ASSESSMENT OF LIPOSOMAL FORMULATIONS AND BIOLOGICAL ACTIVITIES OF EGGPLANT GLYCOALKALOIDS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
Engin TATLIDİL

July 2019
İZMİR

We approve the thesis of **Engin TATLIDİL**

Examining Committee Members:

Prof. Dr. Anne FRARY
Department of Molecular Biology and Genetics, İzmir Institute of Technology

Asst. Prof. Dr. İbrahim ÇELİK
Department of Agricultural and Livestock Production, Pamukkale University

Assoc. Prof. Dr. Gülistan MEŞE ÖZÇİVİCİ
Department of Molecular Biology and Genetics, İzmir Institute of Technology

12 July 2019

Prof. Dr. Anne FRARY
Supervisor, Department of Molecular Biology and Genetics,
İzmir Institute of Technology

Prof. Dr. Volkan SEYRANTEPE
Head of the Department of
Molecular Biology and Genetics

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of
Engineering and Science

ACKNOWLEDGMENTS

First of all, I would like to express my sincere appreciation and gratitude to my supervisors Prof. Anne FRARY and Prof. Sami DOĞANLAR for giving me the unique opportunity to be part of their research team. Their supervision, guidance and support enlightened my path throughout my time in their lab. They are and will always be the model researchers that I hope to be one day.

Secondly, I would like to thank Dr. Nergiz GÜRBÜZ for her incredible support throughout this thesis study. Her guidance has helped me out to cross over hard paths of biochemistry and always gave me a hope when I fell in despair.

I would like to thank all my friends and fellow colleagues with whom I have worked in the Doganlar/Frary Plant Molecular Genetics and Breeding Laboratory for creating the collaborative environment that made laboratory easier to work in.

I also would like to thank IZTECH for providing us with financial support for this project.

Finally, I would like to express deepest love and gratitude to my dearest family, who has always been there for me through my entire life. Their support and never-ending love were an incredible boost that kept me focused on my work even in hard times.

ABSTRACT

ASSESSMENT OF LIPOSOMAL FORMULATIONS AND BIOLOGICAL ACTIVITIES OF EGGPLANT GLYCOALKALOIDS

Billions of dollars are spent every year in the world for cancer treatments and research. In recent years, bioactive compounds are being tested as promising therapeutics. Among these compounds, eggplant glycoalkaloids: solasonine and solamargine are known to be effective against skin cancer and diseases. However, these compounds are water insoluble. This reduces transdermal drug delivery and the efficacy of solasonine and solamargine. Nanocarriers are used for transdermal drug delivery of water insoluble molecules. In this study, liposomes were used as nanocarriers to increase drug delivery of solasonine and solamargine. In the first stage of the study, empty liposomes produced from four different lecithin types with hydration temperatures of 40°C, 45°C and 50°C were evaluated according to their physical, chemical stability and drug loading capacity criteria at three different storage temperatures (4°C, 25°C, 37°C). The liposome formulation which was most suitable for the continuation of the study was determined. In the second stage of the study, solasonine and solamargine loaded liposomes were produced according to the formulation determined in the first stage and these liposomes were evaluated according to their physical, chemical stability, zeta potentials and drug leakage rate criteria for 3 months and it was determined that the drug loaded formulation was stable during the monitoring process. Furthermore, the release profiles of the drugs in different release media were determined and also the efficacy of the free and encapsulated states of solasonine and solamargine were tested in HaCaT and SCC-25 cell lines and IC_{50} values were determined.

ÖZET

PATLICAN GLİKOALKOLOİDLERİNİN LİPOZOMAL FORMÜLASYONLARININ VE BİYOLOJİK AKTİVİTELERİNİN DEĞERLENDİRİLMESİ

Dünyada her yıl kanser tedavileri ve araştırmalarına milyarlarca dolar harcanmaktadır. Son yıllarda, biyoaktif bileşikler umut verici terapötikler olarak test edilmektedir. Bu bileşikler arasında patlıcan glikoalkaloidleri: solasonin ve solamarjinin cilt kanseri ve hastalıklara karşı etkili olduğu bilinmektedir. Bununla birlikte, bu bileşikler suda çözünmezdir. Bu durum, transdermal ilaç dağıtımını ve solasonin ve solamarjinin etkinliğini azaltmaktadır. Suda çözünmeyen moleküllerin transdermal ilaç iletimi için nanotaşıyıcılar kullanılmaktadır. Bu çalışmada solasonin ve solamarjinin ilaç iletimini arttırmak için nanotaşıyıcı olarak lipozomlar kullanılmıştır. Çalışmanın ilk aşamasında 4 farklı lesitin tipinden 40°C, 45°C ve 50°C hidrasyon sıcaklıkları ile üretilen boş lipozomlar 3 farklı saklama sıcaklığında (4°C, 25°C, 37 °C) fiziksel, kimyasal stabiliteleri ve ilaç yükleme kapasiteleri kriterlerine göre değerlendirilip çalışmanın devamı için en uygun olan lipozom formülasyonu belirlenmiştir. Çalışmanın ikinci aşamasında ilk aşamada belirlenen formülasyona göre solasonin ve solamarjin yüklü lipozomlar üretilmiş bu lipozomlar 3 ay boyunca fiziksel, kimyasal stabiliteleri, zeta potensiyelleri, ilaç kaçak oranı kriterlerine göre değerlendirilmiş ve ilaç yüklü formülasyonun gözlem süreci boyunca stabil kaldığı belirlenmiştir. Ayrıca ilaçların farklı salım ortamlarındaki salım profilleri belirlenmiş ve aynı zamanda solasonin ve solamarjinin serbest ve enkapsüle hallerinin etkinliği HaCaT ve SCC-25 hücre hatlarında denenmiş ve IC_{50} değerleri tespit edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

EGFR: Epidermal Growth Factor Receptor

EPR: Enhanced Permeability and Retention

HPLC: High Pressure Liquid Chromotography

GUV: Giant Unilamellar Vesicles

LUV: Large Unilamellar Vesicles

PEG: Polyethylene glycol

PI: Polydispersity Index

SEM: Scanning Electron Microscopy

SM: Solamargine

SS: Solasonine

STTP: Sodium tripolyphosphote

SUV: Small Unilamellar Vesicles

CHAPTER 1

INTRODUCTION

1.1. Nanocarriers and Drug Delivery

Nanotechnology encompasses many products and processes varying from simple products that we use in everyday life all the way up to industrial production and scientific applications thanks to developments in the last few decades. Advancements in molecular medicine and pharmacology have been enormous since the 1990s with increasing use of nanotechnology in studies allowing us to make significant progress in the prevention, diagnosis and treatment of diseases (Couvreur & Vauthier, 2006). Investigation of biological processes at the molecular level has provided a better understanding of disease mechanisms. A better understanding of diseases has allowed us to develop new drugs, but delivery of these drug molecules and their targeting have become major challenges for drug developers. In the last few decades, chemically-roduced therapeutic agents have been replaced with biomacromolecules that are more efficient and less immunogenic. Although they show an excellent therapeutic effect in vitro, the major challenge with these new drugs is that the majority of them are water insoluble lipophilic molecules which leads researchers to focus their studies on carrier systems (Porter, Trevaskis, & Charman, 2007). By 2011 about 70 % of drug candidates were made of water insoluble molecules while in the market, 40 % of orally-administered immediate release drugs are made of water insoluble molecules. Water insoluble drug molecules have some challenges to overcome. For example, since they are water insoluble, they have poor bioavailability. Also, the excipients that are used to increase the water solubility of drug molecules may decrease their effectiveness. Another issue is precipitation of drug molecules after administration which again decreases the bioavailability of the drug or, even worse, precipitates may cause a toxic effect at the site of precipitation (Kawabata, Wada, Nakatani, Yamada, & Onoue, 2011). Nanocarriers offer enhanced solubility for both hydrophilic and lipophilic molecules and therefore improve their bioavailability and overcome almost all problems related to water insolubility.

Nanocarriers offer promising therapeutic interventions to diseases by improving the therapeutic effects of active drug molecules and decreasing the undesired side effects that result from high dosage (Vahed, Salehi, Davaran, & Sharifi, 2017). Using nanocarriers as a drug delivery medium provides many benefits including enhancement of drug accumulation at the disease site, increased cellular uptake, and enhanced stability of drug molecules (Ganta, Devalapally, Shahiwala, & Amiji, 2008).

Nanocarriers can be made using many organic and inorganic materials like, metals, degradable and non-degradable biomaterials, amphiphilic molecules, and inorganic nanocrystals (Moghimi, Hunter, & Murray, 2005). The choice of constituents depends on the goal of treatment, the physical and chemical properties of targeted tissue or organ (pH, temperature, redox microenvironment), route of administration, and material toxicity (Moghimi et al., 2005; Torchilin, 2006). An ideal nanocarrier has to carry enough dose of the drug and should be stable enough to leak the drug molecule with a predetermined release profile, not all at once. It should keep the drug concentration in the therapeutic window and should avoid the "peaks and valleys" of conventional drugs. Thus, it should target and accumulate at only the disease site (Tibbitt, Dahlman, & Langer, 2016). Also, an ideal nanocarrier should not create an autoimmune response. Stimuli responsive nanocarriers can be tailored to enhance the pharmacokinetics of the candidate drug. Such stimuli can be a chemical such as polyethylene glycol (PEG) or biological like the pH or temperature of the environment or hypoxic conditions (Ganta et al., 2008).

There are two major targeting strategies for nanocarrier systems: active and passive targeting. Targeting is crucial in drug delivery system development in order to achieve a systemic treatment.

Passive targeting uses the chemical and physical interactions between the delivery system and disease environment to provide drug accumulation at the disease site and prevent nonspecific binding and accumulation of the therapeutic agent. Passive targeting mechanisms generally rely on a phenomenon called "enhanced permeability and retention" (EPR) effect of tumor tissues. Basically, EPR effect speculates that macromolecules like liposomes, nanoparticles and drug molecules have a higher accumulation at the disease site due to the hyper-permeability of newly formed blood vessels via angiogenesis (Maeda & Matsumura, 1989; Ohtsuka, Konno, Miyauchi, & Maeda, 1987). Van Vlerken et al. showed that administration of a therapeutic agent as a polymer drug conjugate has higher accumulation ranges from 10 to 100 fold compared

with administration of therapeutic agent without a carrier (van Vlerken, Duan, Seiden, & Amiji, 2007). Passive targeting can be used not only in cancer, but also in chronic inflammations and some infections that show the EPR effect. This means that nanocarriers that act via passive targeting can be used in a wide range of diseases.

Passive targeting can also be used in infectious diseases involving macrophages like candidiasis, leishmaniasis and listeria. Nanocarriers have a tendency to accumulate at the reticuloendothelial system where macrophages also accumulate (Davis, 1997).

One of the major passive targeting approaches is stimuli responsing in which the carrier system releases the drug only in the presence of the stimulus to which the carrier itself is sensitive. Stimuli responsive delivery is the choice of drug developers because it is produced for a specific target and responds only to the pathological triggers (pH, temperature, redox environment) of the target of interest (Torchilin, 2007).

After infection, inflammation and/or cancer, pathological tissues tend to have different pH character. In the case of a tumor, a lack of oxygen results in a hypoxic environment which causes an increase in lactic acid production and ATP hydrolysis. The tumor mass eventually ends up with an acidic environment. These pathological regions tend to be more acidic with a pH around 6.5 for cancerous tissue while the pH of normal tissues is around 7.4 at normal body temperature (Vaupel, Kallinowski, & Okunieff, 1989). Not only tissues but also cellular organelles display differential pH profiles. For example, the pH of lysosomes is around 4.5 while the pH of mitochondria is 8. This differential pH profile can be used for intracellular delivery and targeting of therapeutic macromolecules (Gerweck & Seetharaman, 1996). Shenoy et al. showed that usage of pH sensitive nanoparticles like poly beta amino ester (PbAE) results in higher drug accumulation at the tumor site compared with usage of PCL nanoparticles which are pH insensitive (Shenoy, Little, Langer, & Amiji, 2005). Another pathological trigger is temperature. Hyperthermia has been used as a supportive treatment for cancer patients alongside chemotherapy and radiotherapy because tumor cells are more susceptible to heat than normal tissues. It has been shown in SKOV-3 (human ovarian carcinoma) cells that some nanocarriers including liposomes have a higher extravasation from circulation towards tumor mass after heating to 42 °C (Meyer, Shin, Kong, Dewhirst, & Chilkoti, 2001). In nanocarrier systems for gene delivery, redox potential difference (up to 1000 fold) among the intracellular and extracellular spaces can be used as a pathological trigger (Saito, Swanson, & Lee, 2003).

One other approach in passive targeting is using the size and surface charge of the nanocarrier. Positively charged nanocarriers and nanocarriers with a diameter smaller than 200 nm more efficiently accumulate at the tumor site than neutral or positively charged nanoparticles and larger nanoparticles (van Vlerken et al., 2007).

Another major targeting strategy is active targeting which involves alteration of the nanocarrier surface to introduce new ligands that can be recognized at the disease site. Tumor cells rapidly multiply which requires them to over-express some receptors for increased uptake of certain nutrients including sugars, vitamins and folic acid. If a nanocarrier surface is designed with these nutrients, the tumor cells can be targeted via receptors related to them. For example, an FDA approved targeted TNF inhibitor antibody Adalimubab© which is used as an immune suppressor for autoimmune diseases including rheumatoid arthritis, increases the risk of tuberculosis and some cancers due to suppression of immune system (Hochman & Wolff, 2006). This antibody can be used on nanocarriers to specifically target only the disease site. In the same manner, HER2 expressing tumor cells can be targeted with the HER2 receptor targeting antibody Trastuzumab© to treat breast cancer (Kirpotin et al., 2006) or any ligand that targets for the epidermal growth factor receptor (EGFR) can be used to treat prostate cancer (Blessing, Kursa, Holzhauser, Kircheis, & Wagner, 2001).

Nanocarriers are categorized into two categories based on their production material which are polymer-based and lipid-based nanocarrier systems (Amoabediny et al., 2018).

Polymer based nanocarrier systems are made of biodegradable polymer molecules and are used to carry a wide range of molecules including low molecular weight therapeutic agents, proteins, and nucleic acids (Nasir, Kausar, & Younus, 2015). Polymer based nanocarriers are quite stable due to a low polymer degradation rate and therefore they are advantageous for long storage and in vivo circulation stability. On the other hand, this low degradation rate leads to some problems in vivo including toxicity

Lipid based nanocarrier systems are made of lipids instead of polymers. The use of lipids overcomes the toxicity problem created by polymers. Today frequently used lipid based nanocarrier systems are liposomes, micelles, cubosomes, niosomes, and lipid nanoparticles (Weber, Zimmer, & Pardeike, 2014).

1.1.1. Liposomes

The field of biomedicine recently focuses on biomacromolecules as potential carriers because they are non-toxic, biocompatible, biodegradable, renewable, self-targeting and have longer blood circulation time than synthetic carriers. Other advantages of biomacromolecules are their stability, half-life, safety, and ease of production (Zhang, Sun, & Jiang, 2018).

Liposomes are small spherical vesicles that are made up of one or more phospholipid bilayers that contain an aqueous environment within (Figure 1.1). Liposomes has been studied as drug carriers for almost 50 years (Gregoriadis, 1976a, 1976b). Liposomes have become a research focus in targeted drug delivery for their advantages like stabilized therapeutics, enhanced cellular uptake, and increased bioavailability of drug compounds. Liposomes are differentiated from other drug delivery systems due to their nature. The distinctive feature of liposomes is that they are self-assembling vesicles because once phospholipids encounter with an aqueous solution, they tend to form a vesicle due to their amphiphilic property. The amphiphilic nature of the liposomes provides them the ability to encapsulate a wide range of hydrophobic and hydrophilic therapeutic compounds within their lipid bilayer and lumen, respectively (Sercombe et al., 2015). Another advantage of this amphiphilic property is that once the therapeutic agents are entrapped, their interaction with the environment is blocked, thus the off-target effect of the drug compounds are eliminated. Most liposomes are made up of phospholipids and other supportive lipids like cholesterol which is why liposomes are biocompatible and since they are biocompatible, they show no immunogenicity. Using liposomes as a drug carrier has many benefits but like all systems liposomes have some drawbacks. First, liposome-based drugs cost a lot more than conventional drugs. This high price is partly due the cost of ingredients, but the real reason is the equipment cost that is required for liposome manufacture. Most liposomes are non-toxic but some cationic liposomes tend to show cytotoxic effect especially when high doses of the liposome are administered (Sercombe et al., 2015). Liposomes have a relatively short shelf-life compare with polymer-based nanocarrier systems. Therefore, storage stability of the drug-loaded liposome is another challenge to overcome. The physical, chemical and microbiological stability of the liposomes must be protected throughout storage. Stability of drug-loaded liposomes can be affected by

many factors including size distribution and integrity. Some liposomes tend to fuse to each other and form larger liposomes while others lose their integrity over time and leak some of the drug content. The amount of leakage varies with chemical properties of the drug and the liposome formulation.

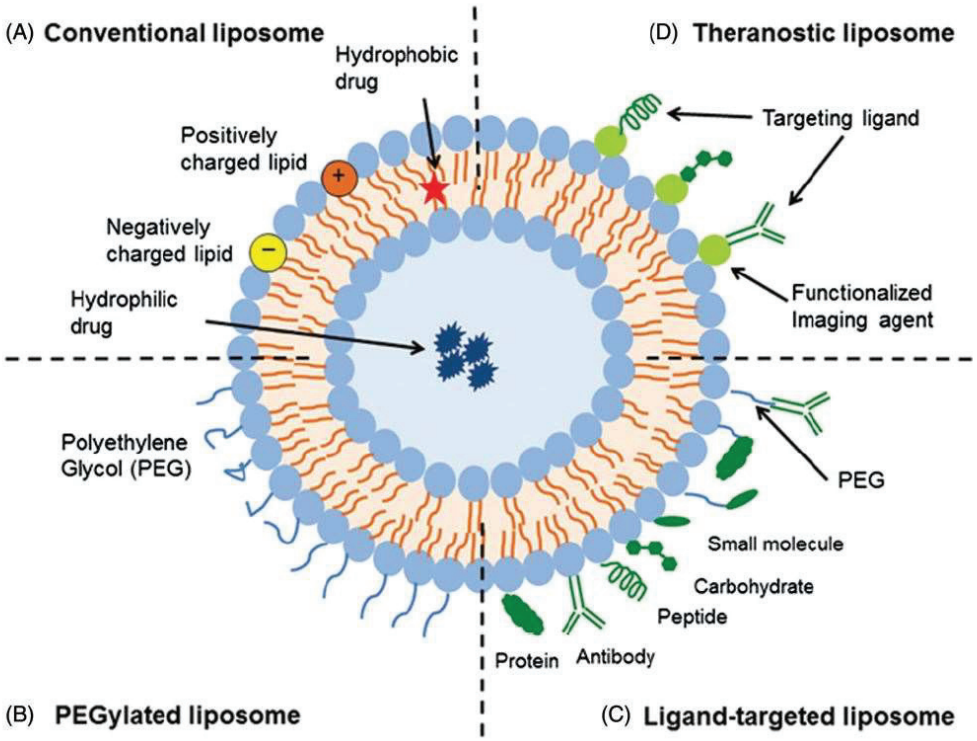

Figure 1.1. Schematic representation of liposome. (Source: Sercombe et al., 2015)

Storage stability can be increased via freezing, lyophilization and other drying methods which are shown to be effective in lengthening the shelf life of drug-loaded liposomes (El-Nesr, Yahiya, & El-Gazayerly, 2010).

1.1.2. Classification of Liposomes

Liposomes are classified into different groups depending on their size and number of bilayer membranes they possess, because size is an important factor in determination of liposome stability and number of bilayers affects the drug loading capacity of the liposomes. Liposomes that are smaller than 100 nm in diameter with a single membrane bilayer are called Small Unilamellar Vesicles (SUV). If the diameters of unilamellar liposomes are between 100 nm and 1000 nm then they are called Large Unilamellar Vesicles (LUV). The ones with diameters higher than 1000 nm are called

Giant Unilamellar Vesicles (GUV). Liposomes that have more than one membrane bilayers layers are called Multilamellar Vesicles. Finally, liposomes that encapsulate many other liposomes are called Multivesicular Vesicles.

1.1.3. Liposome Formulations and Production Methods

Phospholipids are abundant lipid molecules in our body and are the major component of cell membranes. Phospholipids are made up of one hydrophilic head group which carries the phosphate group and two hydrophobic hydrocarbon tails. The phosphate group is key in that it defines the specificity of the phospholipid and is also the source of the polarity of the head group. Different phosphate groups are frequently used by organisms in phospholipid production including serine, choline, and ethanolamine. The hydrocarbon chains in the tails are generally made of fatty acids. If one of the tails has more cis double bonds, it causes bending in the structure. Once placed in an aqueous environment, phospholipids tend to come together through hydrophobic interactions. Hydrophobic tails bind to each other to reduce their interaction with water as much as possible. As a result, a bilayer structure is formed in which the hydrophilic head groups look inward and outward, interacting with the aqueous environment. That is why almost all production methods of liposomes benefit from phospholipids' amphiphilic nature.

The extent of liposome targeting, and delivery can be determined by changing liposomal formulations. Different varieties of liposomes can be produced via changing the component composition. Surface charge, permeability, and fluidity are the main parameters that can be changed via formulation. The components of the liposome should have compatible chemical characteristics to produce a stable liposome. For example, if nucleic acids are the drug of interest, then positively charged phospholipids are needed for liposome production, because nucleic acids are negatively charged. The surface of the liposomes can be modified for better targeting. For example, an unmodified liposome cannot circulate within the body for a long time due to clearance by phagocytosis of the reticuloendothelial system. However, if the liposome is covered with polyethylene glycol (PEG) which forms a barrier between phagocytotic cells and the liposome, it will have a longer circulation time. Also targeting molecules can be

used such as, active targeting ligands like antibodies, proteins, peptides, aptamers, carbohydrates, and some other small molecules.

1.1.3.1. Thin film hydration method

In the thin film hydration technique (Figure 1.2), the phospholipid, cholesterol, and other supportive molecules are dissolved in volatile organic solvents. This solvent-lipid mixture is placed in a boiling glass and then the solvent is evaporated in a rotary evaporator with reduced pressure. Once all the solvent is evaporated, a thin lipid film is formed on the surface of the boiling glass. The aqueous buffer of choice which has a temperature higher than the phospholipid transition temperature, is added onto the lipid film and the hydration step is started. At this point the hydration temperature of liposomes has to be carefully adjusted. Lipids tend to undergo a phase change from a solid state to a gel-liquid phase around their melting temperatures. This temperature is known as phase transition temperature (T_c) (M. R. Mozafari, 2010). The temperature of hydration solution must be higher than the T_c of the phospholipid that is used in the liposomal composition. Otherwise during hydration, phospholipids will not undergo phase transition which leads to lower mobility of lipids and makes it harder for

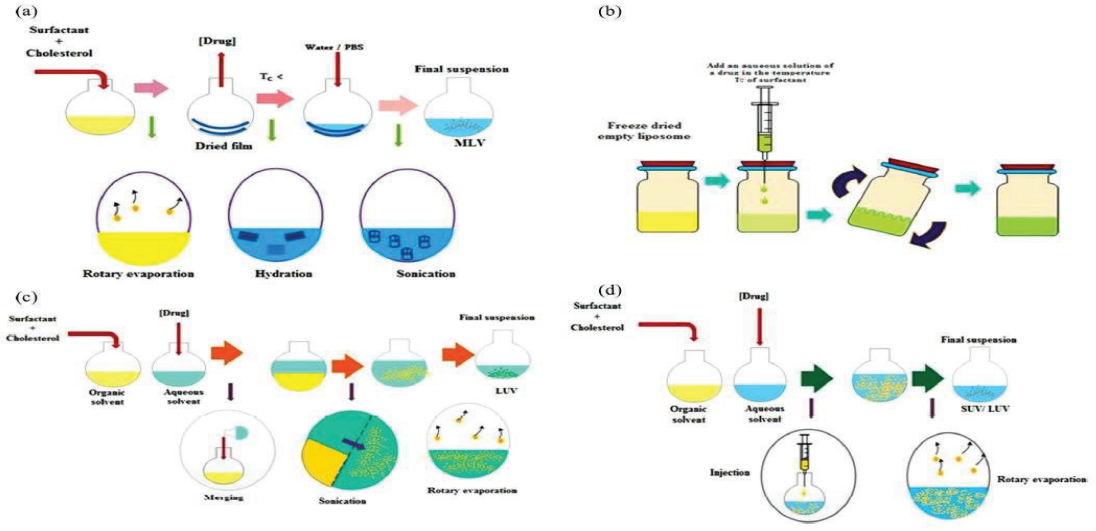

Figure 1.2. Representation of some liposome production methods. (Source: Amoabediny et al., 2018)

hydration of lipids from the sides of the boiling glass (Szoka & Papahadjopoulos, 1980). Depending on liposomal composition T_c can be lower than the melting temperature of phospholipids. Presence of cholesterol has been reported to lower the T_c requirement of

liposomes (Danaei et al., 2018). Other factors that affect liposomal characteristics are hydration duration, hydration buffer and lecithin type. After hydration period the liposomes can be treated with sonicator, filtration, or dialysis for size reduction and homogenization of resulting liposomes. The drug molecule to be entrapped can be dissolved in either in organic solvents at the beginning or in aqueous phase during hydration depending on its polarity and water solubility (Tavano, Muzzalupo, Picci, & de Cindio, 2014).

1.1.3.2. Freeze-drying method

The freeze-drying method is like the thin film hydration method, first lipids and supportive molecules are dissolved in organic solvents but not the drug molecules. After evaporation of solvents, hydration is started but the hydration buffer in this method contains glucose or another cryoprotectant material. Then the empty liposomes are dried in a freeze dryer. The drug loaded liposomes are produced by mixing these freeze-dried empty liposomes with an aqueous phase of drug of interest (Sankar, Ruckmani, Durga, & Jailani, 2010). The limitation of this method is that hydrophobic drugs cannot be efficiently used because the aqueous phase that dissolves the drug can also dissolve the vesicles.

1.1.3.3. Reverse phase evaporation method

Reverse phase evaporation is also similar to other methods. First, lipids and supportive molecules are dissolved in organic solvent. Then the drug molecule is dissolved in the aqueous phase and an emulsion is formed by mixing the organic solvent with the aqueous phase with a sonicator. The organic solvent is removed from the resulting mixture via rotary evaporator under low pressure, in the meantime, large unilamellar vesicles are formed. This method is useful for entrapping water-soluble drugs. The advantage of the method is that the resulting liposomes can entrap a large amount of the drug in their lumen (Junyaprasert, Singhsa, Suksiriworapong, & Chantasart, 2012).

1.1.3.4. Ether injection method

In the ether injection method, lipids and supportive molecules are dissolved in either ether or an ether-ethanol mixture. Then the ether mixture is slowly injected into the aqueous drug solution (at the transition temperature of the phospholipid). Following this the ether is evaporated under low pressure and as the ether is removed unilamellar vesicles are formed (Dufes et al., 2000)

1.1.3.5. Sonication method

In the sonication method, the lipids and supportive molecules are added to the drug solution then the mixture is sonicated with a probe for several minutes at the transition temperature of the phospholipids that are used. This method can be applied to water soluble drugs only (Alam et al., 2013).

1.1.3.6. Microfluidization Method

Compared with other methods, microfluidization produces more repeatable results especially for production of small unilamellar vesicles, but it requires more sophisticated instruments. In this method three adjacent high-speed jet microchannels are used. Lipids that are dissolved in isopropyl alcohol flow through the central microchannel while aqueous solution flows through two adjacent microchannels. Lipids and aqueous solution are condensed at the intersection point, where they create an interface. The aqueous solution penetrates lipid particles and forces them to form self-assembled vesicles (Verma, 2010).

1.1.3.7. Heating method

In this method all lipids and supportive molecules are hydrated separately under nitrogen gas for an hour at room temperature. Then these components are heated at 120 °C for 15 minutes with constant stirring. Following that the temperature is dropped to

the lipid transition temperature and all components are stirred in a container for a predetermined amount of time (Jahn, Vreeland, Gaitan, & Locascio, 2004).

1.1.3.8. Freeze thaw method

This method requires production of empty liposomes prior to encapsulation. The empty liposomes can be produced with any method of choice, but thin film hydration is used most frequently. After formation of the empty liposomes, the drug of interest and liposomes are mixed and kept at -196 °C for 5 minutes. After that the mixture is transferred to a water bath which is pre-heated to the transition temperature of the lipid. The mixture is incubated in the water bath for 5 minutes. These freezing-thawing steps are repeated as much as needed. More repetition results in better encapsulation efficiency (M. Mozafari, 2005)

1.2. Chitosan

Composed of glucosamine and N-catyl glucosamine, chitosan is a natural polysaccharide which is produced via deacetylation of chitin. Chitosan has amine groups in its glycosidic residue which give chitosan its positive charge. One other important characteristic of these amine groups is that when protonated in an acidic environment, chitosan, which is normally water insoluble at pH 6.5, becomes water soluble (Yang et al., 2014) Therefore chitosan can be used as an outer layer to produce a nanocarrier pH-triggered for cancer. Chitosan has the ability to form nanostructures through self-assembly caused by non-covalent interactions and hydrogen bonds (Yang et al., 2014). As a non-toxic, biodegradable, and biocompatible biomacromolecule, chitosan can uniquely adhere to mucosal tissues and also is able to penetrate through the tight junctions of endothelial cells (Wedmore, McManus, Pusateri, & Holcomb, 2006). Chitosan has many unique chemical features that make it a great candidate as a drug carrier and for the same reasons it has already been approved by the FDA for wound dressing applications.

1.3. Nanocarriers and Cancer

Cancer is uncontrolled aberrant growth of cells which results in a cell mass also known as a tumor around or within an organ. Cancer can be seen in different parts of the body and in advance phases, it can migrate from the cancerous origin to other tissues and organs via a process called metastasis. Each year billions of dollars are spent on treatment and research on different cancer types. As a result, a variety of treatments alone or in combination are being tested on human subjects each day. Some of these treatments are chemotherapy, radiotherapy, surgery, immunotherapy (Tran, DeGiovanni, Piel, & Rai, 2017). Chemotherapy is widely used in cancer treatment because of its effectiveness and cheapness, however these advantages come with a cost. First of all, most chemotherapy drugs are not specific and can cause death of healthy cells and tissues. A second drawback is that most chemotherapy drugs do not localize only to a certain target organ or tissue, but they also enter the circulatory system and end up localizing in off-target cells and organs which decreases the drug's bioavailability. As a result of this situation, not only anticancer responses of the drugs are observed but also off-target harmful activities (Albanese, Tang, & Chan, 2012). Conventional chemotherapy drugs mostly target only one of several inhibition mechanisms. In order to increase treatment efficiency, combinations of different drugs also known as combination therapy, are being used nowadays. Combination therapy allows better recovery by inhibiting different cancer pathways using a combination of different chemotherapy drugs (L. Wang et al., 2017). Combination therapy shows many benefits, yet it alone is not enough as the bioavailability of each drug is still low. So, a carrier system is needed to increase bioavailability by increasing localization of lower doses of the active molecule at the tumor site.

An ideal carrier for tumor therapy must stay stable in the blood during circulation and must accumulate mostly at the tumor site. The carrier must be able to penetrate deep into tumor tissue and release the drug both inside and outside of the tumor. Today only a few nanocarriers are able to penetrate through a whole tumor because tumor tissue has a dense stroma composed of many layers and barriers. Also, interstitial fluid pressure and abnormal angiogenesis cause reduced penetration. Although the stability of the carrier is quite important it also creates a dilemma. If a carrier is very stable, then there is a risk of not getting enough drug leakage. For ideal

tumor therapy, there has to be a balance between stability and the leakage of the carrier (Hatakeyama et al., 2007).

Targeting the tumor is a crucial process in the design of a nanocarrier system. As mentioned before, environmental factors like pH can be used as stimuli for nanocarriers to release their content. However, using pH as a stimulus may not always be wise. First of all, blood vessels are far away from the low pH regions of tumor. So, the therapeutic agent may not be able to penetrate into the cancer tissue at all. The same problem occurs for hypoxia. Hypoxic regions of cancer tissue are far away from blood vessels. Also, the pH may not be sufficiently different from normal tissue, in such situations a liposome may not be triggered to release its content (Ganta et al., 2008).

1.3.1. Liposomal Drug Delivery Through Skin

Skin is the largest organ of the body with a surface area of approximately 2 m^2 that represents almost 15% of the body weight (Alexander et al., 2012). Dermis and epidermis are two types of tissues that form the skin (Figure 1.3). They have different structures and embryonic origins. These tissues are separated via the basement membrane. The epidermis itself divides into two layers as viable epidermis (VE) and stratum corneum (SC) which is chemically active but composed of non-viable, large, flat, dead cells called corneocytes (El Maghraby, Barry, & Williams, 2008). The thickness of the viable epidermis is 50 – 100 μm while the non-viable layer is 10 – 15 μm in thickness. The VE is composed of the *stratum basale, stratum spinosum,* and *stratum granulosum,* respectively. About 95% of epidermal cells are self-renewing keratinocytes which migrate from VE to SC and undergo differentiation as the migration happens and forms corneocytes of the SC (Bouwstra, Hofland, Spies, Gooris, & Junginger, 1992). Corneocytes are rich in keratin filaments and water and surrounded with a lipid matrix consisting of keratin, cholesterol, ceramides, and fatty acids. The structure of the SC is explained by a model called “bricks and mortar”. According to the model, the corneocytes represent bricks and the surrounding fatty matrix represents mortar (Riviere & Papich, 2001). This dense formation of SC is the main cause of the failure of drug delivery through skin tissue.

Below the epidermis, a deeper layer is called the dermis and is composed of fibrous proteins, collagens, and elastin (Figure 1.3). The dermis is 1 – 4 nm in thickness.

Dermis hosts many different structures including sweat glands, hair follicles, and nerve endings.

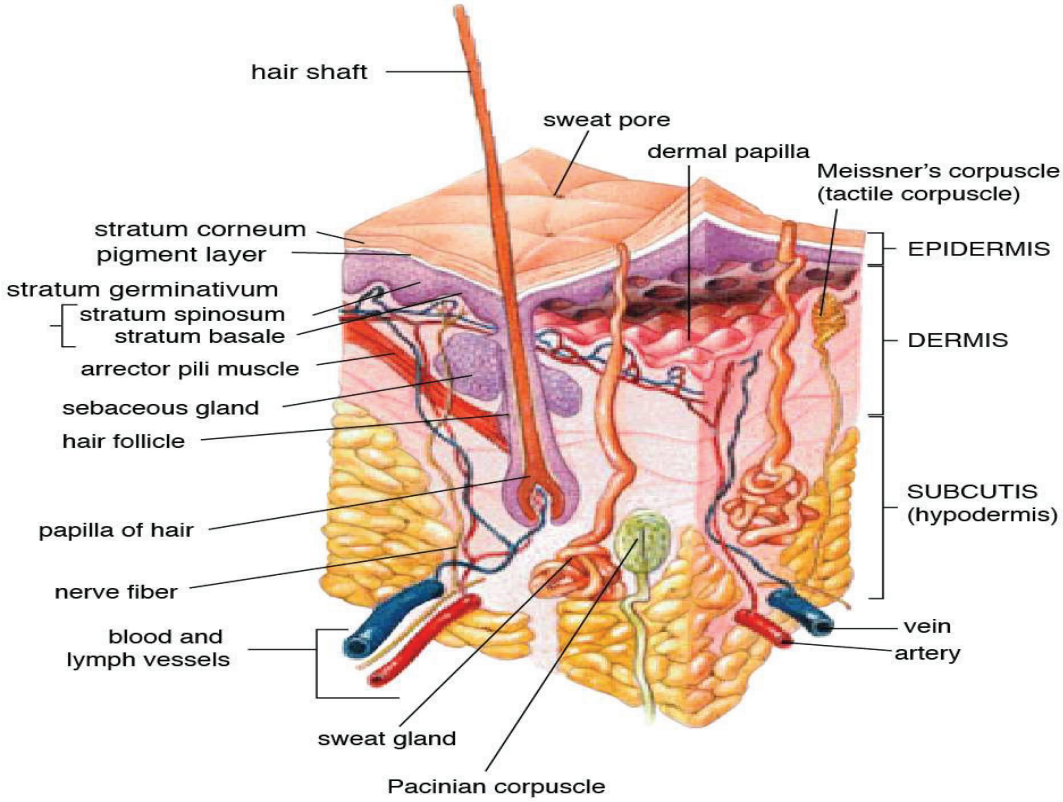

Figure 1.3. A detailed diagram of skin layers. Stratum corneum is the main barrier that prevents passage of drug molecules. (Source: http://training.seer.cancer.gov/ss_module14_melanoma/images/illu_skin01.jpg

Skin has three direct functions: protection, temperature control, and self-repair. Skin can protect the body from mechanical forces due to its elastic structure, its thick structure also protects the body from destructive chemicals. In addition, the skin creates a hostile environment for invasive organisms due to its pH, protective enzymes of VE and hair follicles (Oesch, Fabian, Oesch-Bartlomowicz, Werner, & Landsiedel, 2007). Skin can control body temperature via changing blood flow and rate of sweating (Hayden, Cross, Anderson, Saunders, & Roberts, 2005). Skin also plays a role in sensation, immunity, and secretion by cooperating with other organs (Archer, 2010).

Treatment of skin pathologies has been a significant challenge for drug developers. The difficulty in treatment of skin-related disorders like cancer, inflammatory, and cutaneous disorders comes from insufficient diffusion of drug molecules through SC. The high degree of migration, differentiation, and desquamation of corneocytes hinders effective drug penetration through skin. Moreover, a complete

skin renewal takes 14 days for a healthy person, so to reach an effective dose of the drug at the disease site, conventional drugs must be applied in high doses which frequently results in undesired side effects.

There are three routes for skin penetration of drug molecules: intracellular, intercellular and appendageal routes (Figure 1.4) (Lane, 2013). The intracellular route is more suitable for hydrophilic drugs as these molecules must penetrate through each cell of the layers (Bolzinger, Briançon, Pelletier, & Chevalier, 2012). The intercellular route is chemically more convenient for hydrophobic drugs but although, the intercellular route theoretically exists, it is not widely accepted. Intercellular spaces are smaller than 75 nm which means only non-polar small molecules might penetrate through (Baroli et al., 2007; Kang et al., 2007). Hair follicles are another route for drug administration. Although hair covers a small area on skin, hair follicles grow from dermis and penetrate through all layers of skin which creates an optimal route for drug penetration especially for systemic diffusion of drugs since follicles are close to the capillary bed, a region rich in small blood vessels (Knorr et al., 2009). So, the appendageal route is most suitable for drugs with higher molecular weights.

Lipid-based drug carrier systems have been an effective solution for transdermal skin drug delivery since the lipids in their compositions have similarity with those of the SC. It has been shown that once the liposomes interact with skin, they increase drug delivery by penetrating the epidermis especially the SC via several mechanisms including lipid loosening, lipid exchange, polarity alteration and fluidization (Zhai & Zhai, 2014). There are several penetration mechanisms put forward for liposomal skin penetration. Conventional liposomes are generally disrupted once they interact with the skin surface and the lipids found in these liposomes penetrate the epidermis which allows drug molecules to be more efficiently transferred through skin layers. Also, it has been speculated that conventional liposomes can be transferred deep in the skin through the appendageal route without disruption (Betz, Imboden, & Imanidis, 2001). Another possible mechanism is that liposomes can fused with the SC matrix and release their content within the SC which again increases the permeation of active drug molecules. If liposomes are supported with single chain surfactants in their structure, they can become permeable to skin. Liposomes with such modifications are known as transferosomes. Single chain surfactants like TPGS increase the permeability of these carriers by allowing deformation and reformation of liposomes during the permeation.

Lipid based nanocarrier systems have been used to treat many different skin related diseases including skin cancer. Paolino et al showed (Paolino, Celia, Trapasso, Cilurzo, & Fresta, 2012) that liposomes loaded with paclitaxel are 23-fold more efficient than a suspension of paclitaxel in the treatment of non-melanoma skin cancer.

1.4. Plant Glycoalkaloids and Cancer

The genus *Solanum* consists of almost 2000 species including the economically important crops potato (*S. tuberosum* L.), tomato (*S. lycopersicum*), and eggplant (*S. melongena*). These crops are commonly grown in temperate areas of the world. In traditional medicine, Solanum species has been used as herbal drugs for many diseases like earache, asthma, inflammation, cancer, and haemorrhoids (Ghazanfar & Al-Al-Sabahi, 1993; Sultana, Perwaiz, Iqbal, & Athar, 1995).

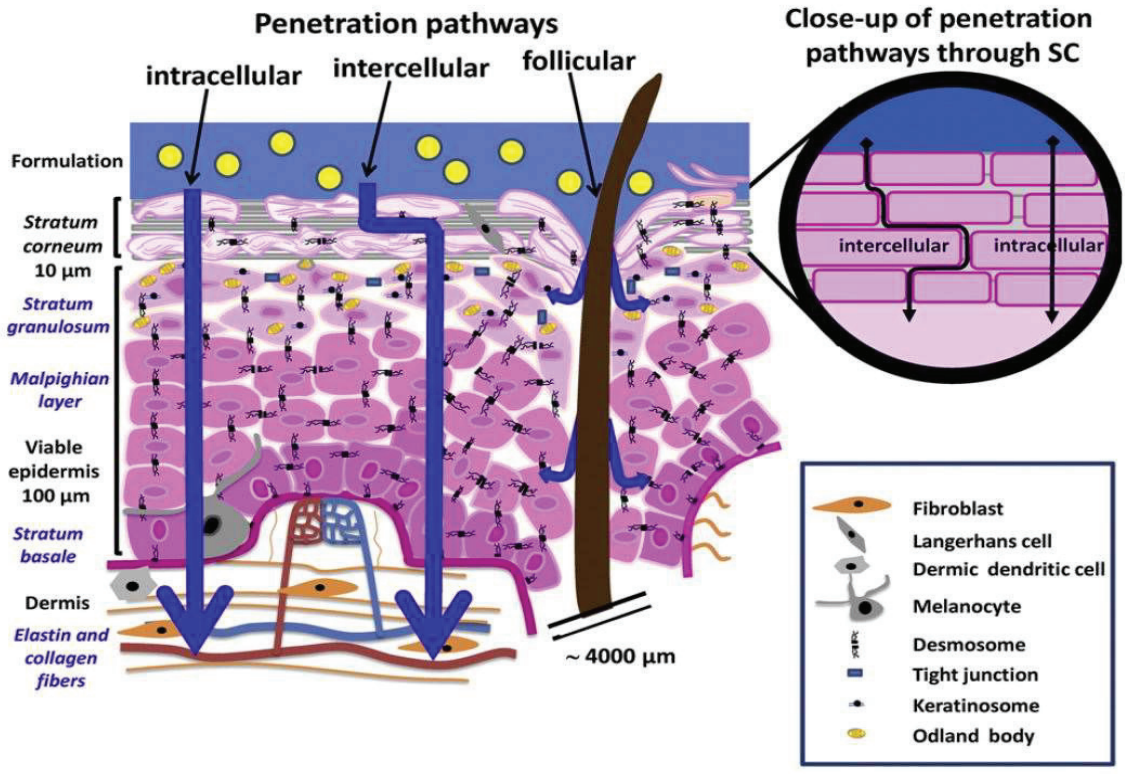

Figure 1.4. Schematic representation of drug penetration pathways through epidermis. (Source: Bolzinger et al., 2012)

Bioactivities of plants are commonly attributed to molecules called secondary metabolites which are produced by plants via secondary reactions that use basic carbohydrates, lipids, and amino acids (Ali Kayani, Masood, Achakzai, & Anbreen,

2007). These secondary metabolites are not fundamental to plant's primary metabolic functions but are mostly required for the plant's survival from biotic or abiotic stress conditions. Some secondary metabolites provide basic defence for the plant as they are antioxidant, antiproliferating compounds while others have more complex mechanism of action like providing feeding deterrence by making the plant's taste bitter or making the plant toxic for herbivores (Kennedy & Wightman, 2011).

Secondary metabolites are divided into three categories according to the biosynthetic pathway that produces them. These groups are terpenoids, phenolics, and alkaloids. Terpenoids are derived from isopentenyl pyrophosphate and have significant roles in plants as toxic compounds. Phenolics are derived from the melonate/acetate pathway or shikimic acid pathway. Alkaloids are synthesized mainly from amino acids, therefore they have at least one nitrogen atom in their structure (Mazid, Khan, & Mohammad, 2011).

Steroidal glycoalkaloids (SGAs) are one of the most known alkaloid groups in plants, produced especially by the Solanaceae family, and have attracted interest as medicinal compounds (Manase et al., 2012). SGAs have been attributed to be a major player in plant-pathogen interactions. There has been a correlation established between high concentrations of SGAs in plants and plant's resistance to bacterial and fungal infections. Solanum glycoalkaloids as the name indicates are glycoalkaloids that are produced by Solanum species. The very first SGA to be discovered was α-solanine of potato in the 1800s. This was followed by discovery of α-tomatine of tomato. Two major SGAs of eggplant, α-solasonine and α-solamargine were discovered much later.

Solanum steroidal glycoalkaloids differ from each other by their chemical structures. The presence of carbon double bonds, sugar and functional groups are used to define new steroidal glycoalkaloids. Solanum SGAs are composed of two groups: a hydrophobic aglycone which contains a C_{27} steroid structure that is the source of hydrophobicity and a hydrophilic carbohydrate side chain. The two groups together create an amphiphilic structure. Cholesterol is thought to be the precursor molecule for aglycone production. Up to date five different aglycone structures has been defined which are solanidanes, spirosolanes, epiminocholestanes, α-epiminocyclohemiketals, and 3-aminospirostanes.

Solasodines are well known SGAs of the Solanaceae family which are water insoluble and have been a research focus for drug development due to their anticancer

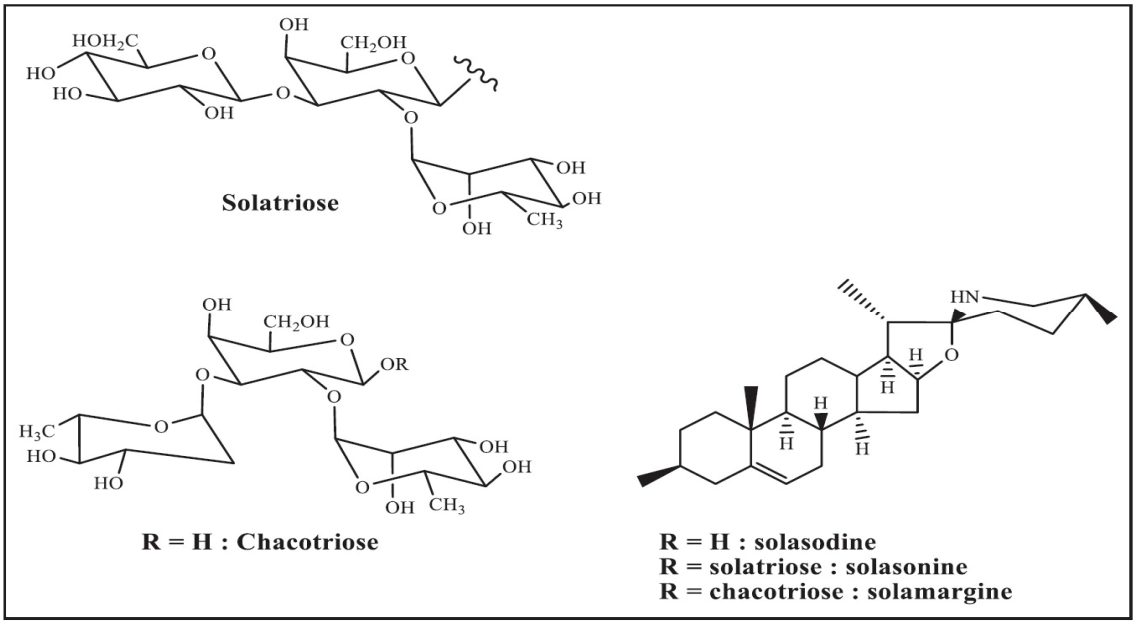

Figure 1.5. Chemical structures of solasonine and solamargine (Source: Tiossi et al.,2012)

activities. Produced by more than 200 species, solasonine (SS) and solamargine (SM) contain identical aglycone groups but differ in their triose groups. Solasonine contains solatriose as triose group while solamargine has chacotriose (Dinan, Harmatha, & Lafont, 2001; Tiossi et al., 2012).

SS and SM have been shown to be effective against pests and toxic against some animals (Jadhav, Sharma, & Salunkhe, 1981; Weissenberg, Levy, Svoboda, & Ishaaya, 1998). Also, a significant cytotoxic effect of SS and SM was shown in many cancer cell lines including skin tumors (Maurya, Gupta, Negi, & Srivastava, 2009). SS and SM have been tested on many epithelial origin healthy and cancer cell lines including VERO p35, MDA-MB-231, MCF-7, SH-SY5Y, SK-BR3, Eahy926, HepG2, SW480, and U87 (Table 1) (Akter, Uddin, Tiralongo, Grice, & Tiralongo, 2015; Burger et al., 2018; Gu et al., 2018; Munari et al., 2014; X. Wang et al., 2017). IC_{50} values among cancerogenic cell lines ranged from 3.965 µM to 23.79 µM for solasonine and from 5.01 µM to 20.68 µM for solamargine, while in healthy cell lines IC_{50} values ranged from 11.18 µM to 16.65 µM for solasonine and from 5.01 µM to 53.94 µM for solamargine. Current data on this topic suggests that both SS and SM are promising drug candidates for many diseases including melanoma. Curaderm© a topical cream used for skin

related diseases that contains SS and SM in 1:1 ratio showed promising results and Phase I and Phase II studies but was not commercialized due to financial problems of the patent owing company, Solbec Pharmaceuticals. SM and SS are also shown to be efficient agents against some other skin related diseases including leishmaniasis and Bowen's disease (Goldberg, Landau, Moody, & Vergilis-Kalner, 2011; Lezama-Davila et al., 2016).

Table 1.1: IC_{50} values of solasonine and solamargine for different cell lines in the literature

Cell Line	Tissue	IC50 value for Solasonine (µM)	IC50 value for Solamargine (µM)	References
VERO	Kidney	16.65	53.94	Akter et al., 2015
AGS	Stomach	94.32	21.19	Akter et al., 2015
HT-29	Colon	>100; 22.67	16.36; 9.88	Akter et al., 2015
MCF-7	Breast	5.09; 22.25	5.01; 18.23	Akter et al., 2015
MDA-MB-231	Breast	8.36	20.68	Akter et al., 2015
SH-SY5Y	Bone Marrow	-	15.62	Burger et al., 2018
SK-BR-3	Breast	-	18.53	Burger et al., 2018
Ea.hy926	Somatic Cell Hybrid	-	8.3	Burger et al., 2018
MGC803	Stomach	17.69	7.02	Gu et al., 2018
HepG2	Liver	22.98; 6.01	12.74; 4,58	Gu et al., 2018; Munari et al., 2013
SW480	Colon	23.79	11.16	Gu et al., 2018
U87	Brain	3.965	-	Wang et al., 2017
U251	Brain	6.675 ; 26.21	8.09	Wang et al., 2017 ; Munari et al., 2013
U118	Brain	13.723	-	Wang et al., 2017
U343	Brain	23.09	16.3	Munari et al., 2013
M059J	Brain	21.72	9.59	Munari et al., 2013
HeLa	Cervix	16.04	7.48	Munari et al., 2013
B16-F10	Skin	24.19	10.15	Munari et al., 2013
GMO7492A	Lung	38.01	26.66	Munari et al., 2013
V79	Lung	33.42	16.75	Munari et al., 2013

1.5. Aim of the Study

Treatment of skin related diseases is considered to be problematic due to the natural barrier function of the skin which leads to low skin penetration of active drug molecules. Thus, in order to achieve therapeutic concentrations of drugs at the disease site, higher concentrations of drugs are applied on skin which eventually cause undesired side effects. Thus, in order to surpass skin penetration problems a carrier system can be a smart solution. The first and main objective of this study was to produce a stable liposome carrier system which will carry drugs of interest. Different formulations with differentiating methodology were tested physically and chemically to reach a stable final liposome formulation. In the second step, the stable liposomes were loaded with drugs of interest, solasonine and solamargine, and further stability tests were conducted. In the final stage, the biological activities of these drug candidates were tested in vitro with HaCaT and SCC-25 cell lines. The outcomes of this study allowed us to create a new drug candidate which we hope can be tested against skin diseases and will not produce significant side effects.

CHAPTER 2

MATERIALS AND METHOD

2.1. Preparation of Liposomes

Liposomes were prepared using the thin film hydration method previously described by Mengoni et al. (2017) with certain modifications. Lecithin (Applichem Egg yolk lecithin, A0893; Natipide II, 510180; Phospholipon 80H, 529200; and Lipoid E, 510000, Germany), cholesterol (Applichem, A0807, Germany) and α-Tocopherol (Applichem, A2232, Germany) were dissolved in 6 mL of solvent mixture (chloroform/methanol (v/v = 1:1)) at various concentrations (Table 2.1). The solvent mixture was evaporated with a rotary evaporator for various times, at various temperatures under 150 mbar vacuum until a thin layer of dried lipids weas formed. The resulting thin layer was hydrated with 10 mL of water (pH = 5.5, 10 mM NaCl) stirring in a shaker incubator (150 rpm) at various temperatures (40°C, 45°C, 50°C) for >1 h. The resulting suspension was sonicated in an ultrasonic bath (Elmasonic S 10 H, Elma, Singen, Germany) for 30 minutes and with a probe sonicator (Sonopuls, Bandelin, Berlin, Germany) for 2 minutes. Empty liposomes were stored at various temperatures (4°C, 25°C, 37°C) in the dark, ready for characterization. For the liposomes loaded with Solasonine (SS) and Solamargine (SM), the same procedure described above was followed but in addition, we added 300 μM of SS SM to initial organic solvent before the evaporation step.

Table 2.1. Ingredients of different liposomal formulations

Lecithin Name	Lecithin (mg)	Cholesterol (mg)	α-Tocopherol (mg)
Applichem	120	19,8	3
Natipide II	600	99	15
Lipoid E	120	19,8	3
Phospholipon 80H	120	19,8	3

2.2. Characterization of Liposomes

Liposomes were characterized for their physical and chemical stabilities for each week or fortnightly.

2.2.1. Physical Stability Measurements

Physical stability of the empty and loaded liposomes was characterized by their size and zeta potential using NanoPlus zeta/nano particle analyser (Particulate Systems, Micrometrics, USA) fitted with a Semiconductor Laser (λ = 660 nm) at 25 °C.

2.2.2. Chemical Stability Measurements

Chemical stability of liposomes was determined via Stewart Assay. The Stewart assay is a colorimetric method that determines stability of phospholipids in the liposome via measuring their capacity to bind ammonium ferrothiocyanate. The method described by Stewart (1980) was slightly changed. Briefly, ammonium ferrothiocyanate solution was prepared in given concentrations as in the method. Standard solution was prepared with not only phospholipids but also with cholesterol and α-tocopherol which we found to interfere with ammonium ferrothiocyanate. The ratio between phospholipids and surfactants was kept constant as in their liposomal formulation. The concentration of phospholipids in the standard solution was set to 2 mg/ml but the final volumes of standard solution, chloroform, and ammonium ferrothiocyanate were doubled. Each sample was vortexed for a minute then centrifuged at 1000 rpm for 10 minutes (Beckman Coulter, USA). After centrifugal separation, the bottom layer of samples was removed with Pasteur pipettes carefully. Absorbance of this layer was read in spectrophotometer (Shimadzu, 1700 UV Visible Spectrophotometer, Japan) at a wavelength of 488 nm. The concentration of stable phospholipids was determined using the standard curve.

2.2.3. Encapsulation Efficiency

Encapsulation efficiency was determined via ultrafiltration tubes with a MWCO of 30K (Sartorious AG, Göttingen, Germany). To separate liposome encapsulated SS

and SM from non-encapsulated SS and SM, 1 mL of loaded liposome solution was placed into ultrafiltration tube and centrifuged for 40 minutes at 4000 x g with Allegra X-12R Benchtop Centrifuge (Beckman Coulter, USA). After centrifugation, the amount of non-encapsulated SS and SM that passed to the bottom of the tube was evaluated using HPLC (Prominence LC-20A Modular HPLC System, Shimadzu, Japan). 0.ereM Na_2HPO_4 pH 7.2 and acetonitrile was used as mobile phase (60% - 40% v/v). C18 column was used and column temperature was set at 30 °C. Flow rate was set at 1 mL/min. Injection volume of sample was 20 µL and UV detection was done at 200 nm.

2.2.4. Determination of Drug Release Profile

Sample and separate method was used to determine drug release profile of SM and SS loaded liposomes. Separate falcon tubes were prepared for each time point (4, 8, 12, 16, 24, 36, 48, 60, 72 hours). Release mediums (Acetate, pH = 5.5; PBS, pH = 7.4) were added to each falcon tube. SS and SM loaded liposomes were added directly to release medium in 1:4 ratio. The mixture was placed into a 15 ml falcon tube and incubated in a shaker incubator at 37°C, 100 rpm. At the end of each designated release period, 2 ml sample was placed into ultrafiltration tube and centrifuged for 40 minutes at 4000 x g with Allegra X-12R Benchtop Centrifuge (Beckman Coulter, USA). The amount of released drug that passed to the bottom of the tube was measured with HPLC instrument (Prominence LC-20A Modular HPLC System, Shimadzu, Japan). The same parameters as mentioned in encapsulation efficiency were used for HPLC measurements. Drug release kinetics of SS and SM were determined by fitting HPLC data to the cumulative drug release model

2.3 Lyophilization

Size analysis of liposome samples was done with NanoPlus zeta/nano particle analyser (Particulate Systems, Micrometrics, USA) prior to lyophilization with the same parameters that were mentioned in the physical stability section. Glucose (Sigma, G7021, Germany), sucrose (Sigma, S5391, Germany), maltose (Sigma, M5895, Germany), and dextrose (Sigma, D9434, Germany) were used as cryoprotectants for

lyophilization tests. Different w/w ratios (8:1, 6:1, 4:1, 2:1, 1:1; 1:2, 1:4) of cryoprotectants and phospholipids (the phospholipid found in the liposome) were tested for each cryoprotectant. For each sample, the amount of phospholipids was kept constant while the amount of cryoprotectant was changed. The cryoprotectants were dissolved in 1 ml liposome aliquot and then placed in a sample tube. All samples were lyophilized for 4 days at -20 °C with 0.1 mbar pressure in Epsilon 1 – 4 LSC freeze dryer (Martin Christ, Germany). After lyophilization the samples were stored at -20 °C for three months and then dissolved in 1 ml water (10 mM NaCl, pH 5.5) and size analysis was repeated.

2.4. Preparation of Chitosan Film and SEM Imaging

For chitosan film, 2% chitosan (Sigma, 448869, Germany) was prepared with 1 % acetic acid. 7.5 ml 2% chitosan, 1 ml glycerol (Sigma, G5516, Germany), and 1.5 ml acetic acid (Riedel-de Haen, 27225, Germany) or liposome sample were put in a 15 ml beaker with constant stirring. Following that the mixture was poured into a 60 mm dish and left for drying in a fume hood for 4 days without covering. For cross-linking, dry chitosan films were incubated in 1% sodium tripolyphosphate (STTP) for a minute and then in 2% NaOH for 30 minutes. Following that films were incubated in water for 30 minutes and then left to dry on a clean surface.

For SEM imaging, films were lyophilized for a day at -20 °C with 0.1 mbar pressure in Epsilon 1 – 4 LSC freeze dryer (Martin Christ, Germany). Prior to imaging samples were gold coated and then imaged with FEI QUANTA 250 FEG scanning electron microscopy with SE detector (FEI Company, Hillsboro, OR, USA).

2.5. Cell Culture

The human keratinocyte cell line HaCaT, and human squamous cell carcinoma cell line SCC-25 were kindly provided by Assoc. Professor Gülistan Meşe Özçivici from Department of Molecular Biology and Genetics at IZTECH in İzmir, Turkey. These cells were used to test the biological activities of Solasonine and Solamargine. The HaCaT cells were cultured using Dulbecco’s Modified Eagle Medium (DMEM)-

High glucose (Sigma, D6429, Germany) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin (10,000 units penicillin and 10,000 units streptomycin) in 60 mm cell culture dishes. The SCC-25 cells were cultured using Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM:F12) (Gibco, 31330038, Thermo Fisher Scientific, USA) supplemented with 400 ng/ml hydrocortisone (Sigma, H0888, Germany), 10% fetal bovine serum, 1% and 1% penicillin-streptomycin (10,000 units penicillin and 10,000 units streptomycin) in 60 mm cell culture dishes. The cultures were kept in an incubator with 5% CO_2 and at 37 °C . During treatments and assays all relevant drugs and solutions were dissolved or diluted in the appropriate medium for the cells.

2.5.1. Cell Viability Assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma, M5655, Germany) test was used to evaluate the biological activities of Solasonine and Solamargine. Cells were seeded in 48-well plates ($1x10^4$ cells per well) or 96-well plates ($2x10^4$ cells per well) with the appropriate medium. After 24 h of incubation, medium was removed and replaced with medium supplemented with free SS and SM or liposome encapsulated SS and SM at predetermined concentrations. As negative controls, medium only and MeOH (for free SS and SM treatment) or empty liposomes (for liposome encapsulated SS and SM treatment) groups were formed. After 24 hours of incubation with treatment materials, medium was removed and replaced with MTT solution (5mg/ml) that was diluted with medium at a ratio of 1:10 and incubated for 4 h at 37 °C. After the incubation, plates were centrifuged at 1800 rpm for 10 minutes in Hettich Universal 30 RF centrifuge (Hettich, Germany). Finally, the culture medium and the MTT solution were removed and replaced with 150 µL (for 96-well plate) or 300 µL (for 48-well plate) DMSO (dimethyl sulfoxide) and wrapped with aluminium foil then put on an orbital shaker at 150 rpm for 5-10 minutes until formazan crystals were completely dissolved. The absorbance of samples was measured at 570 nm with a automated microplate reader (Varioskan Flash ™, Thermo Scientific ™, Massachusetts, USA) after orbital shaking at 300 rpm for 5-10 min. The results were calculated as cell viability relative to negative control.

2.5.2. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, San Diego, CA, USA). All experiments were statistically analyzed using multiple t-test. Differences were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). All biological experiments were conducted at least in duplicate and with at least three technical replicates. All the measurements were compared to the control samples, which were not loaded/treated with SS/SM.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Identification of Suitable Liposomal Formulation

Identification of optimal formulation of liposome is important to reach desired outcome of the carried drug. An ideal liposomal formulation must have physical and chemical stabilities throughout the retention period and must have a high degree of encapsulation efficiency of the drug load. Also, the ideal formulation must provide a stable drug release profile to ensure the drug load stays in the therapeutic window. There are many factors that affect the physical and chemical stabilities of liposomes as well as their retention period and encapsulation efficiency. These factors include type of lecithin, type of hydration solution, hydration temperature, and storage temperature. All of these factors were tested in this study to reach an ideal formulation of the liposomes that were going to be used to carry Solasonine and Solamargine.

3.1.1. Identification of Phase Transition Temperatures of Lecithin

Phase transition temperature of lecithin is one of the key factors that affects the characteristics of liposomes. It has been reported that there is a correlation between the phase transition temperature of a phospholipid and the length of acyl chain it carries (Szoka & Papahadjopoulos, 1980). In our results, Applichem and Lipoid E had similar transition temperatures, 10.83 °C and 10.13 °C (Table 3.1) which supports this notion. Both Applichem and Lipoid E are derived from egg, meaning that the phospholipids they carry are similar in structure and they contain similar amounts of phosphatidylcholine in their formulations which are at least 60% and 70%, respectively. As mentioned in the Introduction, the temperature of the hydration solution must be higher than the T_c of the phospholipid that is used in the liposomal formulation to have higher hydration efficiency but it should be also noted that the presence of cholesterol in the liposomal formulation, reduces the phase transition temperature to some extent if not completely (M. R. Mozafari, 2010). On the other hand, previous studies in the literature that used similar phospholipids to ours, used hydration temperatures ranging

from 40 °C to 60 °C (Albasarah, Somavarapu, Stapleton, & Taylor, 2010; McPhail, Tetley, Dufes, & Uchegbu, 2000; Mengoni et al., 2017; Smith & Kong, 2014). In the light of this knowledge we decided to test three different hydration temperatures: 40°C, 45°C, 50°C.

Table 3.1. Phase transition temperatures of lipids measured with differential scanning colorimetry.

Lipid	Phase Transition Temperature T(m)
Applichem	10.83 °C
Natipide II	91.09 °C
Phospholipon 80H	71.34 °C
Lipoid E	10.13 °C
Cholesterol	148 °C

3.1.2. Determination of Hydration Solution

Hydration solution is one of the key determinants of liposome production as it interferes with many factors including the liposome's size, physical and chemical stabilities, and duration of shelf-life. Three different hydration solutions were used (NaCl, PBS, Sucrose) as they are frequently encountered in the literature. Since this study mainly focuses on increasing permeation of SS and SM across the skin, the main parameter that we consider in choosing a hydration solution was size of the liposome as smaller carriers has a better chance to delivered across the skin barrier. The average size of the liposomes that were created with NaCl pH 4.5 was 114.6 nm; NaCl pH 5.5 was 110.4 nm; PBS pH 7.4 was 348.2; and 10% sucrose was 148.4 nm (Figure 3.1.). PIs of all samples were lower than 0.3 which means that all liposomes were uniform. When compared with others, NaCl pH 5.5 resulted in smaller liposomes. Taking into account this fact, one other advantage of this hydration solution is its pH value which is the same as skin's pH. Another advantage is that this hydration solution is not expected to create an immunogenic reaction on skin as its concentration is quite low (10 mM). Considering all of this information, 10 mM NaCl pH 5.5 was selected as the hydration solution for the rest of the study.

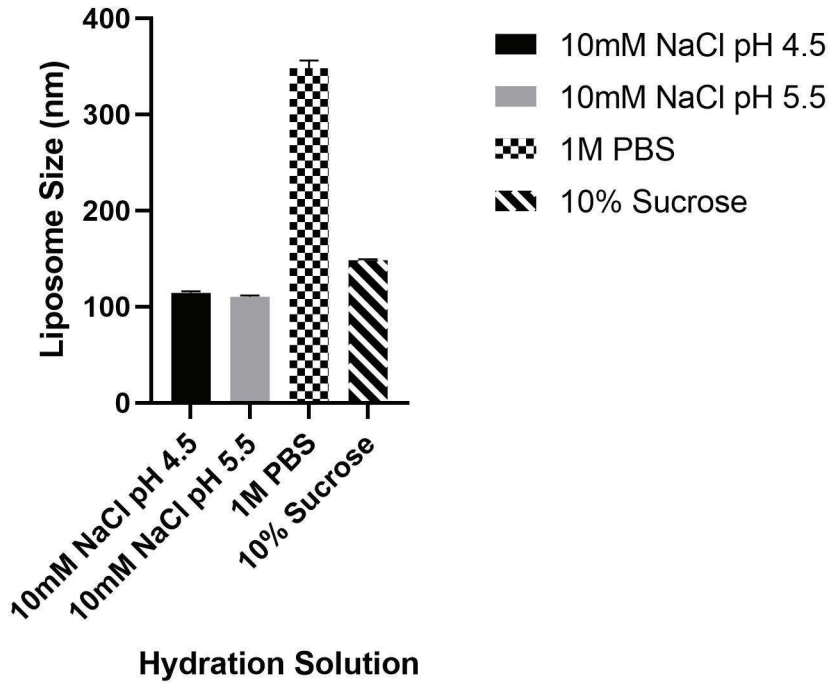

Figure 3.1. Size comparison of liposomes that were prepared with different hydration solutions.

3.1.3. Characterization of Liposomes by Lecithin type, Hydration Temperature and Storage Temperature

Type of lecithin that is used in the liposome is a major factor that affects physical and chemical characteristics of the liposomes. We have evaluated physical and chemical stabilities of liposomes that were made of lecithins of different origins and manufacturers. The lecithin that was used was Applichem A0893, an egg originated lecithin with at least 60% phosphatidylcholine; Lipoid E, an egg yolk originated lecithin with at least 70% phosphatidylcholine; and Phospholipon 80H, a soybean originated lecithin with hydrogenated phospholipids 70% of which are phosphatidylcholines. Also, Natipide II, a pre-liposome concentrate which is a commercially used formulation, was characterized as control. In order to determine the optimal hydration temperature, Applichem and Natipide II liposomes were prepared at different hydration temperatures (40°C, 45°C, and 50°C). All liposomes that were prepared in this batch were also stored at different temperatures (4°C, 25°C, 37°C) to evaluate optimum storage temperatures. Initial size of liposomes prepared at 40°C, 45°C, and 50°C were 109.1 nm, 110.2 nm, and 99.7 nm for Applichem and 89.8 nm, 87.6 nm, and 87.2 nm for Natipide II,

respectively. Polydispersity Index (PI) of these samples were 0.26, 0.26, 0.25 for Applichem and 0.27, 0.27, 0.27 for Natipide II, respectively. PIs of all samples were below 0.5 meaning all liposome samples were uniform. It was noted that initial size of both Applichem and Natipide II liposomes were similar regardless of their hydration temperatures (Figure 3.1. and Figure 3.2.). The physical stabilities of both groups were protected at 4°C storage temperature while change in size was observed by the 35th day at 25°C storage temperature. Sizes of liposomes continuously expanded at 25°C for the rest of the study reaching 2060.6 nm, 1056 nm, and 547 nm for Applichem and 112.1 nm for 40°C and 212.7 nm for 50 °C for Natipide II at 42 days. These results indicate that 25°C is less stable for storage in which liposomes loses their physical stability and start to aggregate much earlier. Also, it was observed that Natipide II liposomes were much more stable than Applichem liposomes at 25 °C storage. Throughout the study a small shrinkage in liposomal size was observed among Applichem liposomes that were stored at 37°C. Initial particle sizes for these liposomes were 109.1 nm (40°C), 110.2 nm (45°C), and 99.7 nm (50°C) while these sizes dropped to 98.6 nm, 98.9 nm and 92.1 nm at the 42nd day, respectively. To the best of our knowledge, this phenomenon was not reported in the literature, and its exact reason is unknown. However, we can speculate that hydration temperature is not a factor that affects this phenomenon as it was observed in all liposomes stored at 37°C regardless of their hydration temperatures.

Chemical stabilities of all liposomes were preserved at 4°C throughout the study regardless of hydration temperature. It was noted that even though physical stabilities of liposomes stored at 37°C were maintained, chemical stabilities of these samples were lost to varying degrees suggesting that 4°C is the best storage temperature for all liposomes. So, the optimal formulation was determined from liposomes that were stored at 4°C. Among those liposomes, hydration temperature did not significantly affect physical stability. On the other hand, it was observed that chemical stabilities of Applichem liposomes prepared at 45°C hydration temperature and Natipide II liposomes prepared at 40°C hydration temperature had higher chemical stabilities compared with liposomes that were prepared at other hydration temperatures (Figure 3.2. and 3.3.). Measurements were terminated after detection of visible precipitates in the liposomal solution. All hydration temperatures (40°C, 45°C, and 50°C) were also tested for Phospholipon 80H and Lipoid E liposomes. After a two-month incubation at 4°C, these liposomes were evaluated by visual observations including color change of

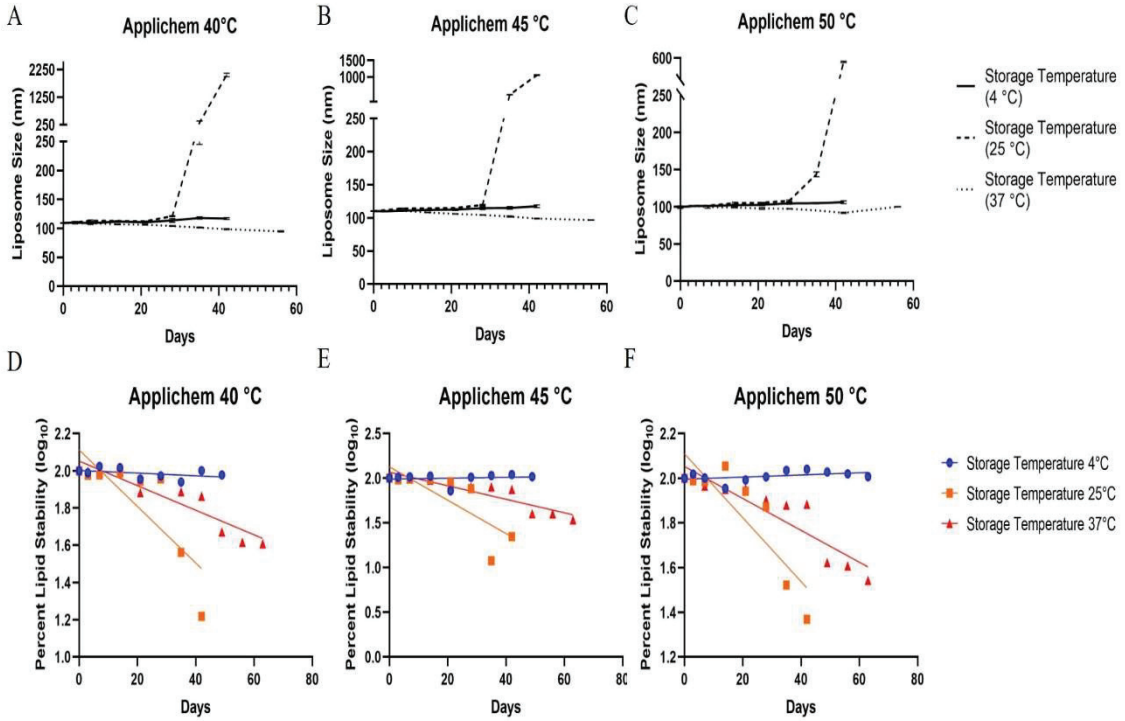

Figure 3.2. Physical and chemical stabilities of Applichem liposomes. A-C, average particle sizes of liposomes obtained by DLS. D-F, percent lipid stability of liposomes obtained from Stewart Assay.

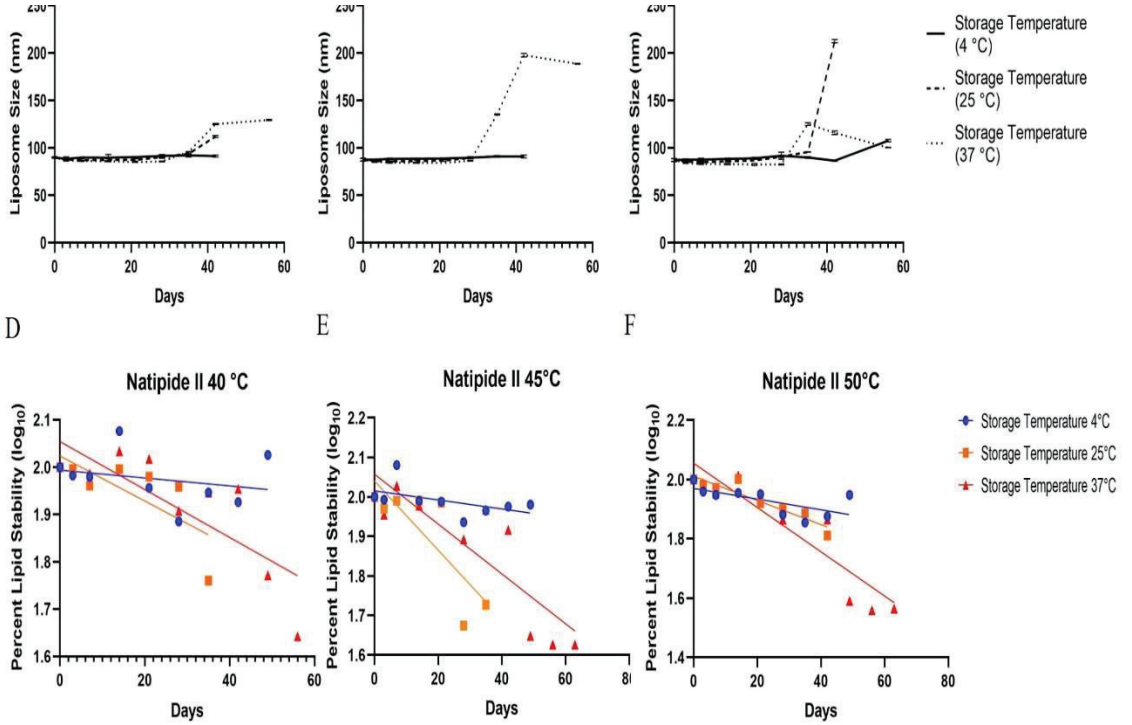

Figure 3.3. Physical and chemical stabilities of Natipide II liposomes. A-C, average particle sizes of liposomes obtained by DLS. D-F, percent lipid stability of liposomes obtained from Stewart Assay.

the liposomal solution and presence of visible precipitates. Based on these observations it was concluded that the ideal hydration temperatures for Phospholipon 80H, and Lipoid E were 40°C and 45°C, respectively (Data is not shown). Following this conclusion, a new batch of these liposomes was prepared at the given temperatures and their physical and chemical stabilities were tested. Initial average particle sizes for these liposomes were 116 nm for Phospholipon 80H and 118.27 nm for Lipoid E with PI values of 0.22 and 0.28, respectively, meaning that liposomes were uniform. It was noticed that initial sizes of these liposomes were relatively larger than those of Applichem and Natipide II. Physical stability of Phospholipon 80H and Lipoid E liposomes were mostly preserved as at the 105th day their average particle sizes were 118.6 nm and 124.7 nm, respectively, with unchanged PI values (Figure 3.4., A-B).

Chemical stabilities of liposomes that were stored at 4°C were preserved in both Phospholipon 80H and Lipoid E liposomes (Figure 3.4., C-D)

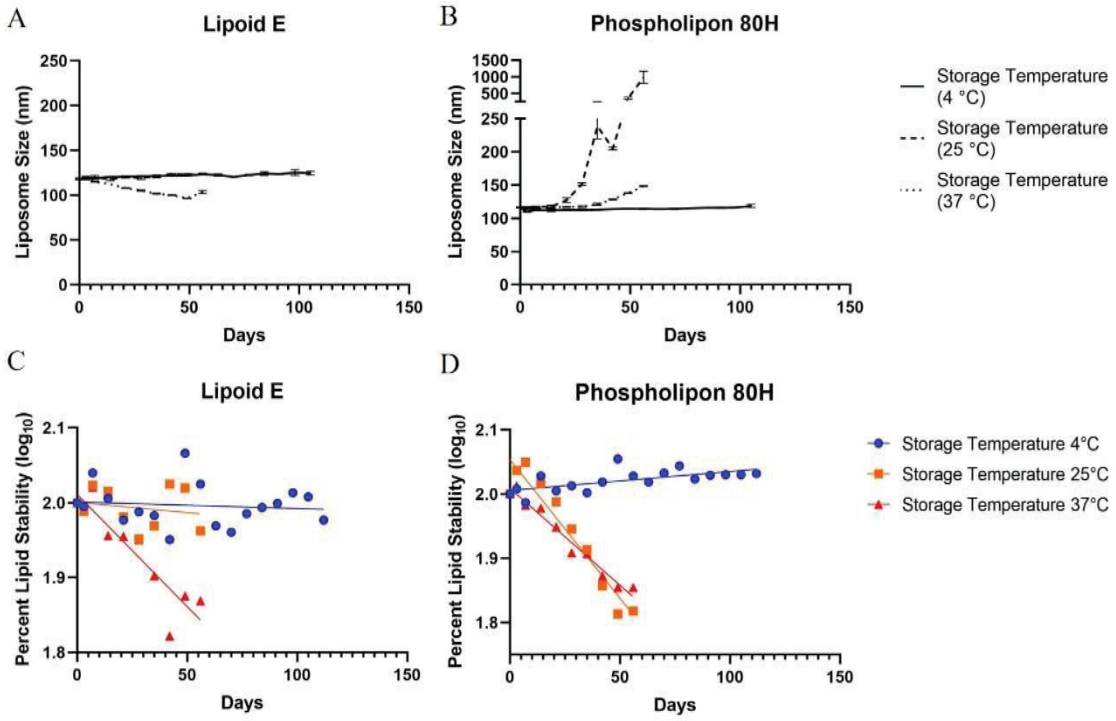

Figure 3.4. Physical and chemical stabilities of Phospholipon 80H and Lipoid E liposomes. A-B, average particle sizes of liposomes obtained by DLS. C-D, percent lipid stability of liposomes obtained by Stewart Assay.

Liposomal shrinkage phenomenon was observed in Lipoid E liposomes that were stored at 37°C. Both Applichem and Lipoid E contain phosphatidylcholine that was originated from egg, so to understand this phenomenon better future research

should focus on physio-chemical properties of this phospholipid. It was noted that chemical stabilities of Phospholipid 80H liposomes that were stored at 25°C and 37°C were lost almost at the same rate (Figure 3.4. C). On the other hand, both physical and chemical stabilities of Lipoid E liposomes were preserved at 25 °C which differentiated it from other samples (Figure 3.4. B and D). Nonetheless, it was concluded that the ideal storage temperature for liposomes is 4°C regardless of lecithin type or hydration temperature.

3.2. Characterization of Solasonine and Solamargine loaded Liposomes

For SS and SM loaded liposomes first lecithin type was determined depending on drug loading capacity of liposomes and physical and chemical stability of their empty versions. Following that physical, chemical stabilities of final formulation were measured and also encapsulation efficiency, drug leakage, and release profile of the final formulation were measured for characterization.

3.2.1. Determination of Lecithin type

As mentioned earlier, all lecithin types that were used in this study had different origins and varying percentages of phosphatidylcholines in their formulations. These differences in chemical compositions and hydration temperatures are expected to change loading capacity of SS and SM. In order to determine the drug loading capacity of each lecithin, empty and drug loaded (300 μM SS and 300 μM SM) liposomes were prepared with each lecithin type. There was a significant difference between sizes of loaded and unloaded liposomes regardless of lecithin type (Figure 3.5.). As SS and SM amphiphilic molecules, both are expected to be encapsulated within the lipid bilayer of liposomes not in the lumen. In the light of this information, the correlation between the drug loading capacity of liposomes and their surface area can be used to determine the most suitable lecithin for SM and SS loading. Surface areas of liposomes were calculated with the following equation: $4*\pi r^2$. The changes in surface area of loaded liposomes from Applichem, Natipide II, Lipoid E, and Phospholipon 80H lecithins were 148.1%, 223.8%, 164.4 %, 226 %, respectively. Compared to their initial size, the highest changes in surface area were in Natipide II and Phospholipon 80H liposomes

which indicates that their drug loading capacity is higher than the other two lecithin types. Although Natipide II and Phospholipon 80H have similar drug loading capacities, Phospholipon 80H has higher physical and chemical stabilities than Natipide II (Figure 3.3., Figure 3.4.). For this reason, Phospholipon 80H was selected for the rest of the experiments.

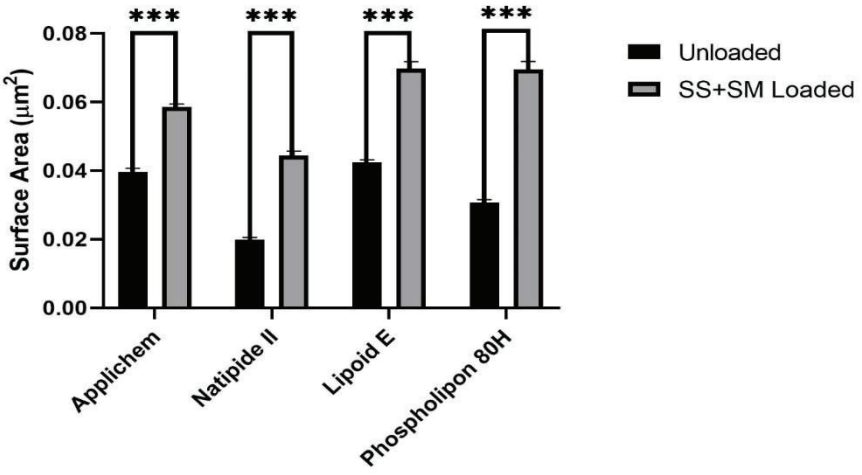

Figure 3.5. Average surface area of loaded and unloaded liposomes that are produced from different lecithin types. *** indicates the significant differences by the multiple t test at $p < 0.001$.

Table 3.2. Average sizes and surface areas of loaded and unloaded liposomes. The letters indicate the significant differences by the multiple t test at $p < 0.001$: different letters indicate significant differences between the loaded and unloaded liposomes with respect to surface area and the same letters indicate the absence of differences.

Lecithin Type	Unloaded liposome (nm)	Surface Area (μm^2)	Loaded Liposome (nm)	Surface Area (μm^2)	Change in %
Applichem	112.2±1.66	0.04±0.001 a	136.53±1.04	0.059±0.001 b	148.1
Natipide II	79.43±1.37	0.02±0.001 a	118.83±1.79	0.044±0.001 b	223.8
Lipoid E	116.2±0.98	0.042±0.001 a	148.97±2.22	0.07±0.002 b	164.4
Phospholipon 80H	98.87±1.32	0.031±0.001 a	148.63±2.57	0.069±0.002 b	226.0

3.2.2. Evaluation of Physical Stability

The Phospholipon 80H formulation was used in drug loaded liposome experiments as it showed the desired physical and chemical properties as well as high drug loading capacity. For the rest of the experiments, the Phospholipon 80H formulation wasnkept constant and liposomes were loaded with same amount of drug (300 μM SS and 300 μM SM). Prepared liposomes were kept at +4 °C as previous experiments showed that it is best storage temperature to keep stabilities of liposomes constant for a longer shelf- life duration. As mentioned earlier due to the amphiphilic nature of SS and SM, these molecules are expected to be docked within the bilayer structure itself. Therefore, after drug loading, changes in the liposome's physical and chemical stability was expected as SS and SM would interfere with lipids and change their stability. In order to assess physical stability of SS and SM loaded liposomes, their size and zeta potential were measured with DLS. A constant increase in liposomal size was detected throughout 3 months of observation. The initial size of drug loaded liposomes was 146.5 nm with a PI value of 0.24. After 3 months of storage, average liposome size increased to 211.83 nm with a PI value of 0.16. The increase in liposomal size was expected as they were losing their physical stability. However, the constant decrease in PI value of our samples was unexpected and indicated that our samples become more uniform as they got bigger. In the literature, it was mentioned that PI can be a good indicator of aggregation of samples. As particles aggregate, their PI value increases. This was confirmed in our experiments too as empty Applichem liposomes that were stored at 25 °C aggregated at day 42 and their PI value increased from 0.26 at day 0 to 0.41. In the light of this information it seems that our drug loaded Phospholipon 80H formulation became more uniform and less likely to aggregate after 3 months of incubation at 4 °C. The reason for this PI phenomenon may be related to the zeta potential of our liposomes. In the literature, it is known that a ± 20 mV zeta potential provides stability to liposomes by preventing aggregation of liposomes via electrostatic repulsion (Honary & Zahir, 2013). The zeta potential of our liposomes was monitored throughout the three month storage. During this period, the zeta potential of the SS and SM loaded liposomes fluctuated between -18.3 mV and -21.9 mV but stayed around -20 mV (Figure 3.6). The zeta potential of our SS, SM loaded liposomes can explain why the PI value decreased over time while their size was increasing. Also the interactions of SS and SM with liposomal lipids might have some effect on this

phenomenon. Further studies must be conducted to reveal the interactions of SS and SM with lipids which would reveal the real reason behind this event.

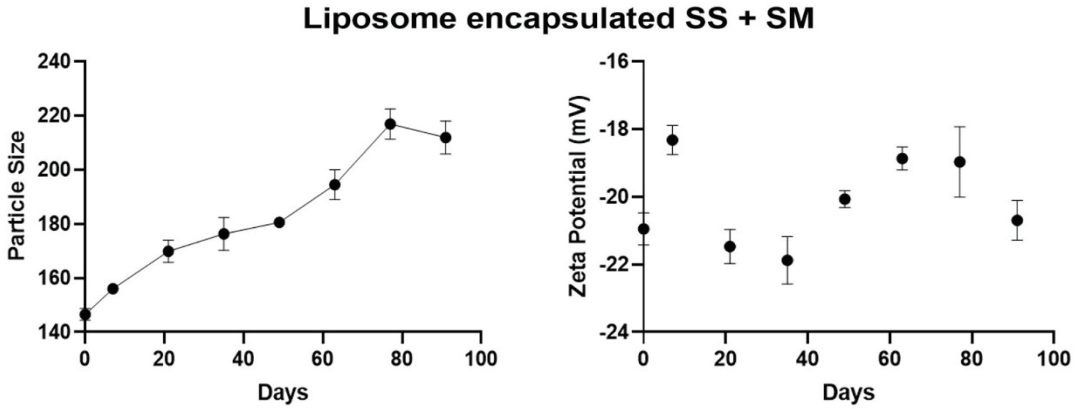

Figure 3.6. Size and zeta potential monitoring of SS+SM loaded liposomes. An increasing trend in size observed in liposomes. Their zeta potential stayed around -20 mV

3.2.3. Evaluation of Chemical Stability

Chemical stabilities of SS, SM loaded liposomes were monitored for 3 months via Stewart Assay. The results indicated that chemical stabilities of liposomes were preserved during three months of storage as in empty liposomes (Figure 3.7; Figure 3.4). Compared with empty liposomes, the presence of SS and SM in the liposomal structure did not change the lipid stability, furthermore they might support the chemical stabilities of lipids by preventing lipid degradation via e.g. hydrolysis of ester bonds.

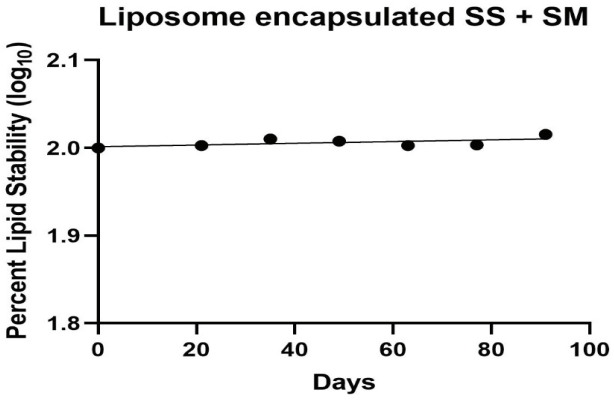

Figure 3.7. Evaluation of chemical stability of SS, SM loaded liposomes by Stewart assay. Chemical stability of liposomes was protected throughout three months

3.2.4. Evaluation of Encapsulation Efficiency and Leakage

One of the major parameters that determines the quality of a liposomal formulation is encapsulation efficiency which shows how much of the total drug is encapsulated by liposomes. After preparation of SS, SM loaded liposomes, unencapsulated SS and SM were separated from SS+SM loaded liposomes via ultrafiltration tubes. The amount of unencapsulated SS and SM was determined with HPLC as described in the Materials and Method. In our loaded liposome samples, encapsulation efficiency of SS was 84.5% and SM was 89.7%. Both of these values are quite high. In addition, we monitored SS and SM leakage from liposomes for three months by separating leaked SS, SM from liposomes via ultrafiltration. Amount of leaked SS, SM was measured with HPLC. There was a small constant leak of both SS and SM observed. Leakage reached up to a 10% for SS and 8% for SM (Figure 3.8). Therefore, after three months of storage at least 90% of the drugs were still encapsulated. Although this value could have been higher, it is an important figure that shows the stability of our loaded liposomes. That rate of leakage of SS and SM were similar, meaning that neither SS nor SM are chemically favoured by the liposomes. This can be explained by the chemical structures of SS and SM which are quite similar to each other.

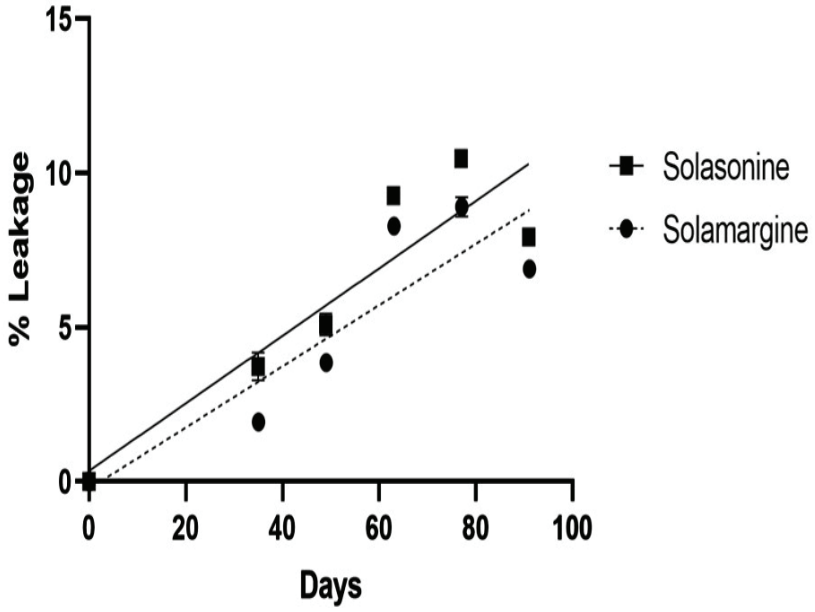

Figure 3.8. % Leakage of Solasonine and Solamargine from loaded liposomes

3.2.5. Determination of Release Profile

As mentioned in the introduction, the release profile of a carrier system is quite important. An ideal carrier system's release profile should avoid the "peaks and valleys" seen with conventional drugs. On the other hand, the ideal carrier system must have a constant sustained release of the drug. In order to examine an accurate release profile, two different release buffers were used: acetate buffer pH 5.5 and PBS pH 7.4. Acetate buffer mimics the pH of skin while PBS mimics the pH of cell culture environment. The SS and SM release from liposomes in acetate buffer started with an initial burst which took 12 to 20 hours. During this period almost 20 % of Solamargine and 15 % of Solasonine were released. Then release stopped for the next 2 days (Figure 3.9.A).

During the same period, release of SS and SM in PBS pH 7.4 was about 5% for each and after the initial release, it stopped in this buffer, too (Figure 3.9.B). By comparing release amounts into acetate pH 5.5 and PBS 7.4, it can be speculated that our loaded liposomes are pH sensitive as they released more SS and SM in the more acidic environment.

One of the future directions of this study is to form a chitosan patch that is embedded with SS, SM loaded liposomes. So, the release pattern of SS and SM from chitosan film needed to be investigated. SS and SM release to acetate pH 5.5 was monitored for 60 hours. Unlike the other samples, the initial burst phase occurred in a shorter time, less than 8 hours. During this period about 18% of SS and 15% of SM was released (Figure 3.9.C). The reason why chitosan released such high amounts of SS and SM faster is that chitosan film contained not only liposomes but also the unencapsulated SM and SS. So the initial burst might came from the unencapsulated drug while the encapsulated drugs have to be released from chitosan first, then they have to be released from liposomes which would take more than 8 hours by taking into consideration the release of SS and SM from liposome only (Figure 3.9.A).

Due to the highly stable nature of our liposomal formulation, complete release of SS and SM took longer than expected. Although stability is one of the factors that caused the slow release, there is one more factor which is release environment. In our release buffers we could only mimic the pH and temperature of the release environment. However as mentioned in introduction, the main mechanism of liposomal release through skin is interaction of lipids of the SC and liposome which leads to disruption of

liposomes. In addition, these liposomes will be applied topically, thus environmental factors like moist air and sunlight, other factors that can destabilize liposomes, were not taken into consideration. In order to mimic all of these factors, another release profiling experiment was conducted. This time 10% methanol was added to release buffer as a destabilizing agent. Release of drug was monitored throughout 24 hours. The initial burst took 4 hours and about 50% of both SS and SM was released which indicated that these liposomes may release more when they are applied to skin. Further studies must be conducted with animals to obtain a more realistic release profile (Figure 3.9.D).

One important question at this point was whether the obtained data were valid or not. The reason behind the low release (5%-20%) might have nothing to do with liposome stability, for example maybe 100 % of the drug was released but 80 % of the drug was degraded during incubation at 37 °C. So, in order to confirm SS and SM stability at 37 °C, we incubated free SS and SM for 7 days at 37 °C. The results showed that both molecules were stable during 7 days of incubation which confirmed our data that showed slow release of SS and SM (Figure 3.9.E).

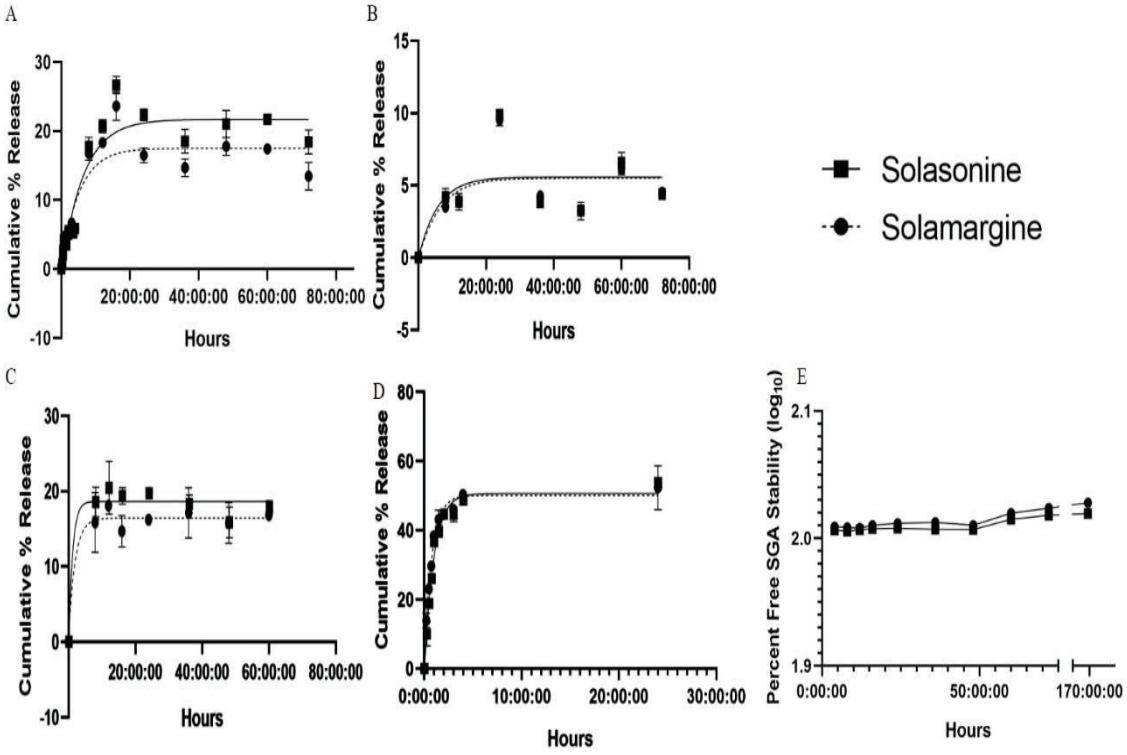

Figure 3.9. Release profile of SS + SM from liposomes (A) to acetate buffer, pH5.5; (B) to PBS, pH7.4; (C) from liposome embedded chitosan film to acetate buffer, pH5.5; (D) to acetate buffer, pH5.5; supplemented with 10% methanol (E) % stability of solasonine and solamargine in their free forms.

3.3. Assessment of Biological Activities of Solasonine and Solamargine

Although SS and SM were tested with many different cell lines previously there were not many studies on skin related cell lines. So, in order to understand the effect of SS and SM on skin, the HaCaT cell line was tested which is an immortalized line of healthy keratinocytes originated from human epidermis. As a diseased line we used SCC-25 which is a squamous cell carcinoma from human tongue. Results indicated that HaCaT cells were more susceptible to SS and SM than SCC-25 cells but, although both cell line are epithelium originated, they still belong to different tissues. So, to reach a conclusion on the effect of SS and SM on healthy and cancerous tissues, both molecules must be tested on a skin cancer line. However it should be taken into account that a previous study in the literature tested SS and SM on the B16-F10 cell line which is skin melanoma cells from *Mus musculus.* This study found IC_{50} values of 24.19 μM for solasonine and 10.15 μM for solamargine which are quite close to the IC_{50} values we detected for SCC-25: 25.83 μM and 9.676 μM, respectively (Figure 3.10). In both of the cell lines examination of IC_{50} values for free SS and free SM suggest that free SM is much more toxic than SS. When applied in combination, SS and SM formed a synergetic effect on SCC-25 cells but not in HaCaT cells (Figure 3.10). When SS and SM are encapsulated, the IC_{50} value rose to 101.1 μM and 182.9 μM for HaCaT and SCC-25 cells, respectively. The reason why the IC_{50} values were so high in encapsulated SS and SM is that, as mentioned in the release profile results, loaded liposomes released only 5% of their content after 20 hours of incubation in PBS pH 7.4 (Figure 3.9.B). The cell culture media, like PBS, have neutral pH, so most of the SS and SM was not expected to be released. In addition, release takes almost 20 hours, so the released SS and SM could not show their effect in less than 24 hours. It is also reported in the literature that the MTT assay shows more viability in liposomal applications. This means that the IC_{50} values of encapsulated SS and SM could have been much lower than were measured. In the light of this knowledge, animal testing must be conducted to observe the actual release profile and biological effects of SS and SM. Also, since SM is much more toxic than SS, in the new formulations the amount of SM can be increased in liposomes. Further cell culture studies must be conducted to reveal the mechanism of action of SS and SM which would be helpful in determining the final formulation of SS and SM loaded liposomes.

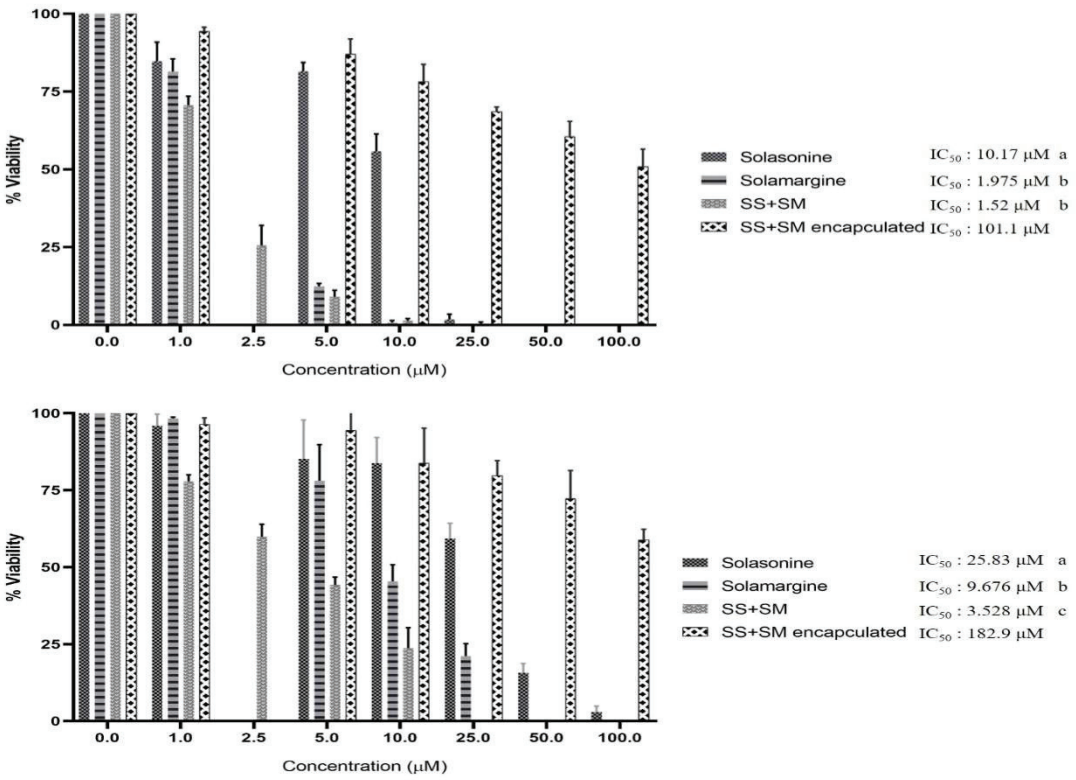

Figure 3.10. Dose dependent Solasonine and Solamargine testing for 24h in (A) Hacat cell line (B) SCC -25 cell line. The letters indicate the significant differences by the multiple t test at $p \leq 0.005$: different letters indicate significant differences between the loaded and unloaded liposomes with respect to surface area and the same letters indicate the absence of differences

3.4. Assessment of Shelf – Life Extending Applications

One of the major struggles in liposome applications is that liposomes have shorter shelf life when compared with polymer-based drug carrier systems. To overcome this issue there are several applications that can extend the shelf-life of liposomes. Among them we tested our liposomes with lyophilization and chitosan film applications.

3.4.1. Lyophilization

As mentioned in the introduction, protecting liposomal stability during storage is one of the most challenging tasks for development of new liposomal formulations. Lyophilization is one of the highly recommended storage applications for liposomes as the method requires removal of all moisture from liposomes which limits chemical

activity of lipids and protects the structure via replacing water molecules between the lipid bilayer with cryoprotectant molecules. In order to determine the optimal lyophilization application, we used different cryoprotectant molecules (Glucose, Sucrose, Maltose, Dextrose) with different cryoprotectant to phospholipid (w/w) ratios (1:8, 1:6, 1:4, 1:2, 1:1, 2:1, 4:1).

The initial particle size measurements of Applichem and Natipide II liposomes were 126.2 nm and 106.7 nm, respectively, and both liposomes had PIs of 0.27, meaning that liposomes were uniform. After lyophilization period, samples were incubated 3 months at -20°C. After 3 month incubation, particle size measurements were done for all cryoprotectants at all concentrations.

The optimal concentration ratio for glucose was 1:4 for Applichem, and 2:1 for Natipide II as particle sizes for these concentrations were 131.8 nm and 96.4 nm, respectively, with PI values of 0.24 and 0.21, respectively. Glucose as cryoprotectant resulted in larger liposomes for Applichem. On the contrary, Natipide II liposomes lyophilized via this cryoprotectant were much smaller than initial liposomes.

Optimal concentration ratio for sucrose were 1:4 for Applichem and 4:1 for Natipide II as particle sizes for these concentrations were 138.6 nm and 107.83 nm, respectively, with PI values of 0.22 and 0.27, respectively. Although sucrose is one of the most used cryoprotectants in the literature, our results showed that it is not the optimal cryoprotectant for our liposomal formulations as both liposomes were larger in size compared to their initial size.

The optimal concentration ratio for maltose were 1:4 for Applichem and 2:1 for Natipide II as particle sizes for these concentrations were 142.07 nm and 103.90 nm, respectively, with PI values of 0.22 and 0.25, respectively. Applichem liposomes were relatively larger than their initial size while Natipide II liposomes were almost the same size.

Finally, the optimal concentration ratio for Dextrose was 1:4 for Applichem and 1:1 for Natipide II as particle sizes for these concentrations were 127.47 nm and 100.43 nm for Natipide II, respectively, with PI values of 0.24 and 0.25, respectively. It was noted that dextrose was the only cryoprotectant that preserved particle size for both liposome groups.

Another remarkable observation in this experiment was that Applichem liposomes were much more stable with lower cryoprotectant to phospholipid ratios while the exact opposite was true for Natipide II liposomes. This situation might be related with the chemical compositions of the lecithins. Applichem A0893 has at least 60% phosphatidylcholine in its formulation while Natipide II has at least 20% of soybean phospholipids of unknown type. In addition, Natipide II has 50% water and around 10-25 % alcohol in its formulation which might be the cause of the higher cryoprotectant requirement for optimal lyophilization process.

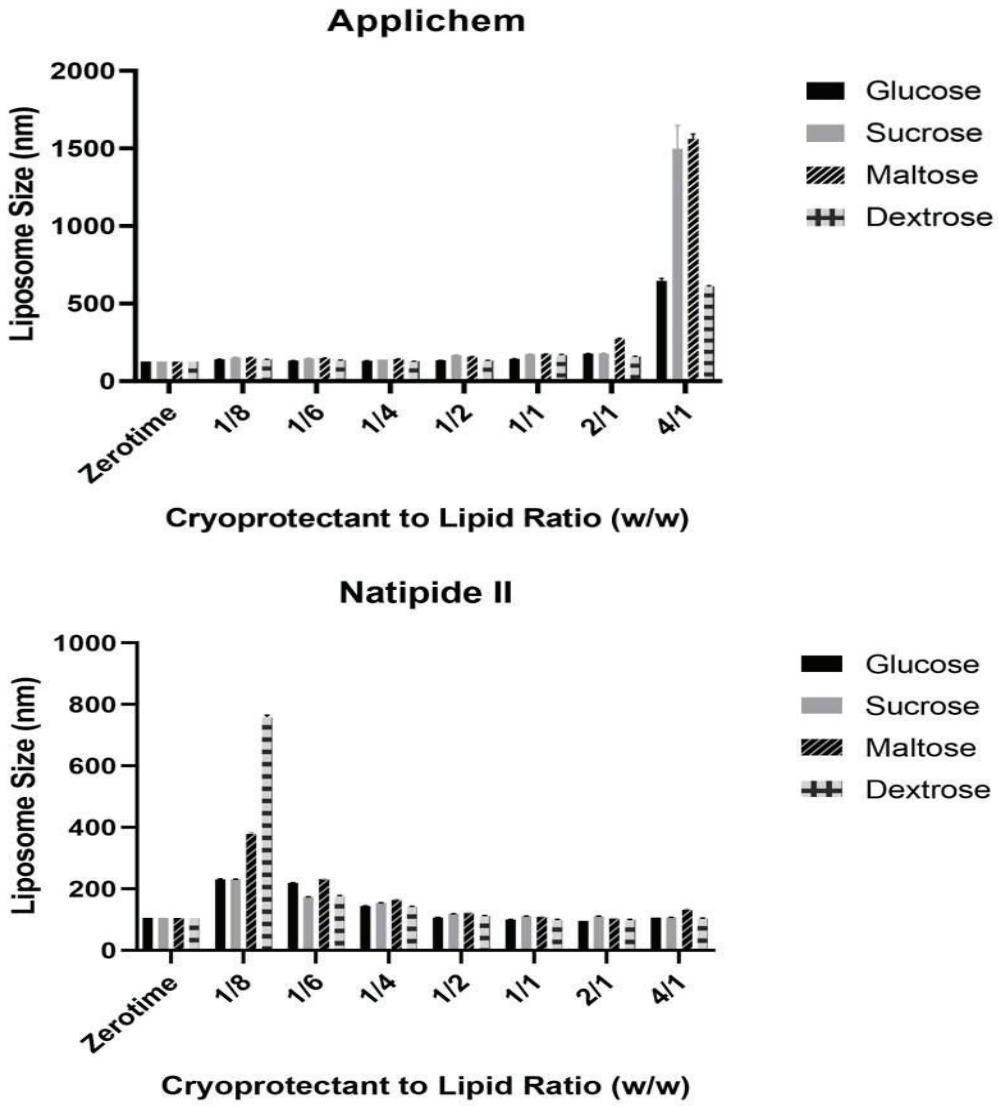

Figure 3.11. Effect of different cryoprotectants and different cryoprotectant to lipid ratio on physical stability of lyophilized liposomes

3.4.2. Chitosan

As a biodegradable material, chitosan offers many solutions to stability issues of liposomes and provides additional benefits like patient friendly usage. In this study we developed a chitosan film formulation and examined its morphology under Scanning Electron Microscopy (SEM). Chitosan film alone, cross-linked chitosan film, and liposome embedded cross-linked chitosan film were examined under SEM. Chitosan film alone had a smooth surface without any surface defects other than pollution that comes from chitosan preparation and damage that was caused by laser beam of SEM (Figure 3.12.A). On the other hand, cross-linked chitosan film had some surface defects like some shrinkage on some parts of the film surface. It was also noted that there were some crystallized structures accumulated on the surface of chitosan filmwhich were probably sodium crystals that might be caused by STTP-NaOH interactions (Figure 3.12.B). Liposome embedded cross-linked chitosan films had similar surface structures with cross-linked chitosan films and the cross-section image from this sample showed that the inside of film was a sponge-like, porous environment. Also, we detected a spherical body in this image that we believed to be a liposome as the size of the body was around 100 – 150 nm which is within the range of sizes of liposomes that we used in this study.

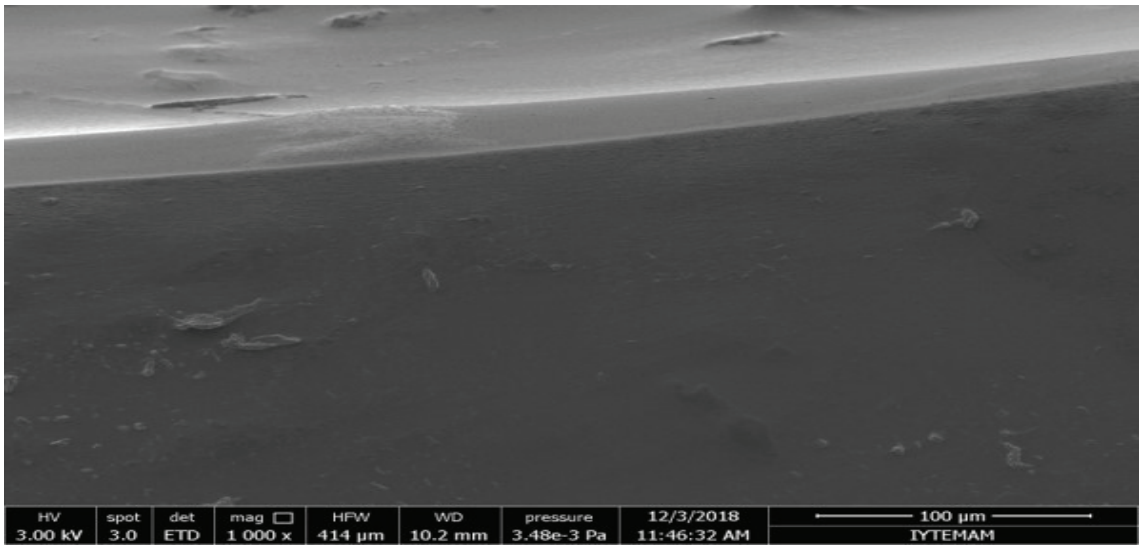

Figure 3.12. SEM image of chitosan film

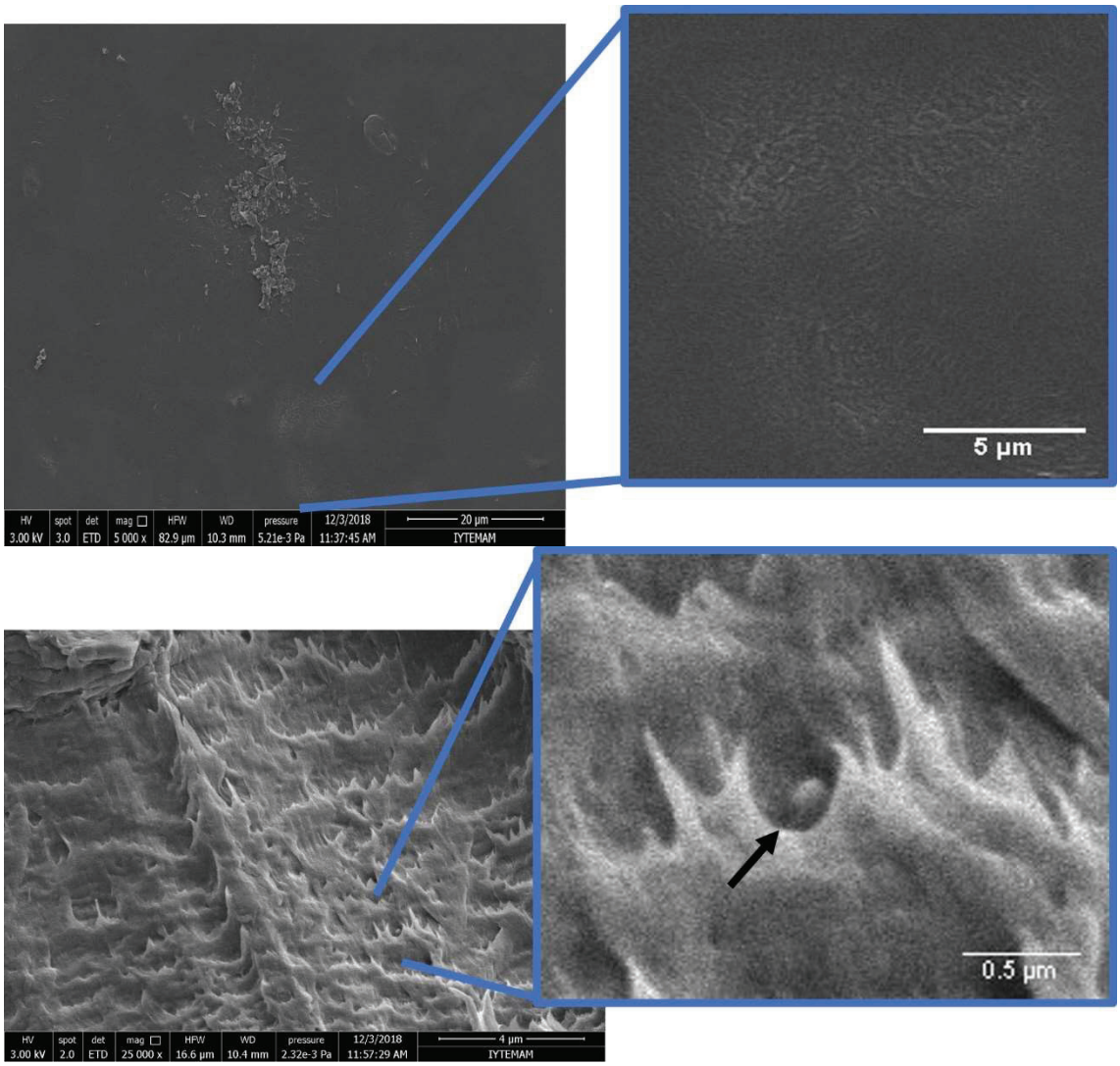

Figure 3.13. SEM images of (A) cross-linked chitosan; (B) liposome embedded cross-linked chitosan

CHAPTER 4

CONCLUSION

Based on the results of this study, the liposomal formulation that was developed with Phospholipon 80H was stable for almost 4 months at 4 °C. In fact, this stability might be a lot longer as we had to finish our observations at the 16th week due to limited time. It was also shown that lyophilization does protect the stability of the liposomes for longer periods, so, by looking at the current physical and chemical stability data we can suggest that the shelf-life of our liposomes can be extended for at least a year or more. The liposomes that were developed in this study are anionic. The formulation can be modified using some cationic phospholipids to increase its targeting towards tumor tissues. The physical and chemical stabilities of SM and SS loaded liposomes did not change much during 3 months of incubation at 4 °C and had quite high encapsulation efficiency with small leakage over three months. Therefore, the formulation developed in this work is promising to carry amphiphilic molecules other than SS and SM. Although the release profile of loaded liposomal formulation was shown in this study, further studies must be conducted with franz diffusion cells and with animal testing to get more accurate release profiles. According to those results, the concentration of SS and SM can be adjusted in the liposome. The MTT results showed the effectiveness of both molecules while solamargine was shown to be more effective than solasonine. Further studies can be conducted with skin melanoma lines or with other skin related diseases like leishmaniasis. According to these results, liposomal formulations that contain solasonine or solamargine individually can be developed for the specific needs of the patient and/or disease. To do so, further cell culture studied must be conducted to find determine the mechanism of action of SS and SM. The chitosan film formulation we developed in this study is promising to create a patch that would contain SM and SS loaded liposomes which would be much more patient friendly and accurate in terms of than applied concentration of drug than topical cream formulations.

REFERENCES

Akter, R., Uddin, S. J., Tiralongo, J., Grice, I. D., & Tiralongo, E. (2015). A New Cytotoxic Steroidal Glycoalkaloid from the Methanol Extract of Blumea lacera Leaves. J Pharm Pharm Sci, 18(4), 616-633.

Alam, M., Zubair, S., Farazuddin, M., Ahmad, E., Khan, A., Zia, Q., Mohammad, O. (2013). Development, characterization and efficacy of niosomal diallyl disulfide in treatment of disseminated murine candidiasis. Nanomedicine, 9(2), 247-256. doi:10.1016/j.nano.2012.07.004

Albanese, A., Tang, P. S., & Chan, W. C. (2012). The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng, 14, 1-16. doi:10.1146/annurev-bioeng-071811-150124

Albasarah, Y. Y., Somavarapu, S., Stapleton, P., & Taylor, K. M. (2010). Chitosan-coated antifungal formulations for nebulisation. J Pharm Pharmacol, 62(7), 821-828. doi:10.1211/jpp.62.05.0002

Alexander, A., Dwivedi, S., Ajazuddin, Giri, T. K., Saraf, S., Saraf, S., & Tripathi, D. K. (2012). Approaches for breaking the barriers of drug permeation through transdermal drug delivery. Journal of Controlled Release, 164(1), 26-40. doi:10.1016/j.jconrel.2012.09.017

Ali Kayani, S., Masood, A., Achakzai, A., & Anbreen, S. (2007). Distribution of secondary metabolites in plants of Quetta-Balochistan (Vol. 39).

Amoabediny, G., Haghiralsadat, F., Naderinezhad, S., Helder, M. N., Kharanaghi, E. A., Arough, J. M., & Zandieh-Doulabi, B. (2018). Overview of preparation methods of polymeric and lipid-based (niosome, solid lipid, liposome) nanoparticles: A comprehensive review. International Journal of Polymeric Materials and Polymeric Biomaterials, 67(6), 383-400. doi:10.1080/00914037.2017.1332623

Archer, C. B. (2010). Functions of the Skin. In S. B. T. Burns, N. Cox and C. Griffiths (Ed.), Rook's Textbook of Dermatology (pp. 1-11).

Baroli, B., Ennas, M. G., Loffredo, F., Isola, M., Pinna, R., & Lopez-Quintela, M. A. (2007). Penetration of metallic nanoparticles in human full-thickness skin. J Invest Dermatol, 127(7), 1701-1712. doi:10.1038/sj.jid.5700733

Betz, G., Imboden, R., & Imanidis, G. (2001). Interaction of liposome formulations with human skin in vitro. Int J Pharm, 229(1-2), 117-129.

Blessing, T., Kursa, M., Holzhauser, R., Kircheis, R., & Wagner, E. (2001). Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. Bioconjug Chem, 12(4), 529-537.

Bolzinger, M.-A., Briançon, S., Pelletier, J., & Chevalier, Y. (2012). Penetration of drugs through skin, a complex rate-controlling membrane. Current Opinion in Colloid & Interface Science, 17(3), 156-165. doi:https://doi.org/10.1016/j.cocis.2012.02.001

Bouwstra, J. A., Hofland, H. E. J., Spies, F., Gooris, G. S., & Junginger, H. E. (1992, 1992//). Changes in the Structure of the Human Stratum Corneum Induced by Liposomes. Paper presented at the Liposome Dermatics, Berlin, Heidelberg.

Burger, T., Mokoka, T., Fouche, G., Steenkamp, P., Steenkamp, V., & Cordier, W. (2018). Solamargine, a bioactive steroidal alkaloid isolated from Solanum aculeastrum induces non-selective cytotoxicity and P-glycoprotein inhibition. BMC Complement Altern Med, 18(1), 137. doi:10.1186/s12906-018-2208-7

Couvreur, P., & Vauthier, C. (2006). Nanotechnology: Intelligent design to treat complex disease. Pharmaceutical Research, 23(7), 1417-1450. doi:10.1007/s11095-006-0284-8

Danaei, M., Kalantari, M., Raji, M., Fekri, H. S., Saber, R., Asnani, G. P., Taheriazam, A. (2018). Probing nanoliposomes using single particle analytical techniques: effect of excipients, solvents, phase transition and zeta potential. Heliyon, 4(12). doi:10.1016/j.heliyon.2018.e01088

Davis, S. S. (1997). Biomedical applications of nanotechnology--implications for drug targeting and gene therapy. Trends Biotechnol, 15(6), 217-224. doi:10.1016/S0167-7799(97)01036-6

Dinan, L., Harmatha, J., & Lafont, R. (2001). Chromatographic procedures for the isolation of plant steroids. J Chromatogr A, 935(1-2), 105-123.

Dufes, C., Schatzlein, A. G., Tetley, L., Gray, A. I., Watson, D. G., Olivier, J. C.. Uchegbu, I. F. (2000). Niosomes and polymeric chitosan based vesicles bearing transferrin and glucose ligands for drug targeting. Pharmaceutical Research, 17(10), 1250-1258. doi:Doi 10.1023/A:1026422915326

El-Nesr, O. H., Yahiya, S. A., & El-Gazayerly, O. N. (2010). Effect of formulation design and freeze-drying on properties of fluconazole multilamellar liposomes. Saudi Pharm J, 18(4), 217-224. doi:10.1016/j.jsps.2010.07.003

El Maghraby, G. M., Barry, B. W., & Williams, A. C. (2008). Liposomes and skin: from drug delivery to model membranes. Eur J Pharm Sci, 34(4-5), 203-222. doi:10.1016/j.ejps.2008.05.002

Ganta, S., Devalapally, H., Shahiwala, A., & Amiji, M. (2008). A review of stimuli-responsive nanocarriers for drug and gene delivery. Journal of Controlled Release, 126(3), 187-204. doi:10.1016/j.jconrel.2007.12.017

Gerweck, L. E., & Seetharaman, K. (1996). Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. Cancer Res, 56(6), 1194-1198.

Ghazanfar, S. A., & Al-Al-Sabahi, A. M. (1993). Medicinal plants of Northern and Central Oman (Arabia). Economic Botany, 47(1), 89-98. doi:10.1007/BF02862209

Goldberg, L. H., Landau, J. M., Moody, M. N., & Vergilis-Kalner, I. J. (2011). Treatment of Bowen's disease on the penis with low concentration of a standard mixture of solasodine glycosides and liquid nitrogen. Dermatol Surg, 37(6), 858-861. doi:10.1111/j.1524-4725.2011.02014.x

Gregoriadis, G. (1976a). The carrier potential of liposomes in biology and medicine (first of two parts). N Engl J Med, 295(13), 704-710. doi:10.1056/NEJM197609232951305

Gregoriadis, G. (1976b). The carrier potential of liposomes in biology and medicine (second of two parts). N Engl J Med, 295(14), 765-770. doi:10.1056/NEJM197609302951406

Gu, X. Y., Shen, X. F., Wang, L., Wu, Z. W., Li, F., Chen, B., . . . Wang, M. K. (2018). Bioactive steroidal alkaloids from the fruits of Solanum nigrum. Phytochemistry, 147, 125-131. doi:10.1016/j.phytochem.2017.12.020

Hatakeyama, H., Akita, H., Kogure, K., Oishi, M., Nagasaki, Y., Kihira, Y., Harashima, H. (2007). Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. Gene Ther, 14(1), 68-77. doi:10.1038/sj.gt.3302843

Hayden, C. G., Cross, S. E., Anderson, C., Saunders, N. A., & Roberts, M. S. (2005). Sunscreen penetration of human skin and related keratinocyte toxicity after topical application. Skin Pharmacol Physiol, 18(4), 170-174. doi:10.1159/000085861

Hochman, D., & Wolff, B. (2006). Risk of serious infections and malignancies with anti-TNF antibody therapy in rheumatoid arthritis. Jama-Journal of the

American Medical Association, 296(18), 2203-2203. doi:DOI 10.1001/jama.296.18.2203-a

Honary, S., & Zahir, F. (2013). Effect of Zeta Potential on the Properties of Nano-Drug Delivery Systems - A Review (Part 2). Tropical Journal of Pharmaceutical Research, 12(2), 265-273. doi:10.4314/tjpr.v12i2.20

Jadhav, S. J., Sharma, R. P., & Salunkhe, D. K. (1981). Naturally Occurring Toxic Alkaloids in Foods. CRC Critical Reviews in Toxicology, 9(1), 21-104. doi:10.3109/10408448109059562

Jahn, A., Vreeland, W. N., Gaitan, M., & Locascio, L. E. (2004). Controlled vesicle self-assembly in microfluidic channels with hydrodynamic focusing. J Am Chem Soc, 126(9), 2674-2675. doi:10.1021/ja0318030

Junyaprasert, V. B., Singhsa, P., Suksiriworapong, J., & Chantasart, D. (2012). Physicochemical properties and skin permeation of Span 60/Tween 60 niosomes of ellagic acid. Int J Pharm, 423(2), 303-311. doi:10.1016/j.ijpharm.2011.11.032

Kang, L., Yap, C. W., Lim, P. F., Chen, Y. Z., Ho, P. C., Chan, Y. W., Chan, S. Y. (2007). Formulation development of transdermal dosage forms: quantitative structure-activity relationship model for predicting activities of terpenes that enhance drug penetration through human skin. Journal of Controlled Release, 120(3), 211-219. doi:10.1016/j.jconrel.2007.05.006

Kawabata, Y., Wada, K., Nakatani, M., Yamada, S., & Onoue, S. (2011). Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: Basic approaches and practical applications. International Journal of Pharmaceutics, 420(1), 1-10. doi:10.1016/j.ijpharm.2011.08.032

Kennedy, D. O., & Wightman, E. L. (2011). Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. Adv Nutr, 2(1), 32-50. doi:10.3945/an.110.000117

Kirpotin, D. B., Drummond, D. C., Shao, Y., Shalaby, M. R., Hong, K. L., Nielsen, U. B., Park, J. W. (2006). Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Research, 66(13), 6732-6740. doi:10.1158/0008-5472.Can-05-4199

Knorr, F., Lademann, J., Patzelt, A., Sterry, W., Blume-Peytavi, U., & Vogt, A. (2009). Follicular transport route--research progress and future perspectives. Eur J Pharm Biopharm, 71(2), 173-180. doi:10.1016/j.ejpb.2008.11.001

Lane, M. E. (2013). Skin penetration enhancers. Int J Pharm, 447(1-2), 12-21. doi:10.1016/j.ijpharm.2013.02.040

Lezama-Davila, C. M., McChesney, J. D., Bastos, J. K., Miranda, M. A., Tiossi, R. F., da Costa Jde, C., . . . Isaac-Marquez, A. P. (2016). A New Antileishmanial Preparation of Combined Solamargine and Solasonine Heals Cutaneous Leishmaniasis through Different Immunochemical Pathways. Antimicrob Agents Chemother, 60(5), 2732-2738. doi:10.1128/AAC.02804-15

Maeda, H., & Matsumura, Y. (1989). Tumoritropic and lymphotropic principles of macromolecular drugs. Crit Rev Ther Drug Carrier Syst, 6(3), 193-210.

Manase, M. J., Mitaine-Offer, A. C., Pertuit, D., Miyamoto, T., Tanaka, C., Delemasure, S., . . . Lacaille-Dubois, M. A. (2012). Solanum incanum and S. heteracanthum as sources of biologically active steroid glycosides: confirmation of their synonymy. Fitoterapia, 83(6), 1115-1119. doi:10.1016/j.fitote.2012.04.024

Maurya, A., Gupta, S., Negi, S., & Srivastava, S. K. (2009). pH-Zone-refining centrifugal partition chromatography for preparative isolation and purification of steroidal glycoalkaloids from Solanum xanthocarpum. J Sep Sci, 32(18), 3126-3132. doi:10.1002/jssc.200900323

Mazid, M., Khan, T. A., & Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants (Vol. 3).

McPhail, D., Tetley, L., Dufes, C., & Uchegbu, I. F. (2000). Liposomes encapsulating polymeric chitosan based vesicles--a vesicle in vesicle system for drug delivery. Int J Pharm, 200(1), 73-86.

Mengoni, T., Adrian, M., Pereira, S., Santos-Carballal, B., Kaiser, M., & Goycoolea, F. M. (2017). A Chitosan-Based Liposome Formulation Enhances the In Vitro Wound Healing Efficacy of Substance P Neuropeptide. Pharmaceutics, 9(4). doi:10.3390/pharmaceutics9040056

Meyer, D. E., Shin, B. C., Kong, G. A., Dewhirst, M. W., & Chilkoti, A. (2001). Drug targeting using thermally responsive polymers and local hyperthermia. Journal of Controlled Release, 74(1-3), 213-224. doi:Doi 10.1016/S0168-3659(01)00319-4

Moghimi, S. M., Hunter, A. C., & Murray, J. C. (2005). Nanomedicine: current status and future prospects. FASEB J, 19(3), 311-330. doi:10.1096/fj.04-2747rev

Mozafari, M. (2005). A new technique for the preparation of non-toxic liposomes and nanoliposomes: The heating method (pp. 91-98.).

Mozafari, M. R. (2010). Nanoliposomes: preparation and analysis. Methods Mol Biol, 605, 29-50. doi:10.1007/978-1-60327-360-2_2

Munari, C. C., de Oliveira, P. F., Campos, J. C., Martins Sde, P., Da Costa, J. C., Bastos, J. K., & Tavares, D. C. (2014). Antiproliferative activity of Solanum lycocarpum alkaloidic extract and their constituents, solamargine and solasonine, in tumor cell lines. J Nat Med, 68(1), 236-241. doi:10.1007/s11418-013-0757-0

Nasir, A., Kausar, A., & Younus, A. (2015). A Review on Preparation, Properties and Applications of Polymeric Nanoparticle-Based Materials. Polymer-Plastics Technology and Engineering, 54(4), 325-341. doi:10.1080/03602559.2014.958780

Oesch, F., Fabian, E., Oesch-Bartlomowicz, B., Werner, C., & Landsiedel, R. (2007). Drug-metabolizing enzymes in the skin of man, rat, and pig. Drug Metab Rev, 39(4), 659-698. doi:10.1080/03602530701690366

Ohtsuka, N., Konno, T., Miyauchi, Y., & Maeda, H. (1987). Anticancer effects of arterial administration of the anticancer agent SMANCS with lipiodol on metastatic lymph nodes. Cancer, 59(9), 1560-1565.

Paolino, D., Celia, C., Trapasso, E., Cilurzo, F., & Fresta, M. (2012). Paclitaxel-loaded ethosomes(R): potential treatment of squamous cell carcinoma, a malignant transformation of actinic keratoses. Eur J Pharm Biopharm, 81(1), 102-112. doi:10.1016/j.ejpb.2012.02.008

Porter, C. J. H., Trevaskis, N. L., & Charman, W. N. (2007). Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nature Reviews Drug Discovery, 6(3), 231-248. doi:10.1038/nrd2197

Riviere, J. E., & Papich, M. G. (2001). Potential and problems of developing transdermal patches for veterinary applications. Adv Drug Deliv Rev, 50(3), 175-203.

Saito, G., Swanson, J. A., & Lee, K. D. (2003). Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. Adv Drug Deliv Rev, 55(2), 199-215.

Sankar, V., Ruckmani, K., Durga, S., & Jailani, S. (2010). Proniosomes as drug carriers. Pak J Pharm Sci, 23(1), 103-107.

Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., & Hua, S. (2015). Advances and Challenges of Liposome Assisted Drug Delivery. Front Pharmacol, 6, 286. doi:10.3389/fphar.2015.00286

Shenoy, D., Little, S., Langer, R., & Amiji, M. (2005). Poly(ethylene oxide)-modified poly(beta-amino ester) nanoparticles as a pH-sensitive system for tumor-targeted delivery of hydrophobic drugs: part 2. In vivo distribution and tumor localization studies. Pharm Res, 22(12), 2107-2114. doi:10.1007/s11095-005-8343-0

Smith, C. E., & Kong, H. (2014). Cross-linkable liposomes stabilize a magnetic resonance contrast-enhancing polymeric fastener. Langmuir, 30(13), 3697-3704. doi:10.1021/la500412r

Sultana, S., Perwaiz, S., Iqbal, M., & Athar, M. (1995). Crude extracts of hepatoprotective plants, Solanum nigrum and Cichorium intybus inhibit free radical-mediated DNA damage. Journal of Ethnopharmacology, 45(3), 189-192. doi:https://doi.org/10.1016/0378-8741(94)01214-K

Szoka, F., Jr., & Papahadjopoulos, D. (1980). Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu Rev Biophys Bioeng, 9, 467-508. doi:10.1146/annurev.bb.09.060180.002343

Tavano, L., Muzzalupo, R., Picci, N., & de Cindio, B. (2014). Co-encapsulation of antioxidants into niosomal carriers: gastrointestinal release studies for nutraceutical applications. Colloids Surf B Biointerfaces, 114, 82-88. doi:10.1016/j.colsurfb.2013.09.058

Tibbitt, M. W., Dahlman, J. E., & Langer, R. (2016). Emerging Frontiers in Drug Delivery. J Am Chem Soc, 138(3), 704-717. doi:10.1021/jacs.5b09974

Tiossi, R. F. J., Miranda, M. A., de Sousa, J. P. B., Praça, F. S. G., Bentley, M. V. L. B., McChesney, J. D., & Bastos, J. K. (2012). A Validated Reverse Phase HPLC Analytical Method for Quantitation of Glycoalkaloids in Solanum lycocarpum and Its Extracts. Journal of Analytical Methods in Chemistry, 2012, 8. doi:10.1155/2012/947836

Torchilin, V. P. (2006). Multifunctional nanocarriers. Adv Drug Deliv Rev, 58(14), 1532-1555. doi:10.1016/j.addr.2006.09.009

Torchilin, V. P. (2007). Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J, 9(2), E128-147. doi:10.1208/aapsj0902015

Tran, S., DeGiovanni, P. J., Piel, B., & Rai, P. (2017). Cancer nanomedicine: a review of recent success in drug delivery. Clinical and Translational Medicine, 6. doi:10.1186/s40169-017-0175-0

Vahed, S. Z., Salehi, R., Davaran, S., & Sharifi, S. (2017). Liposome-based drug co-delivery systems in cancer cells. Materials Science & Engineering C-Materials for Biological Applications, 71, 1327-1341. doi:10.1016/j.msec.2016.11.073

van Vlerken, L. E., Duan, Z., Seiden, M. V., & Amiji, M. M. (2007). Modulation of intracellular ceramide using polymeric nanoparticles to overcome multidrug resistance in cancer. Cancer Res, 67(10), 4843-4850. doi:10.1158/0008-5472.CAN-06-1648

Vaupel, P., Kallinowski, F., & Okunieff, P. (1989). Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res, 49(23), 6449-6465.

Verma, S. S., S. K.; Syan, N.; Mathur, P.; Valeche, V. (2010). Nanoparticle vesicular systems: A versatile tool for drug delivery. J. Chem. Pharm. Res., 2, 496–509.

Wang, L., Shi, C., Wright, F. A., Guo, D., Wang, X., Wang, D., . . . Luo, J. (2017). Multifunctional Telodendrimer Nanocarriers Restore Synergy of Bortezomib and Doxorubicin in Ovarian Cancer Treatment. Cancer Res, 77(12), 3293-3305. doi:10.1158/0008-5472.CAN-16-3119

Wang, X., Zou, S., Lan, Y. L., Xing, J. S., Lan, X. Q., & Zhang, B. (2017). Solasonine inhibits glioma growth through anti-inflammatory pathways. Am J Transl Res, 9(9), 3977-3989.

Weber, S., Zimmer, A., & Pardeike, J. (2014). Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC) for pulmonary application: a review of the state of the art. Eur J Pharm Biopharm, 86(1), 7-22. doi:10.1016/j.ejpb.2013.08.013

Wedmore, I., McManus, J. G., Pusateri, A. E., & Holcomb, J. B. (2006). A special report on the chitosan-based hemostatic dressing: experience in current combat operations. J Trauma, 60(3), 655-658. doi:10.1097/01.ta.0000199392.91772.44

Weissenberg, M., Levy, A., Svoboda, J. A., & Ishaaya, I. (1998). The effect of some Solanum steroidal alkaloids and glycoalkaloids on larvae of the red flour beetle, Tribolium castaneum, and the tobacco hornworm, Manduca sexta. Phytochemistry, 47(2), 203-209. doi:https://doi.org/10.1016/S0031-9422(97)00565-7

Yang, Y., Wang, S., Wang, Y., Wang, X., Wang, Q., & Chen, M. (2014). Advances in self-assembled chitosan nanomaterials for drug delivery. Biotechnol Adv, 32(7), 1301-1316. doi:10.1016/j.biotechadv.2014.07.007

Zhai, Y., & Zhai, G. (2014). Advances in lipid-based colloid systems as drug carrier for topic delivery. Journal of Controlled Release, 193, 90-99. doi:10.1016/j.jconrel.2014.05.054

Zhang, Y. J., Sun, T., & Jiang, C. (2018). Biomacromolecules as carriers in drug delivery and tissue engineering. Acta Pharmaceutica Sinica B, 8(1), 34-50. doi:10.1016/j.apsb.2017.11.005