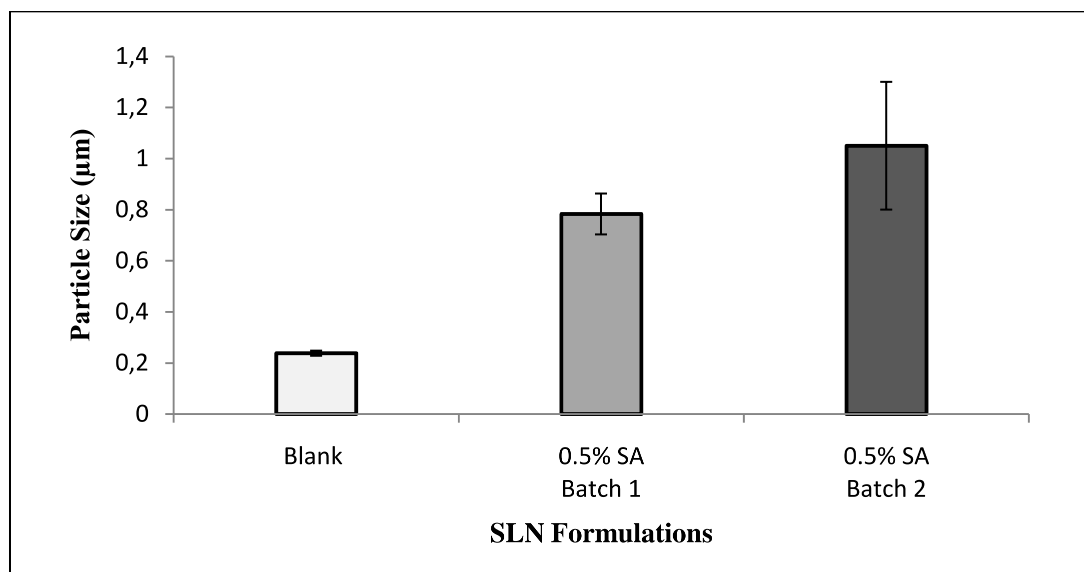


Figure 4.8. Particle size ($d_{0.5}$) of blank and SA loaded SLN formulations (n=5).

It can be concluded that particle size and distribution of the NLC formulations show differences according to the difference in their oil/fat ratio and also different loaded amounts of salicylic acid affected the particle size and distribution, zeta potential and the peak size intensity of the formulations. Depending on the amount of the oil/fat ratios of NLC, in which the oil fraction decreased, particle size and distribution of the formulations increased. However this increase in particle size is more obvious in blank and SA loaded SLN, which does not contain liquid lipid and also shows a broad size distribution.

As obtained for NLC formulations, zeta potential of SLN was also significantly decreased after SA loading ($p<0.05$). There were no statistical difference in zeta potential values between 0.5% SA-NLC(70:30), 0.5% SA-NLC(50:50), 0.5% SA-NLC(40:60) and 0.5% SA-SLN ($p>0.05$).

4.2.5.3. Viscosity

The viscosity of SA loaded and the blank formulations were measured by Brookfield DV-II cone/plate viscometer using CPE-40 and CPE-52 cones at 25°C. All measurements were performed in triplicate.

During rheological evaluation it was observed that all blank and SA loaded formulations show pseudoplastic flow type, that the calculated n value (strength) was smaller than 1. Pseudoplastic flow is a type of non-Newtonian systems and this flow type of systems are determined by the logarithmic relation between shear rate and shear stress as it is explained by using the slope of the log (shear stress/shear rate) and n value is obtained which shows the flow type of the systems. Thus when n<1, the system shows a pseudoplastic flow type. The rheogram shown in Figure 4.9 as the curve begins at the origin and there is no yield value and no linear relation observed in the curves. As the shear stress increased the normally disarranged molecules in the system begin to align their long axes in the direction of flow (178) . Because of the higher viscosity of 0.5% SA-SLN, it indicated more non-linear curve and the curve was shown to be more inclined than blank SLN, blank NLCs, and SA loaded NLCs. Nevertheless, 1% SA loaded NLC(40:60) and NLC(50:50) also had higher viscosity than the blank and 0.5% SA loaded NLC and blank SLN. 1% SA-NLC(40:60) had higher viscosity than 1% SA-NLC(50:50). Thus the obtained curve from these two 1% SA loaded NLCs were found to be more inclined than blank NLC and 0.5% SA loaded NLCs and blank SLN.

Moreover, because of the lower viscosity value of the blank NLCs, 0.5% SA loaded NLCs and blank SLN than 0.5% SA-SLN and 1% SA loaded NLCs, they seemed to be more linear curves but they also indicated pseudoplastic flow type. So, as the viscosity of formulations decreased, their curve observed to be more linear, but at the same time they showed pseudoplastic flow type due to the nonlinear relation between shear rate and shear stress values. Blank NLC formulations indicated similar curves in the rheogram shown in Figure 4.9.

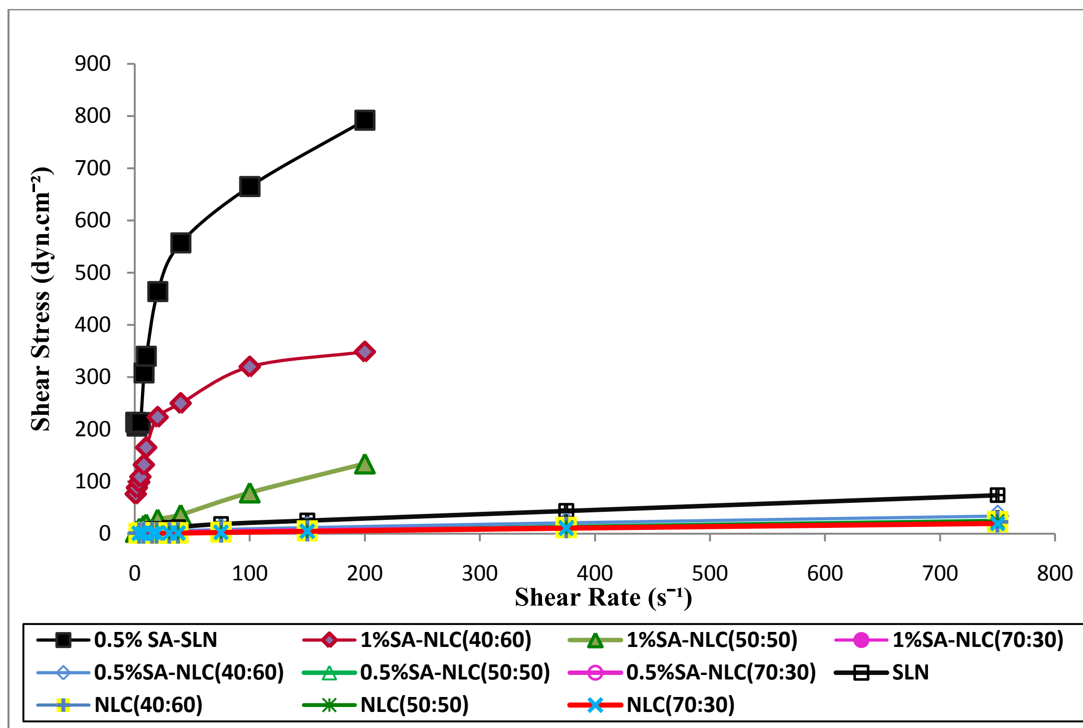


Figure 4.9. Rheological behaviour of blank and SA loaded NLC and SLN (n=3).

Due to the variation of oil/fat ratio in formulations, the viscosity of 0.5% SA-SLN, 1% SA-NLC(40:60) and 1%SA-NLC(50:50) were measured using CPE-52, while the blank formulations and 0.5% SA loaded NLCs and 1% SA-NLC(70:30) were measured using CPE-40. Therefore, the viscosities were given at fixed rpm value (100 rpm) at two different shear rates (Table 4.17). The viscosity value obtained from the formulations decreased with increase in the liquid lipid content in formulations. The viscosity results show that no significant difference in viscosity values were obtained as oil content changes in blank NLC(70:30) and NLC(50:50) formulations ($p>0.05$). However because of the lower liquid lipid content of the blank NLC(40:60) obtained higher viscosity value than NLC(70:30) and NLC(50:50) and was statistically significant ($p<0.05$) and the viscosity of blank SLN was found to be statistically different from blank NLC formulations ($p<0.05$). The viscosity of blank formulations in increasing order is NLC(70:30) = NLC(50:50) < NLC(40:60) < SLN.

Table 4.17. Viscosity of blank and SA loaded NLC and SLN (n=3, at 100 rpm).

Formulation	**Viscosity (cP)** $\bar{X} \pm$ **SE**
* SLN	11.90 ± 0.30
** 0.5% SA-SLN	449.6 ± 0.60
* NLC(40:60)	4.00 ± 0.07
* 0.5% SA-NLC(40:60)	4.48 ± 0.01
** 1% SA-NLC(40:60)	176 ± 0.06
*NLC(50:50)	3.10 ± 0.03
* 0.5% SA-NLC(50:50)	3.25 ± 0.03
** 1% SA-NLC(50:50)	17.9 ± 0.09
* NLC(70:30)	2.10 ± 0.01
* 0.5% SA-NLC(70:30)	2.62 ± 0.01
* 1% SA-NLC(70:30)	3.00 ± 0.01

(*): Viscosity at shear rate 750 s^{-1} , (**): Viscosity at shear rate 200 s^{-1}.

It was observed that SA loading caused an increase in viscosity values especially for the SLN which is only prepared with solid lipid that increased it`s viscosity more than NLCs. 0.5% SA loading increased the viscosity of SLN and was statistically significant from the blank SLN ($p<0.05$). Viscosity of 1% SA loaded NLC(40:60) was found to be higher than the blank NLC(40:60) ($p<0.05$) and 0.5% SA-NLC(40:60) ($p<0.05$). No significant difference were found between blank NLC(50:50) and 0.5% SA loaded NLC(50:50). SA loading show no effect on the NLC(70:30) formulation and no statistical difference was found between the blank NLC(70:30), 0.5% SA-NLC(70:30) and 1% SA-NLC(70:30) ($p>0.05$).

4.2.5.4. pH Value

As described in section 3.3.2.1 4. pH value of blank and SA loaded formulations were measured by direct immersion of electrode of pH-meter in formulations. pH value of the blank formulations were found to be higher than the loaded formulations and they were statistically significant ($p<0.05$). Salicylic acid loaded formulations show an acidic pH value and is believed that this decrease in pH value is because of the acidic character of SA (156) that cause a decrease in the pH value of formulations (Table 4.18). pH value of blank NLC(70:30) was found to be

5.52±0.04, for NLC(50:50) 5.53±0.02, NLC(40:60) 5.48±0.01 and for SLN 5.45±0.04 and they were statistically insignificant (p>0.05).

Table 4.18. pH value of formulations (n=3).

	pH $\overline{X}$ ± SE		
Formulation Code	**Blank**	**0.5% SA**	**1% SA**
NLC(70:30)	5.52 ± 0.04	3.30 ± 0.03	3.28 ± 0.03
NLC(50:50)	5.53 ± 0.02	3.32 ± 0.01	3.27 ± 0.05
NLC(40:60)	5.48 ± 0.01	3.23 ± 0.05	3.30 ± 0.01
SLN	5.45 ± 0.04	3.28 ± 0.16	-

4.2.6. Encapsulation Efficiency and Loading Capacity

As described in 3.3.3. the encapsulation efficiency and loading capacity of the 0.5% and 1% SA loaded NLC(50:50), NLC(40:60) and 0.5% SA loaded SLN formulations were determined. The results of the analysis are given in Table 4.19. In this section NLC(70:30) was not evaluated because of that in vitro release studies were evaluated with SLN, NLC(50:50) and NLC(40:60).

Table 4.19. Encapsulation efficiency and loading capacity of the formulations (n=5).

Formulation Code	Encapsulation Efficiency (%) $\overline{X}$ ± SE	Loading Capacity (%) $\overline{X}$ ± SE	CV (%)
0.5% SA-NLC (50:50)	43.24 ± 0.24	1.80 ± 0.01	1.22
1% SA-NLC (50:50)	34.84 ± 0.24	2.91 ± 0.02	1.55
0.5% SA-NLC (40:60)	36.94 ± 0.13	1.54 ± 0.01	0.77
1% SA-NLC (40:60)	29.76 ± 0.23	2.48 ± 0.02	1.71
0.5% SA-SLN	21.45 ± 0.10	0.89 ± 0.01	1.10

The results indicated that generally as the oil content increased in formulations a significant increase in encapsulation efficiency as well as in loading capacity were observed ($p<0.05$). The encapsulation efficiency of formulations were increased in the following order: 0.5% SA-NLC(50:50) > 0.5% SA-NLC(40:60)> 1% SA-NLC(50:50) > 1% SA-NLC(40:60) > 0.5%SA-SLN. 0.5% SA-NLC(50:50) exhibited higher encapsulation efficiency than 1%SA-NLC(50:50) ($p<0.05$). The same result is also obtained for the NLC(40:60), by increasing loaded amount of SA the encapsulation efficiency significantly decreased ($p<0.05$). Encapsulation efficiency of SLN was significantly lower than NLC formulations ($p<0.05$) (Figure 4.10). This is thought to be due to the liquid lipid absence and also because of the solid lipid matrix core of SLN that lead to reduce incorporation of active compound to SLN.

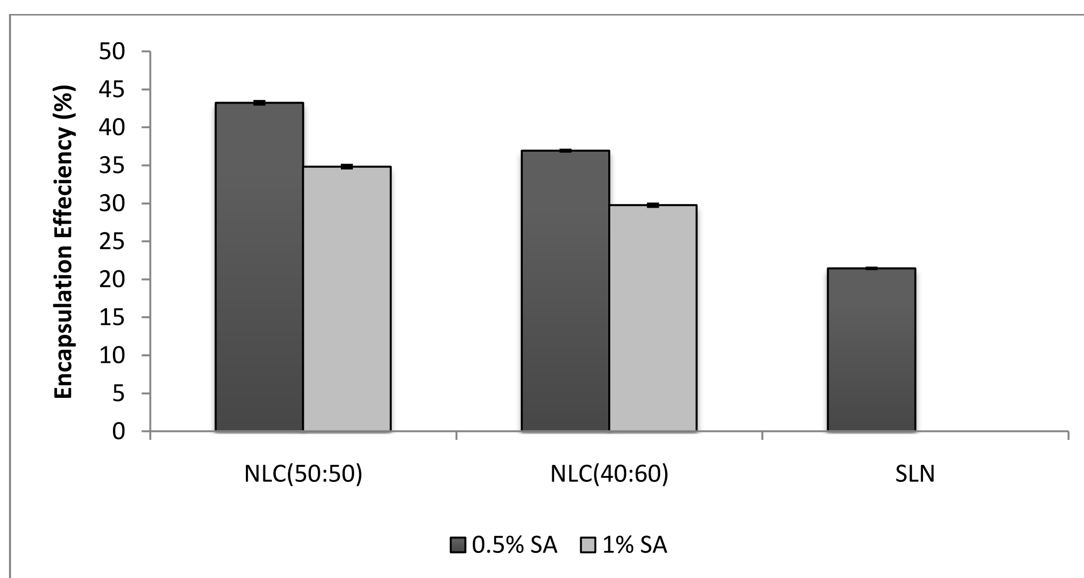


Figure 4.10. Encapsulation efficiency of the formulations (n=5).

The loading capacity increased with the increasing oil content and also in the SA concentration in all formulations ($p<0.05$). 1% SA-NLC (50:50) has obtained the higher loading capacity in comparison to the other formulations ($p<0.05$), followed by 1% SA-NLC(40:60) > 0.5%SA-NLC(50:50) > 0.5% SA-NLC(40:60) > SLN and these values were statistically significant from one another ($p<0.05$) (Figure 4.11).

This is because of the solubility of active compound is thought to be higher in liquid lipid and it could enhance the loading capacity in NLC formulations with the increasing liquid lipid content.

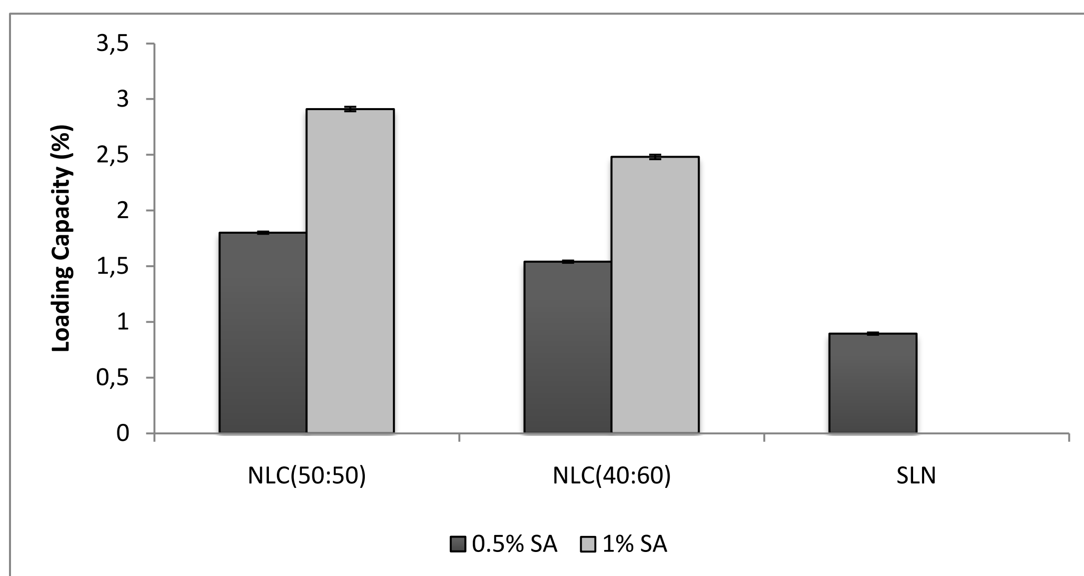


Figure 4.11. Loading capacity of the formulations (n=5).

4.2.7. In Vitro Release Studies

In vitro release studies of 0.5% and 1% salicylic acid loaded NLC(50:50) and NLC(40:60) formulations were performed as described in section 3.3.4. Release study of 0.5% SA loaded SLN was performed in order to compare with NLC formulations.

4.2.7.1. Stability of SA During the In Vitro Release Study

As described in section 3.3.4.1, stability of SA in the diffusion medium was important in order to conclude whether PBS pH 7.4 can be used as release medium. As a result there was found no difference between the obtained concentrations of salicylic acid during analysis in the diffusion medium ($p>0.05$). Thus the results proved that SA is stable during the release period (Table 4.20).

Table 4.20. SA stability in PBS pH 7.4 during in vitro release study (20 µg/mL) (n=6).

Time (h)	Absorbance λ_{max} = 296nm	Calculated Concentration $\overline{X}$ (µg/mL) ± SE	CV (%)
0	**0.515**	20.10 ± 0.03	0.30
1	**0.513**	20.00 ± 0.01	0.12
3	**0.512**	19.96 ± 0.02	0.25
6	**0.513**	20.10 ± 0.06	0.73
10	**0.511**	19.92 ± 0.03	0.37
12	**0.512**	19.96 ± 0.07	0.86
24	**0.510**	19.88 ± 0.04	0.50

4.2.7.2. In Vitro Release Studies of Formulations

NLC(50:50) and NLC(40:60) containing 0.5% and 1% salicylic acid were used for the in vitro release studies. Release study of 0.5% SA from SLN was also performed for comparison with the NLCs. The in vitro release studies were performed as described in section 3.3.4.5 and the results of the SA release from formulations were given in Figure 4.12 and Table 4.21.

An initial burst release observed in the first 30 min in NLC and at 15 min in SLN formulations then the release was slowed down followed by a steady release profile after 8 hours for NLC formulations, and 12 hours for SLN. This burst effect can be explained by the oil/fat ratio, encapsulation efficiency and the viscosity of the formulations. For the 1% SA-NLC(50:50) and 1% SA-NLC(40:60) release profile of SA reaches to plato at 8 h, for 0.5%SA-NLC(50:50) and 0.5% SA-NLC(40:60) this steady state value is at 6 h of release time, however 0.5% SA-SLN reaches to plato position at 12 h.

SA release from 1%SA-NLC(50:50) exhibited faster release profile in comparison with 1%SA-NLC(40:60). This is due to the low viscosity value which is because of higher oil/fat ratio of NLC(50:50). The released SA from 1%SA-NLC(50:50) indicated higher release amount from 1%SA-NLC(40:60) during 12 h,

however after 24 h the released amount was observed to be the similar for both. Hence the released amounts of SA from 1% SA-NLC(50:50) and 1% SA-NLC(40:60) at the end of the 24h were found to be statistically insignificant ($p>0.05$). Besides, release profile of 0.5% SA loaded NLC(50:50) and NLC(40:60) were also comparable. SA release profile from 0.5%SA-NLC(50:50) was higher than 0.5%SA-NLC(40:60) during the study. SA release from SLN exhibited lowest release profile and reached to the steady state slower than the NLCs. The loaded SA amount was also effective on the release behaviour of SA from the formulations. Because of the higher amount of SA in 1%SA-NLC(50:50), the obtained SA release profile curve from 1%SA-NLC(50:50) was higher than 0.5%SA-NLC(50:50). The same release profile was obtained between 1%SA-NLC(40:60) and 0.5%SA-NLC(40:60) (Figure 4.12).

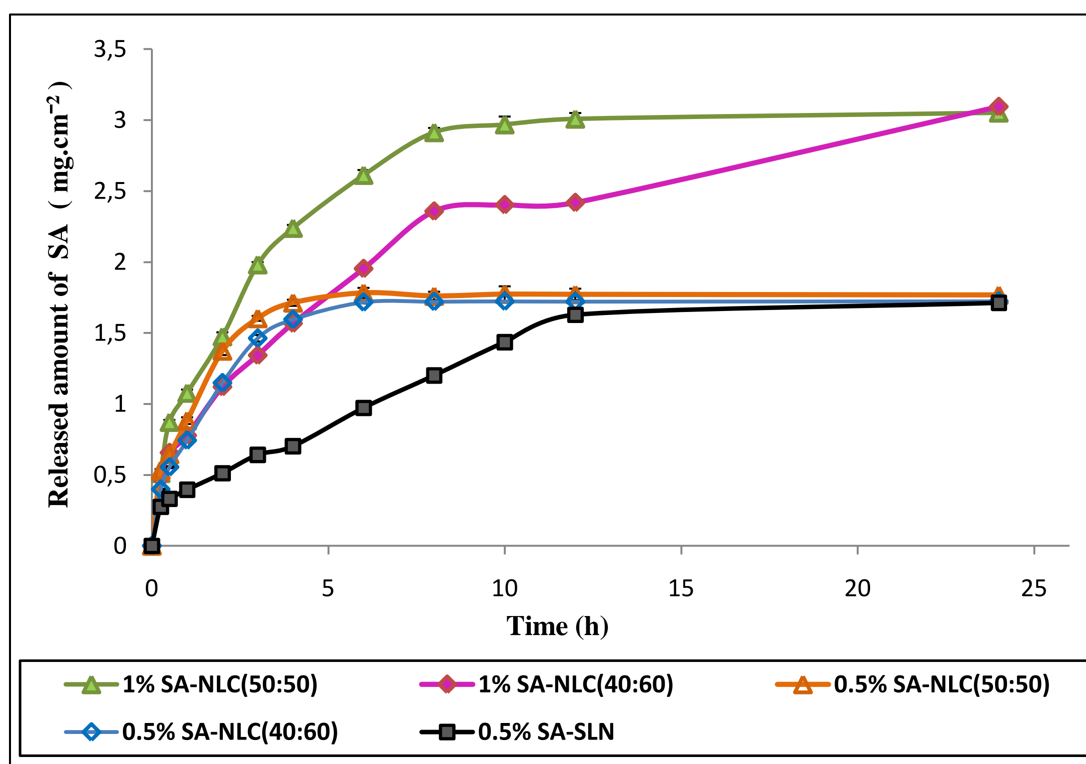


Figure 4.12. In vitro release profile of SA from NLC and SLN formulations (n=6).

The indicated release rate increases with the decreasing viscosity that is because of the increasing of oil content. The increasing release rate order of the formulation were 0.5%SA-SLN < 1%SA-NLC(40:60) < 0.5%SA-NLC(40:60) < 0.5%SA-NLC(50:50) < 1%SA-NLC(50:50).

It can be also explained by the amount of SA loaded into the NLC also affected release rate with regard to the particle size and viscosity. Although 0.5%SA-NLC(50:50) had the smallest particle size and low viscosity, it`s release rate value is lower than 1%SA-NLC(50:50) because of the loaded amount of SA ($p<0.05$). However, in case of NLC(40:60) there is a difference that the release rate of 0.5%SA-NLC(40:60) was higher than that of 1%SA-NLC(40:60). This is thought to be because of the higher viscosity value of 1%SA-NLC(40:60), that made it release slower than 0.5%SA-NLC(40:60) with regard to the loaded amount of SA ($p<0.05$). The SA release profile from the NLC and SLN were fitted into different models. To describe the kinetics of the SA release from the NLC and SLN, mathematical models such as zero-order and Higuchi's model were used (Table 4.21).

Table 4.21. Release amounts and rates of SA from the formulations (n=6).

Formulation Code		**Released Amount ($mg.cm^{-2}$) $\bar{X}$ ±SE**	**Release Rate ($mg.cm^{-2}.h^{-1}$) [a] ($mg.cm^{-2}.h^{-1/2}$) [b] $\bar{X}$ ±SE**	**R^2**
0.5% SA-NLC(50:50)	**Zero Order**	1.776 ± 0.010	0.273 ± 0.002	0.859
	Higuchi Kinetic		0.880 ± 0.001	0.990
1% SA-NLC(50:50)	**Zero Order**	3.053 ± 0.035	0.335 ± 0.003	0.893
	Higuchi Kinetic		1.051 ± 0.004	0.994
0.5% SA-NLC(40:60)	**Zero Order**	1.728 ± 0.004	0.391 ± 0.001	0.889
	Higuchi Kinetic		0.821 ± 0.003	0.996
1% SA-NLC(40:60)	**Zero Order**	3.094 ± 0.010	0.223 ± 0.002	0.920
	Higuchi Kinetic		0.775 ± 0.005	0.995
0.5% SA-SLN	**Zero Order**	1.724 ± 0.010	0.112 ± 0.002	0.971
	Higuchi Kinetic		0.439 ± 0.005	0.982

a : release rate unit for zero order, b: release rate unit for Higuchi model.

Higuchi model explains that drug release from a matrix system is directly proportional to the square root of time (179). The value of R^2 was found to be highest for the Higuchi model ($Q=k.t^{1/2}$). This indicates that the SA release from formulations follows matrix diffusion based release kinetics. Figure 4.13. shows Higuchi model release kinetic of all studied formulations.

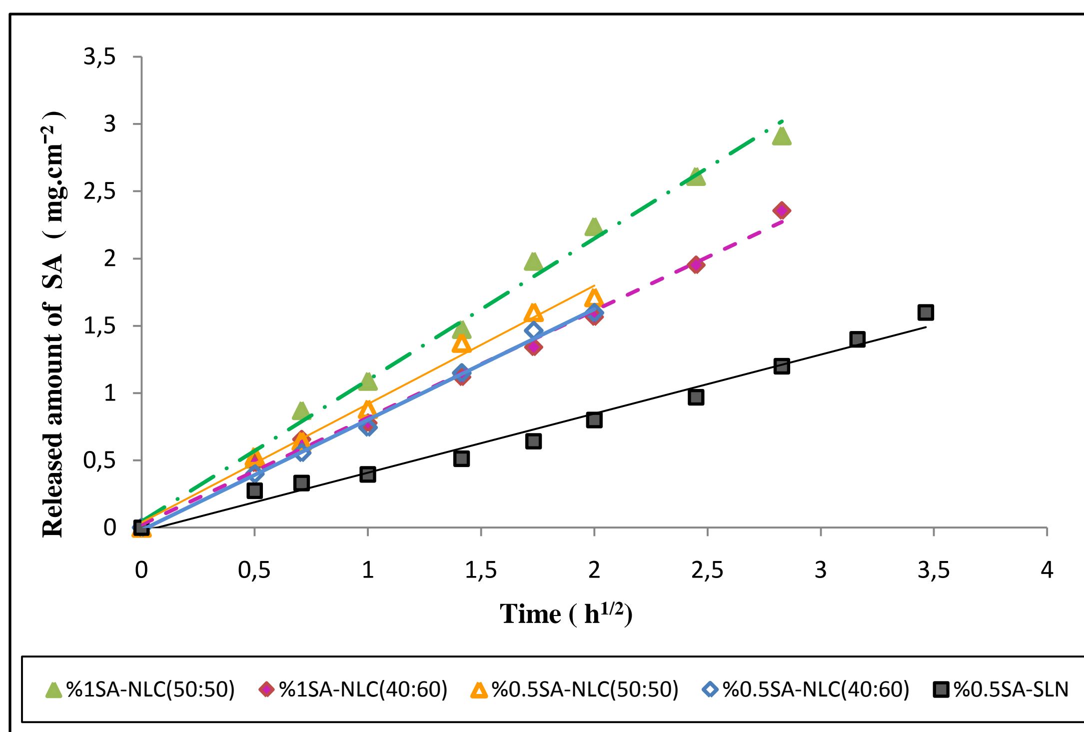


Figure 4.13. In vitro release of SA from NLC and SLN according to Higuchi Kinetic Model (n=6).

As seen from Figure 4.13 the difference in the release rate of formulations were clearly effected by the increase in the oil/fat ratio, viscosity and SA content in formulations. So that SLN had the lowest release rate value due to the absence of liquid lipid and its higher viscosity. The release rate of NLCs were effect with the difference in oil/fat ratio, viscosity and SA concentrations.

Two factors were found to be effective during this release study the first one was viscosity of formulations which was also effected by the change in oil/fat ratio of the formulations, and the second was the loaded SA concentrations into the formulations. Although 0.5% SA loaded NLC(50:50) had the higher encapsulation

efficiency than other formulations as obtained in section 4.3, its the low viscosity value caused to obtain higher release rate. Besides SLN had the lowest encapsulation efficiency and highest viscosity value, after a burst release in the first 30 min the release continued slowly. So it could be concluded that viscosity was the major factor affected the SA release profile and rate from NLC and SLN formulations.

4.3. In Vitro Cell Culture Studies

Cell culture studies were performed as described in section 3.3.5. Studies were performed using blank NLC(50:50), 0.5% SA-NLC(50:50) due to their higher encapsulation efficiency than other studied formulations. Cell viability was determined after 12 h and 24 h using WST-1 assay and also SA solution was used in order to evaluate the LD_{50} value on L929 mouse fibroblast cell line.

4.3.1. Determination of LD_{50} value for SA

Different concentrations of SA solutions were prepared in cell medium and they were were applied on L929 cells for 12 h and 24 h and cell viability were evaluated. (Table 4.22). The results of cytotoxicity tests showed that no cytotoxic effect obtained at concentrations in range 0.000625% - 0.01 % (w/v) after 12 h and 24 h. However at 0.04 % (w/v) concentration and higher, cell viability was decreased significantly after 12 h and 24 h ($p<0.05$). So that the LD_{50} of SA was found to be 0.16 % (w/v) after 24 h.

Table 4.22. Cell viability (%) results and LD_{50} value of SA solutions (n=3).

Concentration of SA Solutions (%, w/v)	Cell Viability (%) $\bar{X}$ ± SE	
	12 h	24 h
0.000625	102.0 ± 0.01	100.3 ± 0.01
0.00125	101.3 ± 0.04	100.4 ± 0.01
0.0025	100.0 ± 0.02	100.0 ± 0.02
0.005	100.0 ± 0.06	100.0 ± 0.03
0.01	101.0 ± 0.05	97.40 ± 0.04
0.04	83.75 ± 0.02	75.50 ± 0.02
0.16	67.60 ± 0.03	48.10 ± 0.05
0.32	69.50 ± 0.05	28.85 ± 0.06
0.5	67.57 ± 0.07	16.35 ± 0.08

4.3.2. Cytotoxicity Studies

Cytotoxicity studies were performed as described in experimental part in section 3.3.5.3. Because of the higher encapsulation efficiency of 0.5% SA-NLC(50:50) (43.24 %) in comparison to the other studied formulations, it was used in this study to evaluate the cytotoxicity effect of NLC formulations before and after loading of SA. NLC concentrations were 0.125%, 0.25%, 0.5%. 1% and 2% (v/v). The equivalent SA concentrations in the loaded NLC(50:50) were 0.000625 %, 0.00125 %, 0.0025 %, 0.005 %, and 0.01 % (w/v). Cell viability results were given in Table 4.23.

Table 4.23. Cell Viability (%) results of the blank and SA loaded NLC(50:50) (n=3).

	Cell Viability (%) $\bar{X}$ **± SE**			
Time	12 h		24 h	
NLC Concentration (%)	**Blank NLC(50:50)**	**0.5% SA NLC(50:50)**	**Blank NLC(50:50)**	**0.5% SA NLC(50:50)**
0.125%	100.0 ± 0.07	100.0 ± 0.04	100.0 ± 0.01	100.0 ± 0.06
0.25%	102.0 ± 0.02	100.0 ± 0.01	100.0 ± 0.01	100.0 ± 0.01
0.5%	100.0 ± 0.01	100.0 ± 0.05	100.2 ± 0.03	100.2 ± 0.04
1%	80.82 ± 0.05	88.12 ± 0.01	75.22 ± 0.03	68.00± 0.08
2%	78.30 ± 0.02	75.70 ± 0.01	70.20 ± 0.03	66.50 ± 0.05

Comparing with control, cell viability results were statistically insignificant between concentrations 0.125% and 0.5% ($p>0.05$) and this result was decreased after 12 h at 1% concentration for both blank and SA loaded NLC(50:50) ($p<0.05$). No significant difference in cell viability results obtained between the blank and SA loaded NLC(50:50) formulations ($p>0.05$). At 2 % NLC concentration the decrease in cell viability result was significant in comparison with control for both blank SA loede NLC(50:50) ($p<0.05$). However no significant difference between blank and SA loaded NLC was observed at this concentration ($p>0.05$) (Figure 4.14).

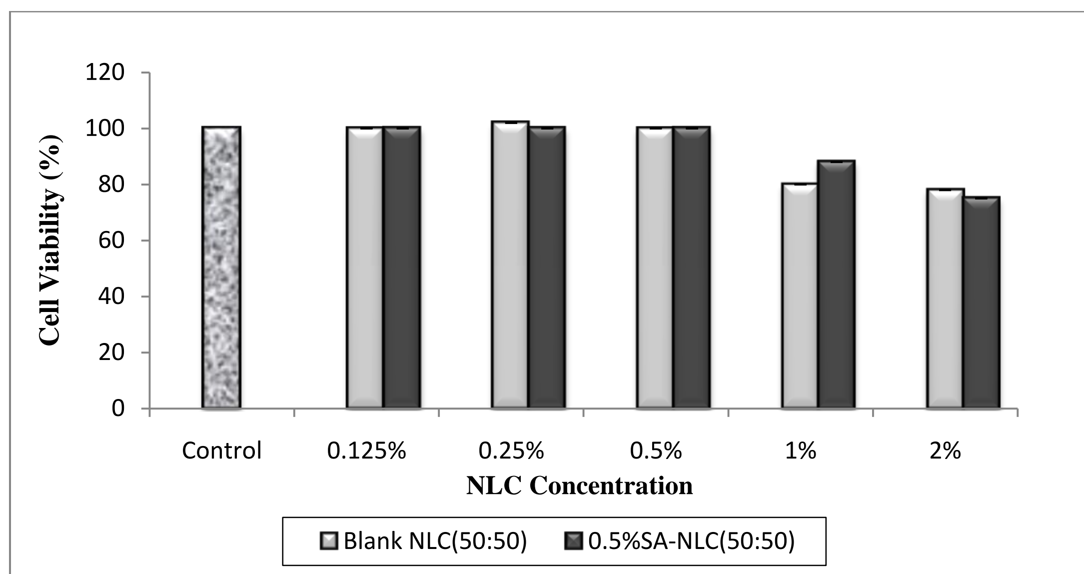


Figure 4.14. Cell viability (%) of L929 cells after 12 h of blank and SA loaded NLC(50:50) formulations (n=3).

It was observed that there was a significant decrease in cell viability at 1% and 2% concentrations after 24 h for both blank and 0.5% SA-NLC(50:50) as compared with control ($p<0.05$). Cell viability results after 12 h, for 0.5% SA-NLC(50:50) was significantly higher than the obtained results after 24 h at both 1% and 2% concentrations ($p<0.05$). After 24 h, the cell viability of blank NLC(50:50) formulation significantly decreased at the 1% and 2 % concentrations as compared with control ($p<0.05$). However, there were no difference between cell viability results of two concentrations (1% and 2%) of blank NLC ($p>0.05$). The cell viability results of both blank and 0.5% SA loaded NLC(50:50) were found to be insignificant as compared with control between concentrations 0.125% and 0.5% ($p>0.05$). However a significant decrease in cell viability obtained with the increasing concentration at 1% for blank NLC(50:50) ($p<0.05$) and 0.5% SA-NLC(50:50) also showed a significant decrease in cell viability with the increasing concentrations to 1% and 2% ($p<0.05$) (Figure 4.15).

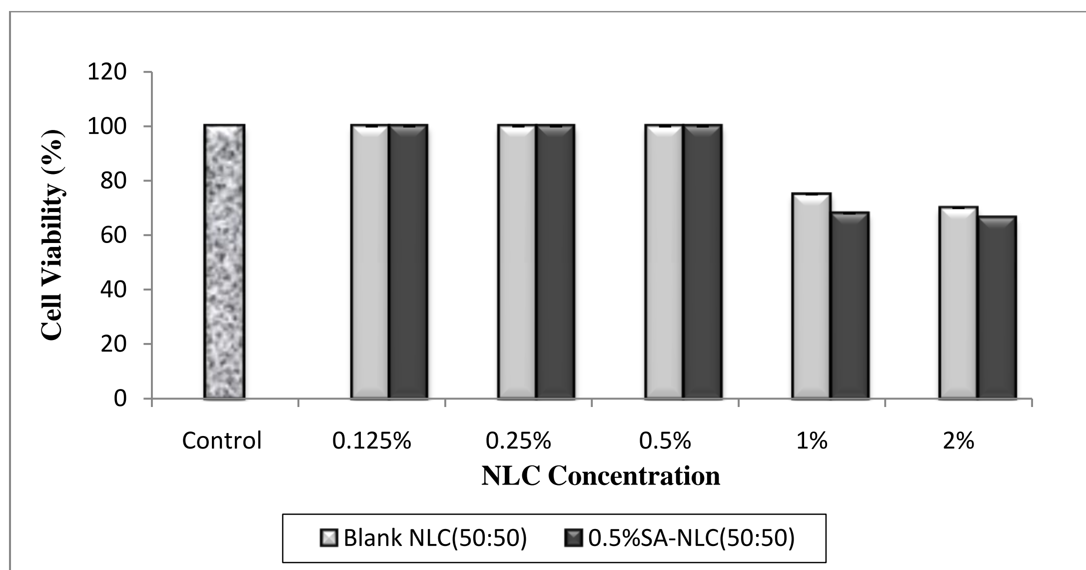


Figure 4.15. Cell viability (%) of L929 cells after 24 h of blank and SA loaded NLC(50:50) formulations (n=3).

As a result of these studies it can be concluded that there were no cytotoxic effect between 0.125% and 0.5% NLC concentrations for all blank and 0.5% SA loaded NLC(50:50) during 12 h and 24h. However it was observed that cell viability results at 1 % and 2 % of blank NLC(50:50) were 80.82 %, 78.30 % after 12 h, and 75.22 %, 70.20 % after 24 h respectively. Cell viability results of 1 % and 2 % concentration of 0.5% SA-NLC(50:50) were 88.22 %, 75.72 % after 12 h and 68 %, 65.50 % after 24 h respectively.

4.4. Stability Studies

Stability of NLC and SLN formulations were performed during 90 days as described in section 3.3.6. Thus the stability study of blank and 0.5% SA loaded NLC formulations were performed with two different forms of various oil/fat ratios [NLC(50:50) and NLC(40:60)] and stability study for blank and 0.5% SA loaded SLN was also performed for comparison.

4.4.1. Macroscopical Evaluation

Physical stability during storage time of the NLC and SLN formulations stored at 4°C and 25°C was evaluated macroscopically in terms of colour, phase seperation, aggregation and sedimentation. The formulations protected their milky colour, and no phase seperation, sedimentation or change in colour observed in blank and SA loaded NLC and SLN stored at 25°C and 4°C. However few aggregations of NLC was observed after 90 day storage at 25°C and 4°C.

4.4.2. Particle Size and Zeta Potential

Particle size, polydispersity index and zeta potential of the studied formulations were measured for NLC and SLN formulations as described in section 3.3.6.2. Particle size measurements obtained from the blank and SA loaded all NLC formulations were found that they protected their monomodal distribution profile at both 25°C and 4°C. During storage of blank NLC(50:50) at 25°C (Table 4.24 and Figure 4.16). The particle size of blank NLC(50:50) increased from 155.6nm to 168.8nm and this increase was statistically significant after 90 days ($p<0.05$). However during storage at 4°C (Table 4.24 and Figure 4.17) there were found no significant increase in particle size and distribution ($p>0.05$). Similar results obtained for the blank NLC(40:60) in which indicated a significant increase in particle size after 90 days at 25°C (Table 4.24 and Figure 4.16) during storage ($p<0.05$) and no significant increase in particle size and distribution were observed 4°C (Table 4.24 and Figure 4.17) ($p>0.05$).

0.5% SA-NLC(50:50) indicated a significant increase in particle size after 90 days at 25°C (Table 4.25 and Figure 4.18) and this increase was statistically significant ($p<0.05$). This increase was not observed for the formulation stored at 4°C (Table 4.25 and Figure 4.19) ($p>0.05$). Also similar results obtained for the 0.5%SA-NLC(40:60), at 25°C a significant increase in particle size at 60 day was obtained ($p<0.05$) but this increase was insignificant at 90 day ($p>0.05$), and no significant difference obtained during storage at 4°C ($p>0.05$).

Table 4.24. Z-Average (PS), Polydispersity Index (PI) and Zeta Potential (ZP) values for blank NLC formulations at 4°C and 25°C during storage (n=5).

	Blank	NLC(50:50)			NLC(40:60)		
	Days of storage	PS(nm) $\bar{X} \pm$ SE	PI $\bar{X} \pm$ SE	ZP(mV) $\bar{X} \pm$ SE	PS(nm) $\bar{X} \pm$ SE	PI $\bar{X} \pm$ SE	ZP(mV) $\bar{X} \pm$ SE
25°C	0	155.60 ± 0.61	0.141± 0.001	-30.90 ± 0.32	168.30 ± 1.30	0.164 ± 0.001	-27.80 ± 0.37
	7	155.21 ± 0.91	0.146 ± 0.001	-27.20 ± 0.60	168.88 ± 0.78	0.172 ± 0.001	-26.40 ± 0.54
	15	157.76 ± 0.50	0.148 ± 0.01	-25.50 ± 0.55	169.72 ± 0.80	0.168 ± 0.030	-24.70 ± 0.36
	30	159.50 ± 0.35	0.165 ± 0.01	-22.10 ± 0.26	172.60 ±0.24	0.198 ± 0.010	-19.50 ± 0.23
	60	159.30 ± 0.20	0.168 ± 0.01	-27.80 ± 0.26	170.10± 0.11	0.169 ± 0.010	-22.40 ± 0.34
	90	168.80 ±1.25	0.220 ± 0.01	28.56 ± 0.50	176.60 ± 3.60	0.192 ± 0.041	-22.70 ± 0.11
4°C	0	155.60 ± 0.61	0.141 ± 0.001	-30.90 ± 0.32	168.30 ± 0.30	0.164 ± 0.001	-27.80± 0.37
	7	155.40 ± 0.05	0.146 ± 0.001	-28.10 ± 0.75	168.36 ± 0.70	0.166 ± 0.000	-27.70 ± 0.26
	15	156.10 ± 0.20	0.144 ± 0.001	-28.80 ± 0.44	169.30 ± 0.50	0.168 ± 0.030	-27.30 ± 0.65
	30	157.70 ± 0.20	0.142 ± 0.01	-25.80 ± 0.50	169.46 ± 0.13	0.177 ± 0.020	-25.81 ± 0.34
	60	158.90 ± 0.13	0.148 ± 0.03	-29.30 ± 0.18	169.80 ± 0.23	0.154 ± 0.020	-26.70 ± 0.23
	90	160.00 ± 0.21	0.156 ± 0.002	-29.70 ± 0.08	174.10 ± 1.60	0.210 ± 0.010	-28.38 ± 0.20

Table 4.25. Z-Average (PS), Polydispersity Index (PI) and Zeta Potential (ZP) values for 0.5% SA loaded NLC formulations at 4°C and 25°C during storage (n=5).

	0.5% SA Loaded	NLC(50:50)			NLC(40:60)		
	Days of storage	PS(nm) $\bar{X} \pm SE$	PI $\bar{X} \pm SE$	ZP(mV) $\bar{X} \pm SE$	PS(nm) $\bar{X} \pm SE$	PI $\bar{X} \pm SE$	ZP(mV) $\bar{X} \pm SE$
25°C	0	168.54 ± 0.40	0.168± 0.001	-8.78 ± 0.54	183.60 ± 2.10	0.172±0.001	-8.50 ± 0.60
	7	168.75± 0.90	0.168 ± 0.002	- 8.50 ± 0.41	183.90 ± 3.50	0.174 ± 0.010	- 8.30 ± 0.20
	15	171.50 ± 0.20	0.170 ± 0.011	-7.80 ± 0.35	186.50 ± 2.70	0.175 ± 0.030	-9.00 ± 0.01
	30	172.00 ± 0.10	0.175 ± 0.020	-8.30 ± 0.02	188.20 ± 2.40	0.186 ± 0.010	-7.30 ± 0.10
	60	173.30 ± 1.20	0.176 ± 0.030	- 7.75 ± 0.20	189.2 ± 0.07	0.187 ± 0.050	- 12.50 ± 0.05
	90	175.10± 0.30	0.199±0.060	-9.85 ± 0.05	187.10 ± 3.50	0.22 ± 0.020	-10.40 ± 0.10
4°C	0	168.54 ± 0.40	0.168 ± 0.001	-8.78 ± 0.54	183.60 ± 2.10	0.172 ± 0.001	-8.50 ± 0.60
	7	168.80 ± 0.50	0.164 ± 0.010	- 8.90 ± 0.50	183.14 ± 1.30	0.171 ± 0.030	- 7.78 ± 0.50
	15	167.50 ± 0.90	0.166 ± 0.010	-8.60 ± 0.30	186.10 ±1.20	0.171 ± 0.020	-7.80 ± 0.32
	30	168.60 ± 0.05	0.165 ± 0.030	-9.20 ± 0.05	187.20 ± 1.50	0.177 ± 0.010	-8.10 ± 0.30
	60	170.20 ± 0.30	0.162 ± 0.010	- 8.00 ± 0.20	185.70 ± 0.80	0.180 ± 0.020	-12.70 ± 0.05
	90	171.20 ± 0.26	0.185 ± 0.010	-10.10 ± 0.01	184.90 ± 1.20	0.199 ± 0.030	-10.70 ± 0.03

Zeta potential values of blank NLC formulations (Table 4.24) were stable during storage. Although there were observed slight influence in zeta potential value of blank NLC(40:60) at 25°C but they were statistically insignificant (p>0.05). The same result is obtained for blank NLC(50:50) which indicated no significant influence in zeta potential value during storage at 25°C (p>0.05). At 4°C storage there were found no significant difference in zeta potential of both blank NLC(50:50) and NLC(40:60) formulations (p>0.05). Thus it could be concluded that the blank NLC formulations indicated a good physical stability during storage at 25°C and 4°C for 3 months.

It can also be seen from Figure 4.16, although the particle size of the blank NLC(50:50) and NLC(40:60) stored at 25°C had obtained a statistical difference after 90 day storage, this increase did not effect the size distribution and zeta potential of formulations. This increase in particle size was not significant until 60 day storage, and after 90 days it was found to be statistically significant. However the polydispersity index of both formulations at both temperature did not increase significantly (p>0.05). The particle size of stored blank NLC(50:50) and NLC(40:60) at 4°C (Figure 4.17) were found to be more stable than the stored ones at 25°C and no influence in polidispersity index of formulations obtained at this period (p>0.05).

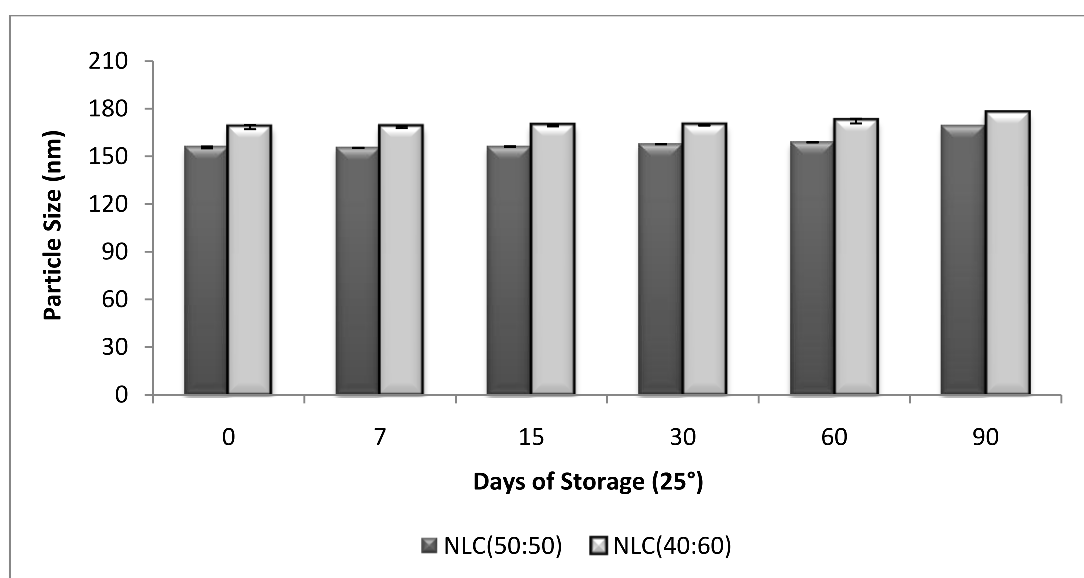


Figure 4.16. The change in particle size of blank NLC formulations during storage at 25°C.

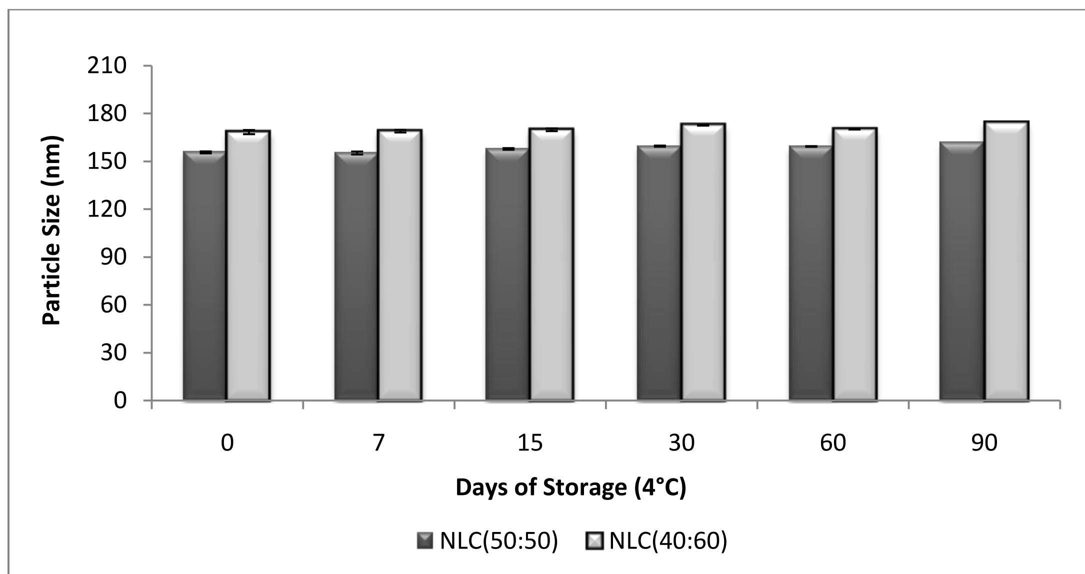


Figure 4.17. The change in particle size of blank NLC formulations during storage at 4°C.

It could be indicated that although the particle size of the 0.5% SA loaded NLC(50:50) at 25°C increased significantly after 90 day storage ($p<0.05$), Figure 4.18 clearly indicates this increase is slower. However, this increase did not effect the size distribution and zeta potential of formulations. This increase in particle size of SA loaded NLC(50:50) was not significant until 60 day storage ($p>0.05$), and after 90 days it was found to be statistically significant. However the polydispersity index of both formulations at both temperature did not increase ($p>0.05$). Both 0.5% SA-NLC(50:50) and 0.5%SA-NLC(40:60) displayed a stable particle size values at 4°C (Figure 4.19) and they were found to be more stable than the stored ones at 25°C and no influence in polidispersity index ($p>0.05$) and zeta potential of formulations obtained at this period ($p>0.05$).

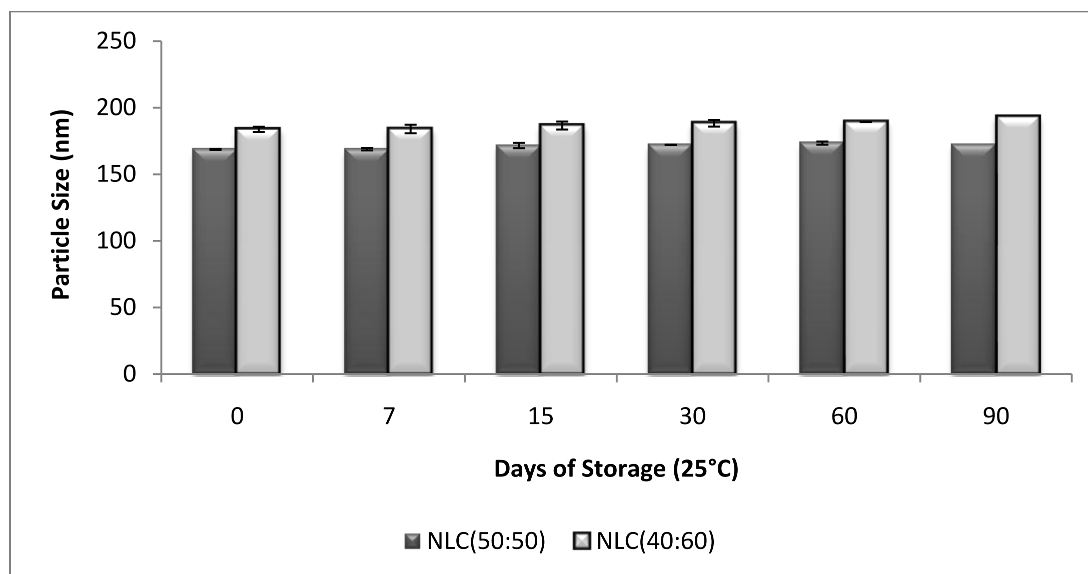


Figure 4.18. The change in particle size of 0.5% SA loaded NLC formulations during storage at 25°C.

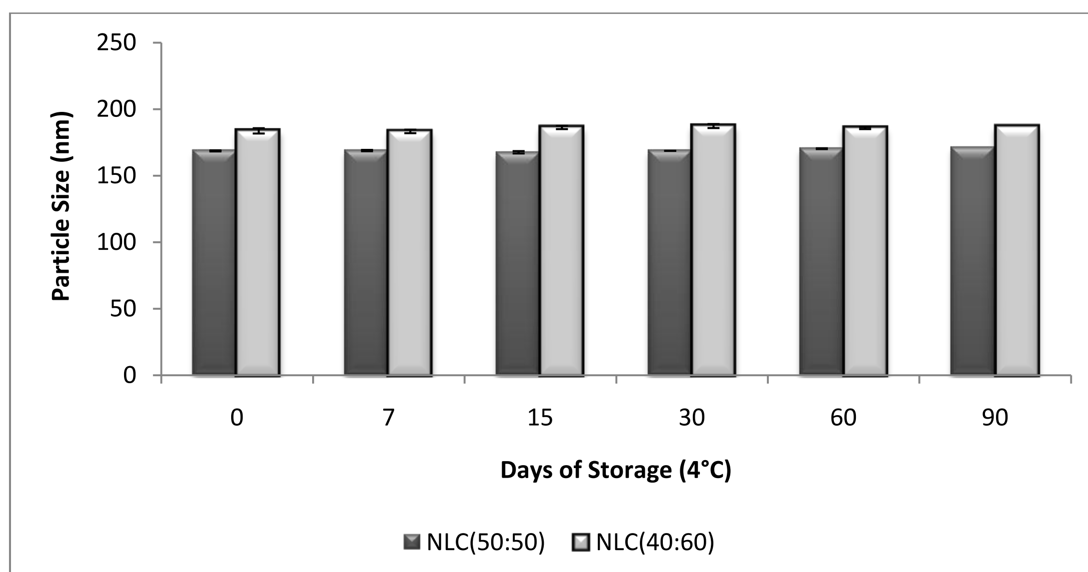


Figure 4.19. The change in particle size of 0.5% SA loaded NLC formulations during storage at 4°C.

The particle size, size distribution and zeta potential values of the blank and SA loaded SLN formulations were investigated in order to evaluated the stability behaviour of NLC formulations versus SLN. The particle size via volume distribution behaviour of blank and 0.5%SA loaded SLN formulations were

obtained, and the zeta potential values were given in Table 4.26. The particle size of blank SLN formulations during storage at 25°C and 4°C indicated a narrower distribution than 0.5%SA-SLN. Thus the increase in particle size of blank SLN was slower than the loaded SLN at both temperatures.

Table 4.26. Particle size, Span and Zeta Potential (ZP) values of SLN formulations during storage (n=5).

			$\bar{X}$ (µm) ± SE			SPAN $\bar{X}$ ± SE	ZP (mV) $\bar{X}$ ±SE
	Formulation	**Day**	$d_{(0.1)}$	$d_{(0.5)}$	$d_{(0.9)}$		
25°C	**SLN**	**0**	0.155±0.001	0.235±0.002	0.571 ± 0.01	1.77±0.03	-26.20 ±1.35
		90	0.194±0.001	0.385±0.010	1.750 ± 0.02	4.00±0.01	-26.40 ±1.02
	0.5 % SA-SLN	**0**	0.273±0.040	0.783±0.080	2.910 ±0.33	3.40±0.46	-8.20 ±0.50
		90	0.301±0.002	1.292±0.600	55.600 ±23.40	44.20 ± 21.9	11.10 ±0.10
4°C	**SLN**	**0**	0.155±0.001	0.235±0.001	0.571 ±0.010	1.77± 0.03	-26.20 ±1.35
		90	0.196±0.005	0.367±0.020	1.730 ±0.20	4.28 ± 0.70	-28.70 ±0.50
	0.5 % SA-SLN	**0**	0.273±0.040	0.783±0.080	2.910 ±0.33	3.40 ± 0.46	-8.20 ±0.50
		90	0.265±0.030	0.798±0.250	37.830 ±22.30	35.15 ± 19.30	-10.10 ±0.30

Blank SLN formulation indicated small particle size ($d_{0.5}$) at the day of production and the size distribution (SPAN) value was found to be 1.77±0.03. However after 90 day storage at 25°C, a significantly high particle size ($p<0.05$) was observed with broad size distribution (SPAN) value ($p<0.05$). This increase in SPAN value was clearly demonstrated from the particle size values of $d_{0.1}$, $d_{0.5}$,and $d_{0.9}$ observed and these were important to obtain the size distribution results. Similar results obtained from the formulation stored at 4°C and although the increase in particle size ($d_{0.5}$) value ($p<0.05$) and this increase was smaller than the one obtained at 25°C. This wide range shows the increase behaviour in particle size and distribution of blank SLN after 90 days. Zeta potential value of blank SLN at both temperatures were obtained similar results and there were no significant influence in the zeta potential values ($p>0.05$).

Although Zeta potential value of blank SLN at both temperatures were obtained that there were no significant influence after 90 days ($p>0.05$), 0.5%SA-SLN indicated a significant increase in particle size and size distributions after 90 day storage at both 25°C and 4°C ($p<0.05$). However this increase was slower at 4°C, due to the obtained particle size values for $d_{0.9}$, it indicated significantly high size distributon results (SPAN) after storage at both temperatures ($p<0.05$). The 90% volume distribution results affected the particle size distribution of SLN formulations. Most of the small sized particles accumulated 50% of particles were smaller particle size at the day of production, they indicated larger values and thus large particles accumulated at the 90% volume distributions of particles. Hence the particle size value of blank and SA loaded SLN formulations reached to higher micron ranges after storage and this was observed from the accumulation of 90% of larger particles at $d_{0.9}$.

The increase in particle size of blank and SA loaded SLN formulations could be evaluated also from Figures 4.20 and 4.21. The increase in particle size is obivious at 25°C for the 0.5%SA-SLN, and it indicates its unstability behaviour after 90 days (Figure 4.20). Although this increase is seemed to be smaller at 4°C for 0.5%SA-SLN formulation, the instability could be observed from the obtained SPAN and the larger particles at micron range from Table 4.26.

From the obtained results it could be concluded that the blank SLN formulations were more stable than SA loaded SLN formulations. Hence blank SLN were observed to have smalle particle size and size distribtion results.

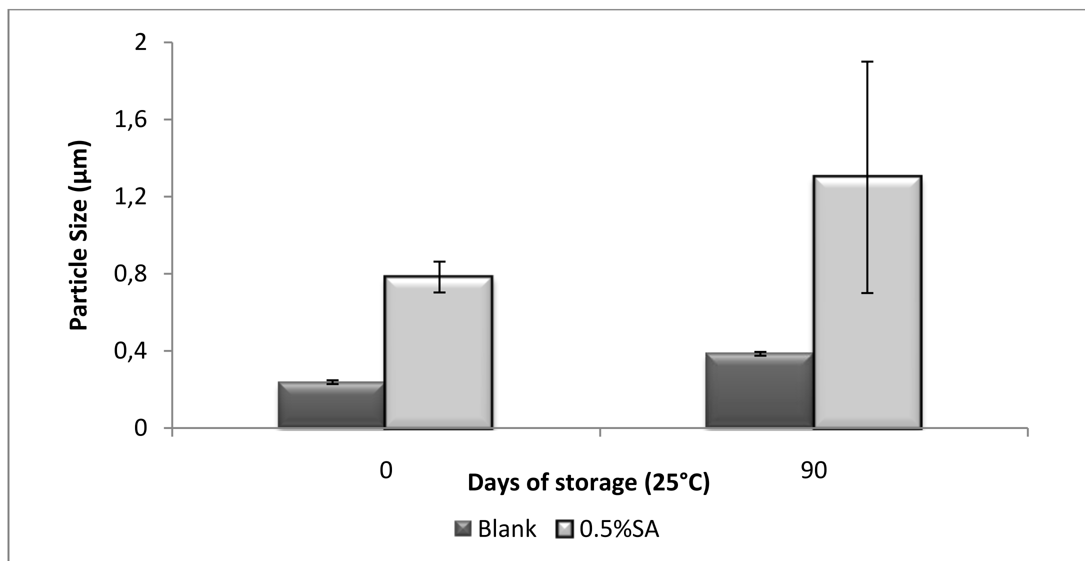


Figure 4.20. The change in particle size ($d_{0.5}$) of blank and SA loaded SLN at 25°C during storage (n=5).

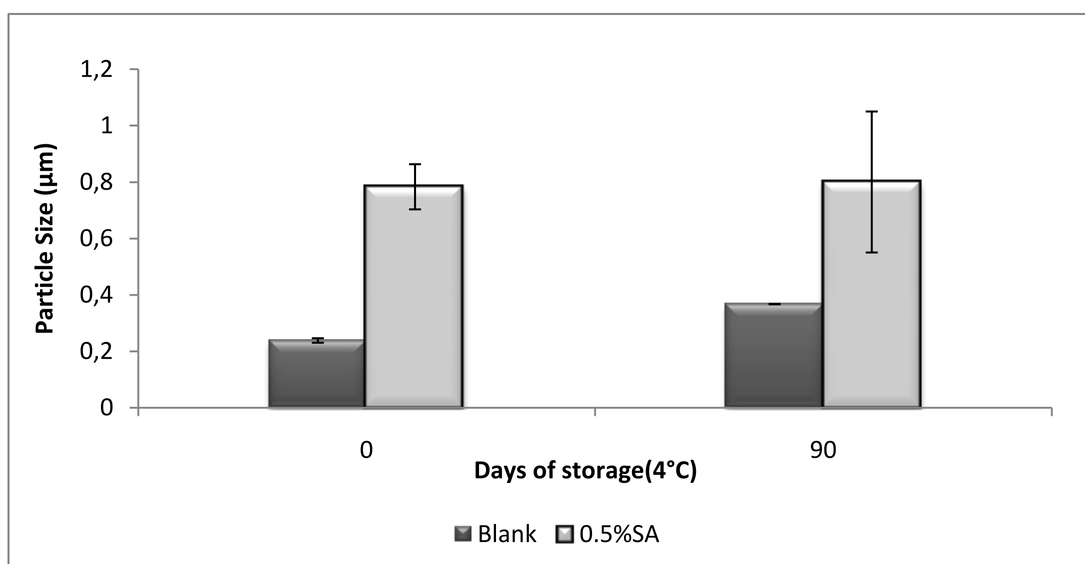


Figure 4.21. The change in particle size ($d_{0.5}$) of blank and SA loaded SLN at 4°C during storage (n=5).

It can be concluded from the obtained results of particle size, size distribution and zeta potential results of all blank and SA loaded NLC and SLN formulations that although the obtained ZP for all formulations did not cause any significant change during storage, NLC formulations were concluded to be more stable than SLN formulations.

4.4.3. Viscosity

Viscosity of blank and SA loaded formulations were evaluated during stability studies. Measurements were performed as described in section 3.3.6.3.

During storage period of all studied formulations at 25°C and 4°C,the most effected formulation was the blank SLN, hereby it`s viscosity was found to be increased in the first week of storage ($p<0.05$) at both temperatures and this was stable until 60 day but at the end of stabilty study analysis it was found a significant increase in viscosity of blank SLN ($p<0.05$). However this increase was slower in the NLC formulations. Blank NLC formulations obtained low viscosity due to the presence of liquid lipid and blank NLC(40:60) indicated a significant increase in viscosity at the end of storage time at both temperatures ($p<0.05$). Besides, no significant increase obtained in viscosity value of blank NLC(50:50) during storage ($p>0.05$) at both temperatures (Table 4.27).

Table 4.27. Viscosity of blank and 0.5% SA loaded formulations during stability studies (n=3).

Viscosity (cP) $\bar{X}$ ± S E							
Days of Storage		1	7	15	30	60	90
T	Formulation Code						
25°C	*SLN	11.98 ± 0.10	19.81 ± 0.01	21.44 ± 0.71	23.33 ± 0.73	23.60 ± 0.15	27.30 ± 0.23
	**0.5% SA-SLN	449.7 ± 0.90	449.8 ± 0.14	451.2 ± 0.70	453.1 ± 0.90	451.3 ± 0.72	455.5 ± 0.71
	*NLC(40:60)	4.00 ± 0.07	5.96 ± 0.07	6.43 ± 0.07	5.99 ± 0.23	6.28 ± 0.05	6.20 ± 0.25
	*0.5% SA-NLC(40:60)	4.48 ± 0.01	5.93 ± 0.08	6.52 ± 0.01	6.10 ± 0.06	6.31 ± 0.20	6.34 ± 0.51
	*NLC(50:50)	3.10 ± 0.03	2.87 ± 0.01	3.14 ± 0.01	3.23 ± 0.08	4.24 ± 0.40	4.24 ± 0.23
	*0.5% SA-NLC(50:50)	3.25 ± 0.03	3.47 ± 0.05	3.77 ± 0.05	3.85 ± 0.10	4.13 ± 0.03	3.90 ± 0.34
4°C	*SLN	12.62 ± 0.32	20.16 ± 0.02	22.57 ± 0.20	26.00 ± 0.50	21.60 ± 0.40	25.70 ± 0.36
	**0.5% SA-SLN	448.7 ± 0.11	448.9 ± 0.54	451.1 ± 0.23	454.4 ± 0.80	454.7 ± 0.45	467.2 ± 0.20
	*NLC(40:60)	3.91 ± 0.03	5.36 ± 0.10	4.49 ± 0.06	4.20 ± 0.15	6.81 ± 0.07	6.55 ± 0.44
	*0.5% SA-NLC(40:60)	5.11 ± 0.40	5.75 ± 0.40	6.51 ± 0.01	6.33 ± 0.06	5.80 ± 0.06	6.38 ± 0.57
	*NLC(50:50)	2.60 ± 0.06	2.88 ± 0.08	3.11 ± 0.03	3.32 ± 0.01	3.31 ± 0.06	3.21 ± 0.25
	*0.5% SA-NLC(50:50)	3.45 ± 0.05	3.72 ± 0.02	3.80 ± 0.07	4.00 ± 0.06	4.43 ± 0.30	5.11 ± 0.60

(*): Viscosity at shear rate 750 s^{-1}, (**): Viscosity at shear rate 200 s^{-1}.

0.5 % SA loaded SLN indicated a significant increase in viscosity value after 90 day storage at 25°C ($p<0.05$). This increase in viscosity of 0.5%SA-SLN was observed to be significant after 60 day storage at 4°C ($p<0.05$).

The viscosity of 0.5% SA-NLC(40:60) also increased significantly ($p<0.05$) at both temperatures after 15 days at 25°C ($p<0.05$). This increase was observed to be insignificant at 30 day ($p>0.05$) then increased significantly after 90 days ($p<0.05$). However at 4°C storage of 0.5%SA-NLC(40:60) there was no significant increase obtained in viscosity during 90 days ($p>0.05$). 0.5%SA-NLC(50:50) show no significant increase in the viscosity value during storage time ($p>0.05$) at both temperatures.

4.4.4. pH Value

The pH of all formulations in both temperatures were measured and the differences between them were evaluated statistically. As indicated in the results, during storage time of all studied formulations the pH values were also evaluated for blank and 0.5% SA loaded NLC and SLN formulations at 25°C and 4°C as described in section 3.3.6.4.

As a result of the storage after 90 days, the change in pH value of all formulations were evaluated at each storage time and temperature and despite of the difference in oil content in formulations, pH values remained stable during storage for both blank and SA loaded NLC and SLN.

These results showed that there were no statistical significance observed in blank and SA loaded NLC and SLN formulations at 25°C during storage ($p>0.05$) (Table 4.28).

Table 4.28. pH value of formulations during storage at 25°C (n=3).

25°C	pH $\overline{X}$ ±SE					
Time (day)	Blank			0.5% SA		
	NLC(50:50)	NLC(40:60)	SLN	NLC(50:50)	NLC(40:60)	SLN
1	5.53 ± 0.02	5.48 ± 0.01	5.45 ± 0.04	3.32 ± 0.01	3.23 ± 0.05	3.28 ± 0.16
7	5.50 ± 0.02	5.50 ± 0.01	5.48 ± 0.02	3.31 ± 0.01	3.30 ± 0.01	2.90 ± 0.01
15	5.51 ± 0.01	5.50 ± 0.02	5.46 ± 0.01	3.33 ± 0.02	3.31 ± 0.01	2.90 ± 0.01
30	5.48 ± 0.01	5.50 ± 0.03	5.47 ± 0.02	3.33 ± 0.01	3.31 ± 0.01	3.10 ± 0.01
60	5.49 ± 0.03	4.47 ± 0.02	5.43 ± 0.03	3.36 ± 0.01	3.30 ± 0.05	2.95 ± 0.03
90	5.36 ± 0.10	4.51 ± 0.04	5.10 ± 0.03	3.27 ± 0.02	3.24 ± 0.03	2.68 ± 0.04

All blank and SA loaded NLC and SLN formulations indicated a stable pH value at 4°C and they were obtained similar pH values from the first day until 90 day storage (Table 4.29). Thus it can be concluded that during storage of the blank and SA loaded NLC and SLN formulations at 4°C did not cause any change in pH value ($p>0.05$).

Table 4.29. pH value of formulations during storage at 4°C (n=3).

4°C	pH $\overline{X}$ ± SE					
Time (day)	Blank			0.5% SA		
	NLC(50:50)	NLC(40:60)	SLN	NLC(50:50)	NLC(40:60)	SLN
1	5.53 ± 0.02	5.48 ± 0.01	5.45 ± 0.04	3.32 ± 0.01	3.23 ± 0.05	3.28 ± 0.16
7	5.49 ± 0.02	5.49 ± 0.01	5.49 ± 0.01	3.31±0.01	3.30 ± 0.02	2.90 ± 0.01
15	5.51 ± 0.01	5.49 ± 0.01	5.47 ± 0.01	3.33 ± 0.01	3.32 ± 0.01	2.91 ± 0.01
30	5.51 ± 0.02	5.50 ± 0.02	5.44 ± 0.03	3.34 ± 0.01	3.32 ± 0.01	2.94 ± 0.03
60	5.52 ± 0.01	5.48 ± 0.02	5.38 ± 0.04	3.36 ± 0.02	3.32 ± 0.02	3.15 ± 0.02
90	5.26 ± 0.04	5.44 ± 0.01	5.28 ± 0.05	3.30 ± 0.02	3.29 ± 0.05	2.98 ± 0.04

4.4.5. Encapsulation Efficiency and Loading Capacity

The encapsulation efficiency and loading capacity of the 0.5% SA loaded formulations were evaluated as described in sections 3.3.3 and 3.3.6.5. Encapsulation efficiency and loading capacity of 0.5% SA loaded NLC(50:50), NLC(40:60) and SLN formulations were studied during 90 days at both 25°C and 4°C (Table 4.30 and 4.31).

Table 4.30. Encapsulation efficiency of 0.5% SA loaded formulations during storage (n=5).

Time (day)	Encapsulation Efficiency (%) $\overline{X}$ ± SE					
	25°C			4°C		
	NLC(50:50)	NLC(40:60)	SLN	NLC(50:50)	NLC(40:60)	SLN
0	43.34±0.24	36.94±0.13	21.45±0.01	43.24±0.24	36.94±0.13	21.45±0.10
7	43.15±0.40	36.79 ±0.2	21.32±0.14	43.18±0.21	36.70±0.21	21.48±0.14
15	43.20±0.20	36.50±0.25	21.30±0.20	43.22±0.20	36.60±0.20	21.42±0.10
30	43.13±0.23	36.35±0.25	21.33±0.12	43.20±0.21	36.52±0.20	21.40±0.15
60	43.30±0.10	36.70±0.30	21.23±0.10	43.40±0.10	36.80±0.20	21.36±0.20
90	43.20±0.10	36.55±0.20	18.54±0.20	43.31±0.10	36.35±0.10	19.06±0.21

The stability of formulations could also be proven by the determination of their encapsulation efficiency and loading capacity. Encapsulation efficiency (Table 4.30) of the studied NLC(50:50) and NLC(40:60) formulations with various oil content were not affected during storage at both temperatures and the results were statistically insignificant with respect to storage time ($p>0.05$). However there was obtained a slight decrease in the encapsulation efficiency of 0.5% SA SLN formulation that stored at 4°C and 25°C but it was statistically insignificant ($p>0.05$) after 60 day storage. However this decrease in encapsulation efficiency value of SLN were significant after 90 days for both temperatures ($p<0.05$). Thus by the obtained results there were no significant changes in the encapsulation efficiency NLC(50:50), and NLC(40:60) after 90 days at both temperatures ($p>0.05$) (Table 4.31).

Loading capacity results during storage were identical with the encapsulation efficiency results at both temperatures (Table 4.31). Both NLC(50:50) and NLC(40:60) formulations that stored at both temperatures were observed no change in their loading capacity value ($p>0.05$). However SLN formulation that stored at 25°C showed a significant decrease after 90 days ($p<0.05$), but this decrease was insignificant at 4°C ($p>0.05$).

Table 4.31. Loading capacity of 0.5% SA loaded formulations during storage (n=5).

	Loading Capacity (%) $\overline{X}$ ± SE					
Time (day)	**25°C**			**4°C**		
	NLC(50:50)	**NLC(40:60)**	**SLN**	**NLC(50:50)**	**NLC(40:60)**	**SLN**
1	1.80±0.01	1.54±0.01	0.89±0.004	1.80±0.01	1.54±-0.01	0.89±0.01
7	1.80±0.01	1.53±0.01	0.89±0.01	1.80±0.01	1.53±0.01	0.90±0.01
15	1.81±0.01	1.52±0.01	0.89±0.01	1.80±0.007	1.53±0.01	0.89±0.002
30	1.80±0.01	1.51±0.01	0.89±0.01	1.80±0.008	1.52±0.01	0.89±0.01
60	1.80±0.01	1.53±0.02	0.89±0.01	1.81±0.01	1.53±0.01	0.89±0.01
90	1.81±0.02	1.52±0.04	0.77±0.06	1.80±0.03	1.52±0.03	0.79±0.05

NLC formulations indicated more stable profile than SLN formulation with respect to the results obtained from encapsulation efficiency and loading capacity and this was thought to be because of the oil content in NLC which improved storage stability and prevented SA leackage from nanoparticles by giving it a flexible structure to enhance loading capacity during storage.

4.4.6. Salicylic Acid Stability in Formulations

The chemical stability of the active compound is important to be known for the determination of such carrier systems` ability to protect the drug from degradation and consider their ability to improve stability of the drug during storage.

The ability of NLC and SLN to improve the stability of the active compounds when loaded, were discussed in literatures (5,68,74,114). NLC is considered to be

suitable carrier systems with their ability to protect the ingredients from degradation. The SA content in NLC and SLN formulations were studied during 90 days. As seen in Table 4.32 the SA amount incorporated into NLC(50:50), NLC(40:60) and SLN formulations was remained stable during storage time at 25 °C and 4 °C and as evaluated there were no statistical significance between the incoporated amount at production day and after 90 day storage (p>0.05). It could be concluded that the chemical stability of SA was provided by incorporation into the NLC and SLN formulations.

Table 4.32. Salicylic acid content in formulations during storage (n=5).

	Salicylic Acid % (w/w) $\bar{X}$±SE					
Time (day)	**25°C**			4°C		
	NLC(50:50)	**NLC(40:60)**	**SLN**	**NLC(50:50)**	**NLC(40:60)**	**SLN**
1	100.1± 0.23	100.0 ± 0.21	100.1 ± 0.15	100.1 ± 0.23	100.0 ± 0.21	100.0 ± 0.15
7	99.98 ± 0.18	99.94 ± 0.17	99.77 ± 0.18	100.1 ± 0.18	100.0 ± 0.20	99.98 ± 0.17
15	99.94 ± 0.34	99.94 ± 0.36	99.81 ± 0.62	99.98 ± 0.38	99.94 ± 0.16	99.98 ± 0.26
30	100.0 ± 0.18	99.94 ± 0.14	99.84 ± 0.30	100.0 ± 0.18	100.1 ± 0.30	99.98 ± 0.26
60	100.0 ± 0.16	99.99 ± 0.31	99.98 ± 0.30	100.0 ±0.20	100.0 ± 0.16	99.98 ± 0.18
90	100.0 ± 0.31	100.1 ± 0.10	98.40 ± 0.21	100.3 ± 0.14	100.1 ± 0.20	100.1 ± 0.10

5. DISCUSSION

A revolution have started in science by the rapid developments in the ability to produce nanoparticles of uniform size, shape, and composition. The development of lipid based drug carriers has increased attention over the last years. Solid lipid nanoparticle is the leader of the rapidly developing field of nanotechnology with many applications in drug delivery, clinical medicine and research. Lipid nanoparticles suggest new therapeutics to develope depending on their size properties. Consequently, nanostructured lipid carriers possess a promise for reaching the goal of controlled of drug delivery systems (180). NLCs are known to be suitable carrier systems for dermal application. The production of these carrier systems as well as for lipid nanoparticle containing topical formulations is possible in laboratory and on large scale. Many features of SLN and NLC that are advantageous for dermal application of cosmetic and pharmaceutical products have been reported like occlusive properties, increase in skin hydration, modified release, increase of skin penetration associated with a targeting effect and avoidance of systemic uptake (7,180).

Salicylic acid is a beta-hydroxy acid, and because it can enter the layer of the sebaceous unit, inducing exfoliation in the oily areas of the skin, it has been used as a keratolytic agent. SA is used as an exfoliant in the treatment of acne in cosmetics at a level of 3% or less. Recently SA has been rediscovered for its ability to improve the appearance of aged skin through exfoliation of the skin surface and within pores (133,181,182). The physicochemical properties of SA, spectorphotometric analysis of SA, analytical validation of the methods, pre-formulation and formulations studies, characterication of formulations, encapsulations efficiency and loading capacity, in vitro release , stability and cytotoxcicty studies were performed.

The physicochemical properties of SA was determined by FTIR and the observed peaks (Figure 4.1) were identical with the literature (175). Melting point of SA was also found in range of 158-161°C identical with the literatures (159). The UV spectra of SA was obtained in both PBS pH 7.4 (Figure 4.2.a) and chloroform:metOH (1:1, v/v) (Figure 4.2.b) systems and the absorbance given by SA in these systems were in agreement to the references (171,176,177). Also calibration

curves were established in both systems and the parameters for the analytical method validation were acceptable for both (152,177).

The investigated studies in this thesis can be explained as follows:

5.1. Formulation Studies

In order to select of suitable solid and liquid lipids and lipid matrix for NLC and SLN, lipid screening process was established first as described in section 3.3.1.2 which was similar to the method used by Xia et al. (183). After visual evaluation at 25°C and 80°C and microscopical evaluation at 25°C in lipid screening studies, Precirol ATO5 and Labrafac Lipophile, Labrafac Hydro, Labrafil were found to be more suitable lipids to dissolve SA and no SA crystals were observed in these lipids visually and microscopically at 1 and 24 h of evaluation (Table 4.12). Based on the selected solid and liquid lipids, Hu et al. (78) studied NLC formulations containing Monostearin as solid lipid and Labrafac Lipophile as liquid lipid and they concluded that NLC with good drug loading capacity and ability to reduce drug expulsion could be formulated by using these lipids. In the same direction, Sanna et al. (19) prepared NLC using Precirol ATO 5 as solid lipid and isopropyl fatty esters with different chain length (C_{13}-C_{23}) and they investigated the econazole nitrate loaded NLC based Precirol ATO 5 as a vehicle for topical application. The NLC were successfully prepared and they concluded that NLC prepared with fatty esters of different chain length could influence the skin permeation effect. On the other hand Teerachaindeekul et al. (18) succeed to prepare ascorbyl palmitate loaded NLC with various amounts of Labrafil as liquid lipid blended with different types of solid lipids (Apifil, Dynasan, glyceryl monostearate, or cetyl aclohol) and stabilized with various types of surfactants (Tween 80, Miranol ultra C32, or Tego care 450). In this work after lipid screening process pre-formulation studies with the matrix of the selected lipids were performed by preparation of blank and loaded formulations with 0.5% and 1% SA. Depending on the results of particle size and distribution of the formulations, the blends of Precirol ATO 5 and the other liquid lipids especially with Labrafac lipophile were found to be the most suitable lipid matrix for blank and SA loaded formulations. However Apifil blends with the liquid lipids with respect to their particle size and distribution results were higher in size and distribution (Table

4.13). In addition, Saupe et al. (184) reported in their study about the structural behaviour of SLN and NLC, that the lower melting point of the lipid is attributed to the α modification form, where the lipid with higher melting point to the most stable polymorphic form β-modification. So it is believed that as the oil/fat ratio of NLC formulations increased, the melting point decreased leading to more structureless type of NLC that dissolves the drugs in oil more than solid lipid. Apifil (59-70°C) has a melting point higher than Precirol ATO 5 (56°C), so it is thought that the formulation with Apifil crystallizes more rapid than Precirol forming a β-modification that increase the drug leackage leading to low degree of incorporation of the drug as well as it`s particle size and distribution shows a higher value. The same results obtained from formulations prepared with blend of Precirol+labrafac hydro, Precirol+labrafil and Precirol+oleic acid but here the dissolution and drug incorporation in Precirol+labrafac lipophile is found to be better.

So Precirol ATO 5 and Labrafac Lipophile were selected as lipid matrix for in this study. These results also depend on the reasons that the melting point of the solid lipids were also effective on sonication and crystallization behaviour of the matrix during cooling process as described by Muller et al. (4). During cooling process of formulations at the last step of production, crystallization occurs as well as causes leackage of the drug.

It is reported that lipid nanoparticles stabilized with surfactant mixtures (Lipoid S75 and Poloxamer 188) have lower particle sizes and higher storage stability compared to formulations with only one surfactant (42). For this reason Lipoid S100 (HLB 3) was preferred to use as surfactant in all formulations with Tween 80 (HLB 15) to provide steric stabilization and to keep the particle surface balance, the combination of these two surfactants were used (32). Besides, Eldem et al. (43) obtained microparticles with smooth surface when the lipid matrix was composed of Triestearin and Compritol 888 ATO. They noted that using lecithin as surfactant could improve the continuity and homogeneity of the walls of microparticles.

Studies on the selected solid and liquid lipids with various oil/fat ratios and the surfactants were decided from the literature reviews to formulate SLN and NLC.

Teerachaindeekul et al. (115) studied Q10 loaded NLC using blend of Cetyl palmitate (solid lipid) various concentrations of Miglyol 812 (liquid lipid) in which they obtained good characterization and stability results of formulations varying with the oil/fat ratios. Also the in vitro permeation and occlusive property of formulations were affected by the oil content. Similarly Wang et al. (185) prepared SLN, lipid emulsion and NLC formulations with various oil/fat ratios (0,25, 50, 75, 100 %. w/w) and thus investigated the effects of oil/fat ratios on particle size , zeta potential and in vitro release behaviour of buprenorphine from NLC. Thus they suggested that NLCs differ from SLN and lipid emulsion by forming a liquid compartment that has interactions with solid lipid. This oil/fat ratio could improve the drug loading and structural behaviour of these systems.

In this thesis blank and SA loaded NLC with various oil/fat ratios and SLN formulations were developed and prepared by the high shear homogenization and ultrasonication technique which was described in section 2.1.4.1. This production technique is one of the easy and initially used techniques for production of NLC and SLN. In which Fang et al. (32) and Sharma et al. (186) used in their study this production technique in their study. They dissolved the drug in the previously melted lipid (80-85°C) and dispersed it in a surfactant solution at the same temperature using high speed stirrer for 1-5 min. Then they sonicated the obtained pre-emulsion using a probe sonicator. The obtained nanoemulsion (O/W) was cooled down in an ice bath to form NLC/SLN. This production was similar to the production technique in this work. The production temperature of SLN and NLC (80°C) was selected over the melting point of Precirol ATO 5 (56°C), depending on the published literatures that it should be at least 5°C over the melting point of the solid lipids (19,115).

It is described by Muller et al. (4) that the main difference between NLC and SLN formulations is their theoretical proposed type and structure modifications that are effective parameters in drug incorporation and stability. As for NLCs because of the liquid lipid content, they indicate less ordered lipid matrix that are solid at room temperature but not crystalline (Figure 2.2). However in case of SLN because of the solid lipid matrix, it tends to form more ordered lipid matrix forming the β-form and that leads to make it crystalline at room and body temperature. In this work three different oil concentrations were selected for NLC formulation evaluations that were

40%, 50% and 70% oil content. It is thought that the one with the tiny oil nanofractions content could be the multiple NLC type (Figure 2.2.). Moreover, imperfect NLC is thought to be formed by controlled mixing of solid and liquid lipid. However, SLN is in ordered form which tends to crystalline after cooling prossess and form the β-modification (1,187).

5.2. Incorporation of Salicylic Acid into Formulations

After obtaining blank NLC formulations, salicylic acid is used to incorporate in NLC at 0.5% and 1% concentrations that is in the accepted range for cosmeceutical use, which has an effect on the skin to provide a smooth exfoliation during daily use as a cosmetic product (156). Salicylic acid is used to incorporate into o/w emulsions (167) and also in liposomes (172), but until this time no literatures were published about incorporating it into SLN and NLC. This work is thought to be the first study for incorporating SA into NLC and SLN.

It was clear that the encapsulation efficiency increased with increasing of oil content in NLC (Table 4.19). In the same direction, Hu et al. (78) investigated the encapsulation efficiency and loading capacity of clobetasol propionate into NLC with various oil concentrations (0, 12.5% and 25% (w/w)) and they observed that as the oil content increased in formulations a significant increase in the encapsulation and loading efficiency were obtained. Thus they concluded that the oil addition into formulations lead to a disturbance in the crystal order of the lipid matrix indicating imperfections to leave more space to load the drug into these systems. In this thesis SLN with no liquid lipid showed the least encapsulation efficiency and loading capacity ($p<0.05$). In NLC, the higher encapsulation efficiency was observed in 0.5%SA-NLC(50:50) followed by 0.5%SA-NLC(40:60) > 1%SA-NLC(50:50) > 1%SA-NLC(40:60) > SLN and their encapsulation efficiency was found to be 43.244% , 36.94%, 34.84%, 29.76% and 21.45% respectively.The loaded amount of SA increases, the loading capacity increased in formulations due to the increase in the incorporated amount of SA ($p<0.05$) (Figure 4.10, 4.11). Factors discussed could be effective on the encapsulation efficiency and loading capacity of formulations:

- Oil/fat ratio : It is noted that as the oil content in formulations increases, the encapsulation efficiency and loading capacity were also increased. This obtains the

fact that because of the imperfect stucture of NLC increases, it causes to have enough and more space to incorporate drugs (1,4). Garcia-Fuentes et al. (53) characterized NLC 1H-NMR to study the composition and structural organization of the components in NLC. The results of NMR analysis indicated a significant incorporation of the oil into solid nanoparticles matrix and suggested the presence of oil in the form of phase separated liquid nanocompartments within the nanoparticles. They observed limited diffusion energy for oil. The crystallisation of solid lipid was found to be unaffected by the incorporation of oil.

- The second factor is the solubility of SA in the lipid matrix, and by increasing oil content it was observed that an increase in the loading capacity was occured (Table 4.19, Figue 4.10 and 4.11). Also it can be concluded that the solubility of SA in the liquid lipid is more than the solid lipid which the increase in oil content leads to a decrease in particle size of the formulations. Moreover, Teeranachaideekul et al. (115) were studied the factors that depend on encapsulation efficiency and loading capacity Q10 loaded SLN and NLC. They concluded that NLC obtained had the higher loading capacity because of the increase of solubility of the loaded drug in liquid lipids and also because of the increase of number of imperfections in NLC providing more space for the incorporating of drugs.However the low encapsulation efficiency and loading capacity of SLN is related to it`s ordered structure form which explains this phenomenon (1,12,89).

5.3. Evaluation and Characterization of NLC Formulations

The developed NLC and SLN formulations characterized by measuring particle size, polydispersity index and zeta potential. The effect of increasing oil content in formulations and loaded amount of SA were also discussed. Particle size of NLC formulations decreased with increasing oil content from 40% to 70% (Table 4.15, Figure 4.7). However there were no statistically significance in zeta potential values between blank NLC formulations ranging between -25.36 and -30.9 (Table 4.15) ($p>0.05$). As for the blank formulations NLC(70:30) had the lowest particle size value (138.64nm±0.36), followed by NLC(50:50) (155.6nm±0.61), NLC(4:60) (168.3nm±1.3). Similar results were obtained by Wang et al. (185) in their study of NLC with different oil/fat ratios as they discussed that the higher oil content lead to

lower size and distribution of the nanoparticles, hence SLN had larger particle size due to the liquid lipid absence but with no difference in zeta potential values. In this work SLN formulations indicated broad particle size distribution and with no repeatablity of obtained distribution result.

The particle size of blank SLN was observed to be higher than blank NLCs ($p<0.05$) (Table 4.15). SA loading also affected the particle size of formulations and zeta potential of all formulations. Thus by loading 0.5% and 1% SA, an increase in the particle size of formulations was observed. So, as the loaded amount of SA increased, higher particle size were obtained in NLC. As the obtained results were increased from 168.5nm to 190.9 nm for the 0.5 and 1 % SA loaded NLC(50:50) respectively. The same results were obtained from NLC(40:60) and NLC(70:30) (Table 4.15, Figure 4.7). Similar results also obtained by Souto et al. (170) and Korting et al. (89) in which they reported that the incorporation of the active compound into these systems could cause an increase in the particle size values in comparison to the studied blank formulations.

Blank SLN formulation indicated smaller particle size and distribution as compared to the 0.5% SA loaded SLN. SA loaded SLN formulations indicated much higher particle size values in micron ranges which were obivious when the 90% of particles were in micron sizes (Table 4.16). After SA loading into SLN significantly high increase in the particle size was observed ($p<0.05$). This obvious increase in particle size in SLN is thought to be because of the low solubility of SA and hence low loading effect in solid lipid matrix in comparison to NLC formulations with imperfect structure propeties. Similarly, Jores et al. (12) studied comparing the SLN, NLC and nanoemulsion formulations by PCS and LD measurements and they reported that there is no clear correlation between LD particle size and oil content, and in PCS. They studied SLN and NLC formulations with various oil content and concluded that larger particles and an increase in the size distribution were observed for both SLN and NLC with low oil contents. Also they found that the particle size of the investigated NLC approaches the size of the nanoemulsion only for high oil contents. However NLC had smaller PI than nanoemulsions. In addition Korting et al. (89) concluded in their study that the incorporation of active compounds into

NLC and SLN could increase the particle size and thus reduce the mobility of the systems.

The obtained zeta potential values in this study were negatively charged in all NLC and SLN formulations. This is described by Fang et al. (32) that the negative zeta potential of SLN and NLC stabilized with soybean lecithin is originated from the anionic fractions of lecithin which contained 80% phosphatidylcholine which is uncharged and other components were 20% are negatively charged. In this work Lipoid S100 containing 94% phosphatidylcholine was used to stabilize formulations and so the remained components were thought to be effective in the zeta potential results. The value of zeta potential was decreased after loading SA into all developed formulations ($p<0.05$) due to the low pH and acidic character of SA (156) caused a significant decrease in zeta potential of SA loaded formulations ($p<0.05$) (Table 4.15, and 4.16). However there were no significant change in zeta potenial values between 1% and 0.5% SA loaded formulations ($p>0.05$). The low pH value of SA in this study lead to a decrease in zeta potential value. Carrillo et al. (188) reported that the zeta potential is related with the pH value of the system and increases with increasing pH. Besides they reported that the zeta potential do not practically depend on the length of the hydrocarbon chain but on the pH of aqueous solutions.

In viscosity measurements, it was concluded that all blank and SA loaded formulations show a pseudoplastic flow type. The rheogram of pseudoplastic flow begins from origin and there is no yield value and no linear relation observed in the curves. Pseudoplastic flow is a type of non-Newtonian systems and this flow type of systems are determined by obtaining the logarithmic relation between shear rate and shear stress. It is explained by using the slope of the log (shear stress/shear rate) and n value is obtained which shows the flow type of the systems. Thus when $n<1$, the system shows a pseudoplastic flow type. As the shear stress increased the normally disarranged molecules in the system begin to align their long axes in the direction of flow. This reduce the internal resistance of the material and allows a greater shear rate at each succeed shear stress (178). Similarly, Junyaparaset et al. (96) were investigated Q10 loaded NLC and nanoemulsion formulation dispersions and gels and they studied the rheological behaviour of the dispersions were indicated pseodoplastic flow type, where shear rate increased with increasing shear stress that

the curve begins from origin with a yield value practically zero, in which the relation between them is not linear. However the gel type formulations showed plastic flow type. In this work it was observed that the increase in oil content caused a decrease in the viscosity of formulations as well as SA loading was also effective factor on viscosity of the formulations especially for SLN and NLC(40:60) (Table 4.17). Because of the used different spindles due to the viscosity difference in formulations, their viscosity were compared at a fixed rpm (100 rpm). The viscosity of blank SLN formulation showed a significant increase when 0.5% SA loaded into it ($p<0.05$). As the loaded amount of SA increased, an increase in the viscosity was observed for 1%SA-NLC(40:60) and 1% SA-NLC(50:50) ($p<0.05$). Based on this, it is described by zur-Muhlen et al. (46) that the interaction between the drug and the lipid phase could affect the viscosity of these systems which is also leading to change in the melting point of the lipid matrix. The increasing order in viscosity of the blank formulations was: NLC(70:30) < NLC(50:50) < NLC(40:60) < SLN, for 0.5% and 1% SA loaded formulations the same order is obtained. However the difference between blank NLC(50:50) and blank NLC(70:30) were statistically insignificant ($p>0.05$), but viscosity of blank NLC(40:60) was significantly higher than both blank NLC(50:50) and NLC(70:30) ($p<0.05$). SLN had the higher viscosity value in comparison with due to the absence of liquid lipid in its content. It is reported by Fang et al. (32) that because SLN formulations lack from liquid lipids, resulting an increased viscosity due to the crystalline state of the inner lipid phase. The SLN showed the highest viscosity, followed by the NLC and lipid emulsion.

Similarly it is observed by Souto et al. (189) that for all antifungal agents loaded SLN and NLC hydrogel formulations used for topical application showed a pseudoplastic flow during rheological measurements. As the difference between the rheological behaviour of SLN and NLC could be explained based on the lipid concentration in which the viscosity of SLN formulations were found to be higher than NLC formulations.

5.4. In Vitro Release Studies

In order to evaluate the release mechanism of SA from NLC, in vitro release from NLCs and SLN were investigated over 24 hours at 37ºC. The release profiles

obtained from NLC were compared according to the difference in their oil/fat ratio and with SLN as described in section 4.2.7.2.

Before perfoming the in vitro release study, diffusion medium for the SA was selected after solubility test in order to evaluate whether it provides the sink condition for SA in the diffusion media. The selection of diffusion medium, the influence of buffers and pH value are important parameters. Here the aim is to provide sink condition, and it is defined as the volume of medium should be at least three times that required to form a saturated solution of drug (169). As a result of solubility test of SA in PBS pH 7.4, it was found that this medium could provide sink condition for the SA and hence for the 0.5% and 1% SA loaded formulations. According to solubility test result of SA in PBS pH 7.4 saturation concentration was found to be 11.955 mg/mL±0.103. This concentration is concluded that the volume of diffusion medium in the receptor compartment to be 10 times of required concentration of SA to dissolve in diffusion medium and thus it provides sink condition (Table 4.11). This medium and pH value were selected according to the published literature for the release study of SA from O/W emulsions (167). Release of SA loaded from liposomes were also studied in this medium and also this pH value for the release study was selected because it is physiological pH. Depending on the release study of SA done by Cao et al. (168) that they indicated release rate of SA from supramolecular hydrogels formed by l-phenylalanine derivatives as hydrogelator in this medium dependent on pH value, and this pH 7.4 could the most suitable for release study of SA from formulations. Stability of SA in PB pH 7.4 also was an important step to study before perfoming release studies. SA stability during 24 h was evaluated in diffusion medium and was found stable (Table 4.20).

Regarding the effect of particle size, encapsulation efficiency and loading capacity of the formulations, the viscosity and the particle size had also effects on the release behaviour. A burst release observed in the first 30 min in NLC and at 15 min in SLN formulations. This burst effect can be explained by the oil/fat ratio, encapsulation efficiency and the viscosity of the formulations. Hence, after this burst effect a slower release followed by steady release from the formulations obtained (Figure 4.12).

The release profile of SA from 0.5% SA-NLC(50:50) and 0.5% SA-NLC(40:60) indicated an initial burst release at the first 30 min followed by a steady release after 6 hours. Because of the lower particle size and viscosity value of 0.5% SA-NLC(50:50) it indicated higher release profile from 0.5% SA-NLC(40:60). Thus the release of SA from SLN was much lower than the 0.5% SA loaded NLCs because of the higher particle size and viscosity value of SLN. Besides, the release of SA from 1% SA-NLC(50:50) found to be higher from 1% SA-NLC(40:60) and this is also was thought to be because of the lower in particle size and viscosity of 1% SA-NLC(50:50). Although 1% SA-NLC(50:50) indicated a higher release compared to 1% SA-NLC(40:60) until 12 hour of release, at the end of release the release profiles of both were got close to each other. Similarly, Joshi and Patravale (6) investigated the in vitro release study from NLC based gel of celecoxib and at the first 15 min the release of drug from NLC showed a burst release then followed by a steady release. They explained release pattern of celecoxib from NLC as diffusion of the unencapsulated drug, then solubilization by the surfactant micelle, diffusion of celecoxib from oil nanodroplets in first 2 h, diffusion from the solid lipid surface and diffusion from the core. Moreover they reported that this release rate was fitted to Higuchi kinetic release model in which indicated higher r^2 value than zero order.

1%SA-NLC(50:50) showed the highest release rate followed by 0.5% SA-NLC(50:50), 0.5% SA-NLC(40:60) and 1% SA-NLC(40:60). This results can be explained as the amount of drug loaded into the NLC also affected release rate with regard to the particle size and viscosity. Although 0.5%SA-NLC(50:50) had the smallest particle size and low viscosity, it`s release rate value is lower than 1% SA-NLC(50:50) because of the loaded amount of SA ($p<0.05$). It is described by Teerachaindeekul et al. (115) the release profile of Q10 from NLC formulations was affected by the Q10 amount loaded and as well as on the oil amount in NLC formulations. A fast release obtained at the initial stage due to the free Q10 and the enrichment of the outer phase of NLC with Q10. The also reported that the different melting behaviour of solid and liquid lipids can lead to accumulation of the liquid oil in the outer the shell of lipid nanoparticles after lipid crystallization due to the presence of Q10 in the oily layer. Concerning the release model of NLC, it was found that the model to describe the release profile of Q10 from NLC is the Higuchi

model. Hence they concluded that higher lipid concentration created the drug enriched core model which showed a prolonged release and , it is possible to achieve the desired release profile by varying the oil/fat ratio in NLC. Similarly, release studies of clobetasol propionate loaded SLN and NLC done by Hu et al. (22,78) was found to be a biphasic release pattern in which a burst release was observed at initial 8 hours then following by a slow release. They described the burst release was because of the clobetasol propionate content in the formulations as well as the liquid lipid content in NLC formulations. After the initial 8 h the drug release rate was found to be fitted to Higuchi kinetic of release model. Hence the release rate was increased with the increasing oil content in formulations.

However, in case of NLC(40:60) there is a difference that the release rate of 0.5%SA-NLC(40:60) was higher than that of 1%SA-NLC(40:60). This is thought to be because of the higher viscosity value of 1%SA-NLC(40:60), that made it release slower than 0.5%SA-NLC(40:60) with regard to the loaded amount of SA ($p<0.05$) (Table 4.21 and Figure 4.13). In addition this drug release pattern is thought probably related with the SA distribution in nanoparticles, as also reported by Hu et al. (22) that the release of clobetasol propoinate from NLC formulations indicated that liquid lipid was not homogenously distributed in nanoparticles matrix. Instead, most of liquid lipid was located at the shell of nanoparticles and left little or no liquid lipid entrapped into the core during the cooling process from melted lipid droplet to solid nanoparticles. Jenning et al. (88) investigated that the NLC obtained by high pressure homogenization technique, the liquid lipid inside was homogenously distributed, and the drug release rate of NLC was affected by liquid lipid throughout the drug release time. Furthermore, the incorporation of liquid lipid into solid lipid matrix caused the NLC became more imperfect and allowed loaded drugs easier to release, thus increased the drug release rate when liquid lipid was included in NLC matrix.

Besides SLN had the lowest encapsulation efficiency and highest viscosity value, after a burst release in the first 30min the release continued slowly. Drug release from NLC and SLN were fitted to Higuchi model release kinetic, hence the R^2 value was found higher than zero order release results for Higuchi kinetic (Table 4.21) which is described the release of drug is diffusional controlled from insoluble matrix as a square root of time dependent process (179). The fast release at the initial

stage of SA loaded NLC appears to be suitable to promote SA penetration due to the higher concentration of SA at the skin surface after applying the formulation, and followed by the prolonged release to supply a certain level of SA in the skin.

After 24 hour release, the cumulative amount of released SA from 1% SA-NLC(50:50) was higher than NLC(40:60), followed by SLN. The release amount from 1% SA-NLC(50:50) was identical with 1% SA-NLC(40:60) ($p>0.05$) and release amount of 0.5% SA-NLC (40:60) was identical with 0.5% SA-SLN ($p>0.05$). However the release drug amount from 0.5% SA-NLC(50:50) was found different from both 0.5% SA-NLC(40:60) and 0.5% SA-SLN ($p<0.05$). Besides release rate between 0.5% and 1% SA loaded NLC(50:50) statistically found to be different ($p<0.05$). Also this is concluded for NLC(40:60), the 0.5% and 1% SA loaded ones were different from each other ($p<0.05$) (Figure 4.13).

As a result of the in vitro release studies; the oil/fat ratio, particle size and viscosity of formulations were found to be major factors affected the release behaviour of SA from NLC and SLN formulations. Hence, the release of SA from NLC could be controlled by the control of oil/fat ratio in NLCs.

5.5. Cytotoxicity Studies

Cytotoxicity studies of NLC and SLN formulations are investigated usually for two different purposes, one of them is the evaluation of cytotoxicity and the other for the determination of bioactivity of several used cell lines after applications of the formulations, such as L929, 3T3 mouse fibroblasts, human keratinocytes, Human promyelotic (HL60) cells and mouse macrophages were reported in literatures (14,47,100,190) . Mouse connective fibroblasts L929 (ATCC cell line CCLl, NCTC clone 929) cell line are reported to be standard model for biocomptability tests. L929 cells are widely used for cytotoxicity studies are recommended by standard institutions (191,192). For determination of cytotoxicity effect some known methods were used and expressed as viability by MTT, or WST tests (99,193).

In this thesis L929 mouse fibroblast cell line was used to evaluate the LD_{50} of SA solution, blank and 0.5% SA loaded NLC(50:50). The cytotoxicity of SA solutions with different concentrations was investigated and the LD_{50} value of SA

solution obtained was 0.16 % (v/w) after 24 h incubation. Thus, it was observed that cell viability results were decreased by the increasing concentrations of SA (Table 4.22).

The cytotoxicity of blank and 0.5% SA loaded NLC was performed as described in section 3.3.5. Both blank and 0.5% SA loaded NLC(50:50) were prepared with 5 different concentrations regarding the concentrations of the NLC (Table 4.23). These results indicated that after 12 h incubation, as compared to control, cell viability results were statistically insignificant between 0.125% and 0.5% NLC concentrations and showed a decrease with the increasing concentrations from 1% to 2% for both blank and 0.5% SA loaded NLC(50:50). Hence, blank and 0.5% SA loaded NLC(50:50) were found to be noncytotoxic between concentrations 0.125% and 0.5% and no significant difference in cell viability results as compared with control ($p>0.05$). Similarly, Hekimoglu et al. (190) studied the cytotoxicity of solid lipid particles with and without DEET incorporated in hydroxyethyl cellulose gel and O/W emulsion base vehicles on L929 cell line and SK-Mel 30 (human melanoma) cell line. They evaluated their results using MTT assay, and they were concluded that the cell viability results were decreased with the increasing concentration of formulations. They reported that gel vehicle was found not cytotoxic, but the O/W emulsion based was found cytotoxic in different degrees. The cytotoxic effect of formulations on both cell line after 18 h incubation were found to be increased by increasing concentration. This cytotoxic effect is thought to be arised from the composition of ingredients in solid lipid particles. Also they studied the irritant effect of formulations on human skin in vivo and it was found that DEET incorporated solid lipid particles in gel and emulsion bases did not show a significant irritant effect on human skin. Thus, it is thought that this effect is produced because of the protective effect of stratum corneum which is lacking in monolayer cell cultures.

After 24 h incubation cell viability results at 2 % NLC concentrations were decreased to 70.20%±0.03 for blank NLC(50:50), and 66.50%±0.05 for 0.5% SA-NLC(50:50). However in blank NLC formulation, the obtained cell viability results between 12 h and 24 h incubation were not significant. Similarly, Ruktanonchai et al. (13) were studied the cytotoxicity effect of alpha lipoic acid loaded NLC and SLN on

foreskin fibroblast cells at concentration ranges 0.01-10µM and they obtained that cell viability results after 48 h were higher than 80 % below 10 µM nanoparticle concentrations, indicating that with increasing concentration of nanoparticles a decrease was observed in cell viability compared with control. Whereas the cell viability results of pure alpha lipoic acid solution was remained unchanged. They thought that this result of decrease in cell viability of nanoparticles is due to the free surfactants in solution or bound to nanoparticle surface, which may then cause possible cell damaging effect. They concluded that these systems could be used as colloidal carriers for cosmeceutical compounds with low cell cytotoxicity.

Thus as a result of cytotoxicity studies for the blank NLC and 0.5% SA loaded NLC(50:50) it could be concluded that they were not cytotoxic on L929 mouse fibroblast cells between 0.125% and 0.5% (v/v) concentrations of blank and 0.5% SA loaded NLC(50:50). The decrease in cell viability results for both blank and SA loaded NLC might be due to the used lipids and surfactants type and concentrations. It is described by Muller et al. (14) that the nature of lipid had no effect on cell viability but cell damaging effect of surfactants depends on the status of the molecules either free in solution or bound to surfaces and also on their conformation on surfaces.

5.6. Stability Studies

Stability studies of NLC and SLN were evaluated by means of particle size, zeta potential, encapsulation efficiency, loading capacity, and chemical stability of the active compound. Stability could be affected by different parameters production technique, temperature, cooling process, drug loading and also used lipids and surfactants (1,18,25,42,194). In this work stability studies for blank and 0.5% SA loaded NLC (50:50), NLC(40:60), and SLN were evaluated during 90 days of storage at 25°C and 4°C. The physical stability of formulations during 90 days at 4ºC and 25ºC were determined macroscopically and by measuring particle size, zeta potential, encapsulation efficiency, loading capacity, and viscosity of formulations (Tables 4.24, 4.25 and 4.26). Besides the chemical stability of the active SA was evaluated by spectrophotometric analysis of SA in formulations at both temperatures.

Macroscopical evaluation results of formulations showed no phase seperations or precipitation of the formulations during 60 days of storage time at 25°C and 4°C. However there were observed few aggregations in the blank and 0.5% SA loaded NLC formulations after 90 day storage at 25°C.

Depending on production technique of NLC, it is reported that one of the disadvantages of high shear homogenization-ultrasonication technique is that like it distributes larger particle size ranging between micrometer range lead to physical instability like particle growth upon storage and also metal contamination due to ultrasonication (194). However good physical and chemical stability were reported during storage for NLC and SLN formulations in published literatures (7,20,58).

Stability studies of NLCs were achievable. During storage of blank NLC(50:50) at 25°C there were observed no increase in particle size and distribution values until 60 day storage ($p>0.05$). However an increase in particle size was obtained at the end of 90 day storage but with keeping their particle size in nanometer ranges with narrow size distribution property. During 90 day storage at 4°C there was observed no increase in the particle size of the blank NLC(50:50) ($p>0.05$) (Table 4.24). SA loaded NLC(50:50) show no difference in particle size and distribution during 60 day storage at 25°C ($p>0.05$). Nevertheless, the particle size of 0.5% SA loaded NLC(50:50) formulations obtained significant increase after 90 days storage at 25°C ($p<0.05$) (Table 4.25) but at 4°C storage no significant increase observed ($p>0.05$). During 90 day storage there was no statistical difference in pH value of blank ($p>0.05$) and loaded NLC(50:50) formulations ($p>0.05$) (Table 4.28 and 4.29). Junyaprasert et al. (96) investigated long term stability of NLC formulations loaded with Q10 at 4°C, 25°C and 40°C for 12 months. They reported that the stability results were influenced by the storage temperature. Also comparing the effect of oil content in NLC, they were concluded that when oil content increased the stability of Q10 in NLC stored at 40°C tended to decrease in which they attributed this result with accumulation of Q10 at the surface of lipid nanoparticles.

During 60 days storage of blank NLC(40:60) at 25°C no change in particle size and distribution was obtained ($p>0.05$), but statistically significant increase in the particle size was observed after 90 days at 25°C ($p<0.05$) (Table 4.24). However

during 90 days storage at 4°C, there were observed no increase in the particle size and distribution of the blank NLC(40:60) ($p>0.05$). 0.5%SA loaded NLC(40:60) indicated an increase in particle size and distribution after 90 days storage ($p<0.05$). For 0.5% SA loaded NLC(40:60) during storage at 4°C no significant increase in their particle size and distribution observed ($p>0.05$) (Table 4.24 and 4.25) and during 90 days storage no significant difference in the pH value ($p>0.05$) of blank and loaded NLC(40:60) formulations ($p>0.05$) was observed (Table 4.28 and 4.29) . Stability studies of NLC and SLN were performed by Souto and Muller (28) and they studied on ketoconazole loaded SLN and NLC. They were concluded that Glyceride based SLN and NLC are both suitable for the incorporation of ketoconazole. However, in light exposed SLN the ketoconazole shows chemical instability, a problem which can be solved by sufficient packaging. In contrast to this, ketoconazole has a higher chemical stability in NLC, but the Compritol 888 ATO based formulations showed a significant increase in particle size during storage time. They thought that this is due to the presence of α-tocopherol, which decreases the crystal ordering of the lipid nanoparticles but increases the chemical stability of the drug under light exposure. This physical instability of NLC should not occur anymore when they are formulated. Consequently, they were concluded that SLN and NLC can be characterized as suitable carriers for topical actives with long term physical stability.

Zeta potential values are important to estimate the physical stability of formulations. The evaluated zeta potential for stability studies show no significant influence from the day of production until 90 days. The blank formulations were obtained a zeta potential in range -26.2 and -30.9 mV at the first day of production, and no statistical difference were obtained after storage at both 25 and 4°C during 90 days ($p>0.05$). This result was obtained also for the 0.5%SA loaded formulations ($p>0.05$). Thus it can be concluded that after SA loading into formulations the decrease in zeta potential value as compared to the blank ones, but did not affect the stability of formulations and they were found to be statistically insignificant ($p>0.05$).

Besides, these NLC formulations stability studies of blank and 0.5%SA loaded SLN were also evaluated. As a result, the stability blank and 0.5% SA loaded

SLN formulations were found to be unstable. The particle size and distribution of blank SLN at 25 °C was found to be unstable and 90% of most particle size increased to micron ranges ($p<0.05$). However at 4°C storage this increase was lower but statistically different from day of production ($p<0.05$) (Table 4.26). For the 0.5% SA loaded SLN at both temperatures because of the wide size distribution of SLN, it indicated a significant increase in particle size and distribution reaching to higher micrometer range ($p<0.05$). Also it should be noticed that the production of SLN formulations were not repeatable by means of the particle size and distribution results. Although the blank and SA loaded SLN were not stable, zeta potential values were found to be statistically insignificant at both temperatures ($p>0.05$).

These results can be matched with other stability studies of NLC formulations as reported by Hu et al. (78) in which they investigated the stability of SLN and NLC formulations loaded with clobetasol propionate at 4°C and 25°C in dark over a period of 30 days. They concluded that the changes in particle size and zeta potential were significantly increased during storage at 25°C for both NLC and SLN. However, this increase was observed to be slower at 4°C. But they reported that surprisingly the rate of particle growth of NLC was higher than SLN. They explained this case is due to the increased viscosity of NLC surface when higher amounts of liquid lipid was added to the formulations and also could be because of the high temperature (25°C) increased kinetic energy of system and consequently increased the possibility of aggregation for nanoparticles. They suggested that storage stability of SLN and NLC formulations could be enhanced by lyophilization or by using cryoprotectants to decrease the aggregation of nanoparticles during freeze drying.

Viscosity of the prepared formulations was also evaluated during storage at both temperatures (Table 4.27). The viscosity of blank NLC(50:50) was remained unchanged during storage at both temperatures ($p>0.05$). The viscosity of 0.5% SA loaded NLC(50:50) at 25°C was also unchanged, but the viscosity at 4°C was showed an increase ($p<0.05$). Viscosity of the blank NLC(40:60) increased at both temperature and found to be statistically significant ($p<0.05$). Besides the viscosity of the blank and SA loaded SLN formulations were increased significantly at both temperatures ($p<0.05$). This increase in viscosity of SLN is thought to be because of the solid lipid matrix which is lacking from liquid lipid and causes a higher increase

in viscosity more than NLCs which contain liquid lipid in its matrix. Because of the oil content in NLCs, the increase in viscosity of SA loaded formulations were found to be slower with the increased oil content. It is reported by Fang et al. (32) as they were developed NLC, SLN and lipid emulsion for topical psoralen delivery, they obtained a good stability of active compound from NLC, and they noted that the solid lipid (Precirol ATO 5) used in the formulations resulted an increase in the viscosity. Thus they reported that the viscosity increased by time with reducing the liquid lipid content in the formulations.

In this work, during stability studies no significant decrease in encapsulation efficiency and loading capacity value observed for all SA loaded NLC(50:50) and NLC(40:60) formulations ($p>0.05$) during storage at both temperatures. However a significant decrease in the encapsulations efficiency and loading capacity of SA loaded SLN was obtained at the end of storage period ($p<0.05$) (Table 4.30 and 4.31). Similar results obtained by Hu et al. (78) in which they investigated the relation between the drug loading and storage time of SLN and NLC. They found that NLC displayed a good stability during storage to reduce drug expulsion after 6 months storage. However during storage drug loading of SLN was reduced from 2.27% to 1.24%. This result also indicates that the stability of the active compound in NLC formulations without any expulsion effect during storage time were achieved for the developed formulations at both 25ºC and 4ºC. This is also important for the chemical stability of the active compound in these carriers. It indicates a protective effect for active compounds during storage time. This is also can be shown by the results of spectrophotometric analysis of SA in formulations. So, no significant decrease obtained in the amount of SA in all NLC (50:50), NLC(40:60) and SLN formulations ($p>0.05$) (Table 4.32). As a result from this study it can be concluded that all NLC formulations with a good physical stability showed also a good chemical stability within the evaluated period. The drug content of all formulations was the same at the day of production and after 90 days of storage.

6. CONCLUSION AND PERSPECTIVE

The new developments in nanocarrier systems provided a great attention especially in recent years for various applications. Nanostructured lipid carriers (NLC) are one of these nanocarrier systems in which involved a rapid development in pharmaceutical and cosmetic formulations. The objective of this work was to develop NLC formulations with various oil/fat ratios having narrow size distribution and loaded with active compound for cosmeceutical use.

- In this thesis NLC formulations with various oil/fat ratios were developed successfully and a cosmeceutical active compound “salicylic acid” which is widely used in cosmetics, was successfully incorporated at 0.5% and 1% concentrations into NLC formulations.

- NLC formulations had different rheological behaviours due to various oil/fat ratio. Thus the viscosity of NLC dispersions could be arranged from milky lotion to semisolid cream by changing the oil/fat ratio.

- The oil/fat ratio could be also an effective factor to improve the encapsulation efficiency and loading capacity of the NLCs.

- The release profile of SA from NLC formulations could be controlled by modifying oil/fat ratio or by changing the loaded amount of SA in the formulations.

- Blank and SA loaded NLC formulations were found to be noncytotoxic on L929 mouse fibroblast cell line between 0.125% and 0.5% concentrations.

- NLC formulations indicated good physical and chemical stability during 90 day storage at 25°C and 4°C.

In conclusion NLC formulations could be good carrier systems for various cosmeceutical and dermal active compounds for topical delivery.

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- **I.T. Anwar**, S. Hekimoglu, Effect of Oil/Fat Ratio on The Physicochemical Properties of Nanostructured Lipid Carriers Containing Salicylic Acid. **15th International Pharmaceutical Technology Symposium (IPTS)**, September 13-15, 2010, Antalya, Turkey.

- **Scientific Areas of Interest**
  - Conrolled Drug Delivery Systems
  - Nanoparticulate Drug Delivery Systems
  - Transdermal Drug Delivery Systems
  - Dermal and Topical Applications of Cosmeceuticals
  - Intellectual Property Rights and Patent in Pharmaceutical Industry
- **Computer Skills**
  - Microsoft Office: Word, Excel, Power point, Front page, Access
  - Programs: Endnote, SPSS
- **Language Skills**
  - Turkmen (Native Language)
  - Turkish (Level: Advanced)
  - Arabic (Level: Advanced)
  - English (Level: Upper Intermediate)
  - Kurdish (Level: Upper Intermediate)

APPENDIX

International Declaration

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EFFECT OF OIL/FAT RATIO ON THE PHYSICOCHEMICAL PROPERTIES OF NANOSTRUCTURED LIPID CARRIERS CONTAINING SALICYLIC ACID

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INTRODUCTION

Nanostructured Lipid Carriers are new generation of colloidal carrier systems which are produced using blends of solid lipids and liquid lipids (1). Solid lipid nanoparticles (SLN) are distinguishable from nanostructured lipid carriers (NLC) by the composition of the solid particle matrix. Both are alternative carrier systems to liposomes and emulsions (2). NLC are produced by controlled mixing of solid lipids with spatially incompatible liquid lipids leading to special nanostructures with improved drug incorporation and release properties (3).

Salicylic acid is a beta hydroxy acid and has keratolytic action on normal human skin and multifunctional properties to help with a variety of skin problems, including acne, pimples and blemishes.These effects are induced according to the concentration of salicylic acid in formulations. In cosmetics salicylic acid's concentration ranging from 0.0008% to 3% (4).

MATERIALS AND METHODS

Materials

Precirol ATO 5, Labrafac Lipophile WL 1349 (Gattefosse, Germany), soybean lecithin-LS100 (Lipoid S100, Lipoid, Germany). Tween 80 (Sigma-Aldrich, Germany), Salicylic acid (SA) (Carlo-Erba, Italy), and double distilled water were used.

Preparation of Formulations

The oil/fat phase 12% (w/w) consist of Labrafac Lipophile WL1349 (liquid lipid), precirol ATO 5 (solid lipid) at different ratios [(40:60), (50:50), (70:30)] and soybean lecithin (Lipoid-S100) as lipophilic surfactant. The aqueous phase consisted of double distilled water and a hydrophilic surfactant (Tween 80). The melted lipid phase was dispersed in the hot hyrophilic surfactant solution (70-80 ^oC) at 11000 rpm for 5 min by high speed stirrer (Ultraturax T25, IKA-WERKE GmbH & Co.KG, Staufen, Germany). The obtained pre-emulsion was ultrasonicated by using Ultrasonic probe (Bandelin Sonopuls HD2200, Germany) at 10% intensity for 10 min. The obtained O/W nanoemulsion was cooled down in an ice bath by continue ultrasonicating for 5 min. to form NLC. SLN is prepared as an alternative

formulation for comparison with NLC. Salicylic acid was dissolved in the melted lipid phase for drug loaded formulations.

Characterization of NLC

Particle size and Zeta potential measurements were carried out on Zetasizer Nano-ZS (Malvern Instruments, UK). Viscosity of the systems were determined by a Brookfield Model DV-II cone and plate viscometer at 25±1 ^{0}C (Brookfield Engineering Laboratories, USA).

RESULTS AND DISCUSSION

The total amount of lipid phase was kept constant being %12 with regard to formulation, while the ratio between oil and solid lipid varied (40:60, 50:50, 70:30). It was found that when oil content of placebo formulations was increased the particle size was decreased ($p<0,05$). On the other side different types of oils were used in this study and it was found that NLC with Labrafac Lipophile gave the smallest particle size ($p<0,05$).

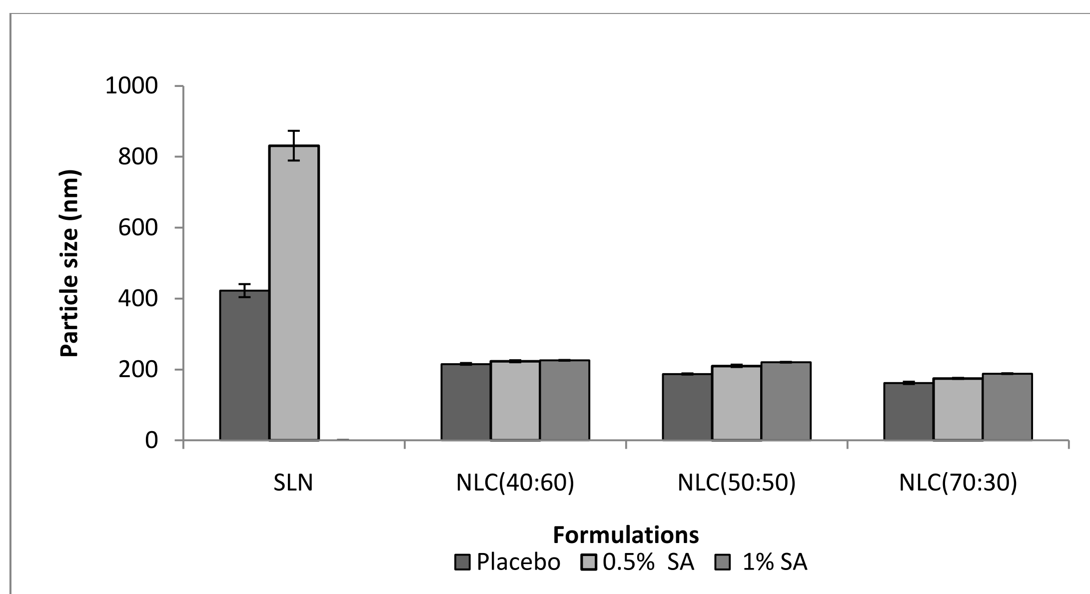

Figure 1. Particle size of SLN and NLC formulations (n=5)

As shown in Figure 1 the physicochemical properties of NLC were affected by varying ratios of oil/fat. Moreover increase in oil content of NLC decreased the particle size ($p<0.05$). After loading of formulations the particle size increased ($p<0,05$). However the obtained results in each type of formulation after loading salicylic acid between 1% and 0.5% showed no influence on the particle size of NLC ($p>0,05$).

After incoporation of salicylic acid into NLC, a significant decrease was obtained in zeta potential ($p<0,05$) as shown in Table1. Polydispersity index of NLC formulations obtained were less than 0,2.

Table 1. Zeta potential (mV) of formulations (n=5)

Formulations	Placebo	0,5% SA	1% SA
SLN	-21,2 ± 0,1	-5,94 ± 0,2	-
NLC(40:60)	-24,9 ± 0,3	-8,8 ± 0,4	-10 ± 0,2
NLC(50:50)	-23,04 ± 0,4	-8,5 ± 0,4	-9 ± 0,7
NLC(70:30)	-23 ± 0,5	-8,7 ± 0,2	-13 ± 0,3

In Table 2 viscosities of all formulations are shown. It can be concluded that as the oil content increases, the viscosities decrease in both placebo and drug loaded formulations ($p<0,05$). Incorporation of salicylic acid caused significant increase in the viscosities of the formulations except NLC(70:30).

Table 2. Viscosities of formulations (n=3)

Formulations	*Viscosity (cP)*
SLN	9,9 ± 0,3
0,5% SA-SLN	394,6 ± 0,9
NLC(40:60)	4 ± 0,07
0,5% SA-NLC(40:60)	4,48 ± 0,01
1% SA-NLC(40:60)	176 ± 0,06
NLC(50:50)	3,1 ± 0,03
0,5% SA-NLC(50:50)	3,25 ± 0,03
1% SA-NLC(50:50)	17,9 ± 0,09
NLC(70:30)	2,1 ± 0,01
0,5% SA-NLC(70:30)	2,62 ± 0,01
1% SA-NLC(70:30)	3 ± 0,01

CONCLUSION

The results obtained in these formulations containing salicylic acid with two different concentrations could be useful for topical and cosmetic applications. Among these NLC (40:60) had the most suitable viscosity and desired particle size for ease of use. Furthermore in vitro release characteristics of these formulations will be studied.

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