UV RESPONSIVE DRUG DELIVERY FROM SUPROFEN INCORPORATED LIPOSOMES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BİRSEN DEMİRBAĞ

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

SEPTEMBER 2011

Approval of the thesis:

UV RESPONSIVE DRUG DELIVERY FROM SUPROFEN INCORPORATED LIPOSOMES

submitted by **BiRSEN DEMiRBAĞ** in partial fulfillment of the requirements for the degree of **Master of Science in Department of Biotechnology, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu _____
Head of Department, **Biotechnology**

Prof. Dr. Vasıf Hasırcı _____
Supervisor, **Biological Sciences Dept., METU**

Prof. Dr. Nesrin Hasırcı _____
Co-Supervisor, **Chemistry Dept., METU**

Examining Committee Members:

Prof. Dr. Mesude İşcan _____
Biological Sciences Dept., METU

Prof. Dr. Vasıf Hasırcı _____
Biological Sciences Dept., METU

Assist. Prof. Dr. Sreeparna Banerjee _____
Chemistry Dept., METU

Assist. Prof. Dr. İrem Erel _____
Molecular Biology and Genetics Dept., METU

Assist. Prof. Fatma Neşe Kök _____
Molecular Biol. And Genetics Dept., ITU

Date: 16.09.2011

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Birsen Demirbağ

Signature :

ABSTRACT

UV REPONSIVE DRUG DELIVERY FROM SUPROFEN INCORPORATED LIPOSOMES

Demirbağ, Birsen
M.Sc., Department of Biotechnology
Supervisor: Prof. Dr. Vasıf Hasırcı
Co-Supervisor: Prof. Dr. Nesrin Hasırcı

September 2011, 78 pages

Drug delivery systems are designed to achieve low, local doses at the target site. Delivery systems can provide the drug in a continuous manner or in response to environmental stimuli such as temperature, pH or UV.

This study aimed to develop photosensitive liposomes that achieve UV-responsive release of their content. The main mechanism was to incorporate a light sensitive molecule into the liposomal bilayer then achieve destabilization of the membrane by exposure to UV. This would result in an on demand release of the bioactive content. Suprofen, a nonstereoidal anti-inflammatory drug, also a light sensitive molecule, was selected to achieve the destabilization in this study. Lipid vesicles were prepared with different ratios of phosphatidyl choline, cholesterol and Suprofen (PC:CHOL:SPF) and characterized in terms of encapsulation efficiency, release rate and responsiveness to UV. Preliminary studies were carried out with calcein (CAL), a fluorescent dye, due to the ease of detection and the *in vitro* studies were carried out with the cancer drug Cisplatin.

Keywords: Responsive drug delivery, Cisplatin, Suprofen, Liposome

ÖZ

SUPROFEN İÇEREN LİPOZOMLARDA IŞIK KONTROLLÜ İLAÇ SALIMI

Demirbağ, Birsen
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Vasıf Hasırcı
Ortak Tez Yöneticisi: Prof. Dr. Nesrin Hasırcı

Eylül 2011, 78 sayfa

İlaç salım sistemleri ilacın hedef bölgede düşük bölgesel dozda salınmasını sağlamak için tasarlanır. İlaç salım sistemleri ilacın sürekli salımını sağlayabildikleri gibi uyarana duyarlı (sıcaklık değişimi, pH değişimi ya da UV) salınmasını da sağlarlar.

Bu çalışma ışığa duyarlı lipozomlardan UV ışığı kontrollü ilaç salım sistemi geliştirmeyi amaçlamıştır. Model, ışık duyarlı bir molekülün lipid çeperin içine dahil edilmesini kapsar. Böylece UV ışığına maruz bırakılması kesecik çeperinin bütünlüğünün bozulmasına ve içeriğinin ortama salınmasına neden olur. Bu biyoaktif maddenin isteğe bağlı salımını sağlar. Suprofen, bir steroid olmayan anti inflamatuvar ilaç olmasının yanında, ışığa duyarlı bir moleküldür ve bu çalışmada UV ışığına maruz bırakılınca çeper bütünlüğünü bozması için seçilmiştir. Lipid kesecikler fosfatidilkolin, kolesterol ve Suprofen (PC:CHOL:SPF) içeriklerinin değişik konsantrasyonlarında hazırlanmış ve kapsüllenme randımanı, salım değeri ve UV ışığına duyarlılık açısından değerlendirilmiştir. İlk çalışmalar saptama kolaylığı açısından bir floresan boya olan kalseinle yapılmıştır ancak in vitro çalışmalar bir kanser ilacı olan Cisplatinle yapılmıştır.

Anahtar kelimeler: Duyarlı ilaç salımı, Cisplatin, Suprofen, Lipozom

Dedicated to my family

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Vasıf HASIRCI for his continuous guidance, advice, support, encouragement and insight throughout the research.

I am also indebted to my co-supervisor Prof. Dr. Nesrin HASIRCI for her support and guidance.

I am especially grateful to Arda BÜYÜKSUNGUR for his valuable advices, his guidance, patience, support and friendship throughout the research.

I would like to express my special thanks to Dr. Pınar YILGÖR HURİ for her guidance, continuous help and friendship during the time we shared in BIOMAT.

I am grateful to my special lab partners and friends Sinem KARDEŞLER, Özge KARADAŞ, and Gökhan BAHÇECİOĞLU for always being with me and their continuous support.

I am also grateful to my lab partners and special friends Gizem ALTAY and Aysu KÜÇÜKTURHAN for their support, friendship and sharings.

I would like to thank all of the members of METU-BIOMAT group and my labmates Dr. Pınar ZORLUTUNA, Dr. Albana NDREU, Hayriye ÖZÇELİK, Aysel KIZILTAY, Tuğba ENDOĞAN, Dr. Beste KINIKOĞLU, Dr. Erkin AYDIN, Dr. Deniz YÜCEL, Dr. Halime KENAR, Cansel IŞIKLI, Elif VARDAR, Damla ARSLANTUNALI, Gözde EKE, Bilgenur KANDEMİR, Menekşe ERMİŞ, Cemile KILIÇ, A. Selcen ALAGÖZ, Sepren ÖNCÜ, Aylin ACUN, Tuğba DURSUN and our technician Zeynel AKIN for their support in this study.

I am also grateful to my very close friends Elif ATICI and Onur GÜLFİDANGİL for “always being there for me”.

I would also like to thank Serap SONGUN, Yurdaer SONGUN, Elif SONGUN and Efe SONGUN for being a family to me in Ankara.

Finally, I would like to express my deepest gratitude to my parents Aysun DEMİRBAĞ and Orhan DEMİRBAĞ for their understanding, endless help and support, their patience and trust in me, not only for this study, but for all my life. I would like to express special thanks to my brother Cem DEMİRBAĞ, who makes my life a better place. I would also like to thank to other members of my family for their support during this research.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

CAL	Calcein
CHOL	Cholesterol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MWCO	Molecular Weight Cut Off
PBS	Phosphate Buffer Saline
SPF	Suprofen
Pen/Strep	Penicillin/Streptomycin
PC	Phosphatidyl choline
TCPS	Tissue Culture Polystyrene

CHAPTER 1

INTRODUCTION

1.1. Drug delivery systems

Drug delivery is administration of a therapeutic agent to the body for treatment of a condition. There are a variety of administration routes including oral, topical, transmucosal, inhalation, and injection. These routes are fast and easy to apply. However, there are drawbacks such as difficulties in regulating the dose of the drug (especially in inhalation and topical delivery), systemic toxicity (especially in injection), and nonspecific application of the drug.

Drug delivery systems have been developed to overcome the problems encountered in administration of the drugs. Systemic toxicity of the drugs and inactivation or degradation of the drugs in the circulation system are the most common problems of drug administration. In addition, nonspecific therapeutic applications and fluctuating drug concentration after administration (Figure 1) also lead researchers to develop delivery systems.

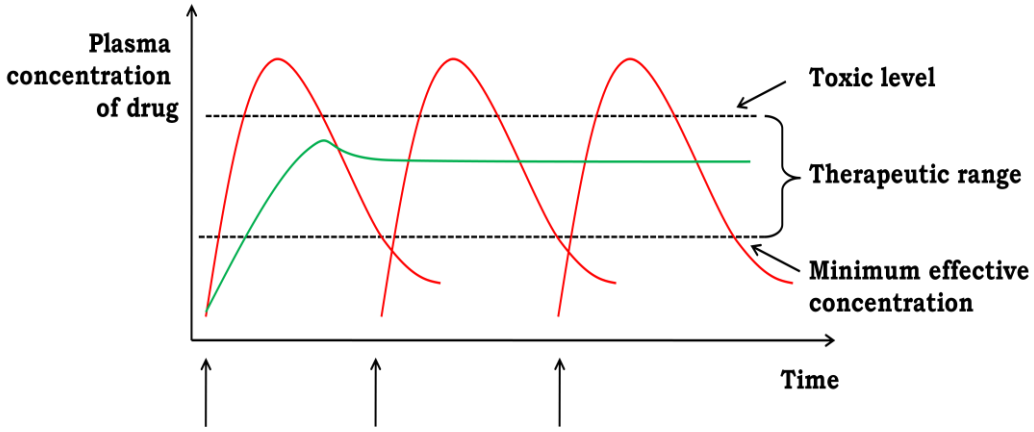


Figure 1. Drug concentration in plasma in drug delivery. Red plot presents conventional delivery and green plot presents drug delivery systems. Arrows indicate administrations.

In conventional drug delivery, drug concentration in the plasma increases beyond the therapeutic level soon after administration, and then drops to an ineffective level and below until the next administration. In order to keep the concentration in the therapeutic range, increasing the frequency of drug administration or increasing the dose of the drug could be attempted. However, this raises the problems of high drug toxicity and high cost of the treatment (Pierigè *et al.*, 2008).

Drug delivery systems allow the administration of the drug for an extended time at a therapeutic level during the treatment (Figure 1). These systems may obey the Zero order, First order or Higuchi kinetics for the delivery of the drugs. Conventional delivery mechanisms generally follow the First order release kinetics while the ideal delivery system is defined as a system that maintains constant drug concentration in plasma, which is represented by Zero order (as observed in Figure 1). Mathematical presentation of the release models are presented in Table 1. (Vemuri and Rhodes, 1995, Hughes, 2005)

Table 1. Mathematical models for release kinetics

Release Kinetic model	Mathematical formula
Zero order	$D_t = k_0 t$
First order	$\ln D_t = \ln D_0 - k_1 t$
Higuchi	$D_t = k_H t^{1/2}$
Hixson-Crowell	$D_t^{1/3} = D_0^{1/3} - k_{HC} t$

Adapted from Dash, *et al.*, 2010

In Table 1, D_t is the amount of drug released at time t, D_0 is the initial amount in the system and k_0 is Zero Order, k_1 is First Order, k_H is Higuchi and k_{HC} is the Hixson-Crowell release constants, respectively.

The Zero Order rate describes a release mechanism where the drug release rate is independent of its concentration (Hadjiioannou *et al.*, 1993). The first order shows a concentration dependent release rate (Bourne, 2002). Higuchi release rate is described as the release of drugs from insoluble matrices as a function of square root of time. The Hixson-Crowell cube root law describes the release from systems where there is a change in surface area and diameter of particles or tablets (Hixson and Crowell, 1931).

Any system developed for administration of drugs is expected to protect the host from the toxic effects of the drug in addition to protect the drug from inactivation or degradation within the circulatory system. Moreover, these systems could be designed to be responsive or targeted to achieve high local concentrations of the drug. In other words, these systems are developed to enable safe and focused administration of the drugs preferably to a particular tissue for a predetermined duration and rate.

Drug delivery research is focused on developing the most appropriate approaches, materials and forms of administration of the specific drug to the specific target (Wang and Thanou, 2010, Brewer *et al.*, 2011, Basel *et al.*, 2011).

1.2. Nanoparticles in drug delivery systems

Nanoparticles are defined as the particles of size between 1 and 100 nm and these particles are designed to be used in many areas such as electronics (Wolff and Hilleringmann, 2011), forensic science (Jessirie *et al.*, 2011), textiles (Montazer *et al.*, 2011), agriculture (Choudhury *et al.*, 2010), etc. Nanoparticles are also important tools for biotechnology (nanobiotechnology) especially in therapeutic applications. They can be used in imaging (Tu *et al.*, 2011), as carriers of bioactive agents embedded in scaffolds in tissue engineering (Yilgor *et al.*, 2009), for delivery of genes (Huang *et al.*, 2011) and growth factors (Basmanav *et al.*, 2008) and other agents (Vashist *et al.*, 2011). Improved bioavailability, solubility and retention time are among the characteristics that nanoparticles impart to the bioactive agents (Kumari *et al.*, 2010). When the drug is encapsulated in nanoparticles, it is protected from inactivation until it is released meanwhile organism is also protected from the toxic effects of the drug. Moreover, nanoparticles can be designed to deliver drugs in a targeted, controlled or responsive manner, and high local doses of the drug can be achieved. (Alexis *et al.*, 2008).

Nanoparticles can be in the form of tubes, wires, fibers, spheres, capsules, or vesicles depending on their applications (Demirbag *et al.*, 2011). Figure 2 is a schematic presentation of all these nanoparticle systems.

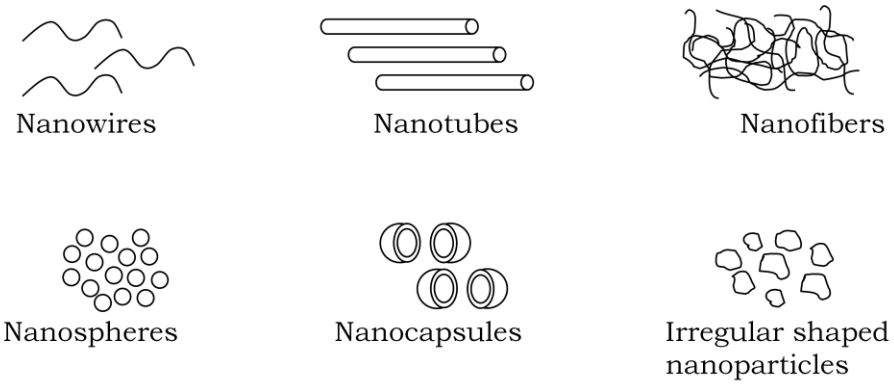


Figure 2. Forms of nanoparticles

Nanoparticles can be prepared using a variety of methods and materials. Among the materials are polymers, lipids, and inorganics.

1.2.1. Polymeric nanoparticles

Polymers have been widely preferred materials in drug delivery systems due to some unique properties such as versatility, variety, ease of process, etc. Polymeric nanoparticles can be produced from a single type of polymer or a blend of several polymers as well as in different forms (fibers, spheres, capsules, etc.). Moreover, the characteristics of the polymer of interest can be chosen to be biodegradable or nondegradable, hydrophilic or hydrophobic, high melting temperature or low, etc. depending on the application area.

There is also the option of modification on the polymeric materials either by changing the bulk or surface properties. Surface of the material is the first part that comes in contact with the living environment and therefore, properties such as biocompatibility, hemocompatibility, hydrophilicity, surface roughness, permeability, etc. play an important role and the surface can be controlled by modification. Modifications of surface can be chemical

or physical (Kumari *et al.*, 2010). Bulk properties of polymers such as solubility, crystallinity, mechanical properties can be modified by blending or copolymerizing with another type of polymer (Pillai *et al.*, 2001).

The polymers used can be of natural or synthetic origin. Among the natural polymers used are proteins like human serum albumin (HSA), collagen, gelatin, hyaluronic acid, chondroitin sulfate, chitosan which are all biodegradable and biocompatible. One limitation in their use is high cost and impurities that might lead to adverse reactions. Synthetic polymers on the other hand, have certain advantages over the natural ones such as high purity, ease of production and reproducibility, nontoxic byproducts, and controllable biodegradability and biocompatibility (Jain 2000, Nair *et al.*, 2006). Synthetic polymers such as poly(glycolic acid), poly (alkyl-cyanoacrylate), poly(3-hydroxybutanoic acid), poly(organophosphazene), poly(ethylene glycol), poly(caprolactone), poly(ethylene oxide), poly(amidoamine), poly(L-glutamic acid), poly(ethyleneimine) and poly(propylene imine) are some examples of synthetic polymers that are widely used in nanoparticle preparation (Domb *et al.*, 2002).

When choosing polymeric nanoparticles for drug delivery, the encapsulation efficiency, the solvent types and release profiles have to be taken into consideration. Polymeric devices (capsules or spheres) generally release drugs by diffusion (through a matrix or across a wall), solvent activation (swelling or osmotic effects) or chemical reaction (degradation or chemical cleavage) (Langer, 1990).

1.2.2. Inorganic nanoparticles

Inorganic materials have been selected for use in drug delivery due to their magnetic properties, fluorescence, or durability (Xu *et al.*, 2006). Calcium phosphate, gold, carbon materials, silicon oxide, iron oxide, layered double hydroxide (LDH), cadmium selenide and zinc selenide are some examples of inorganic nanoparticles. Gold nanoparticles are the most widely used in

drug delivery because they are easy to produce with a certain size and form in addition to their suitability for surface modifications and coating. Like polymeric nanoparticles, inorganic nanoparticles can also be modified to obtain desired characteristics such as absence of immunogenic response or suitability for fluorescence imaging (Liong *et al.*, 2008).

1.2.3. Lipid based nanoparticles

Lipid-based nanoparticles are popular tools in drug delivery not only because they protect the drug from being cleared by the circulatory system and protect the non-target tissues from toxic effects of the bioactive agent, but also because they can be metabolized and removed from the body without leaving any by-products behind. Lipid nanoparticles have advantages like ease of preparation, variety of forms and range of sizes, ability to encapsulate both hydrophilic and hydrophobic compounds (Arias *et al.*, 2011). Figure 3 is a presentation of different forms of lipid nanoparticles.

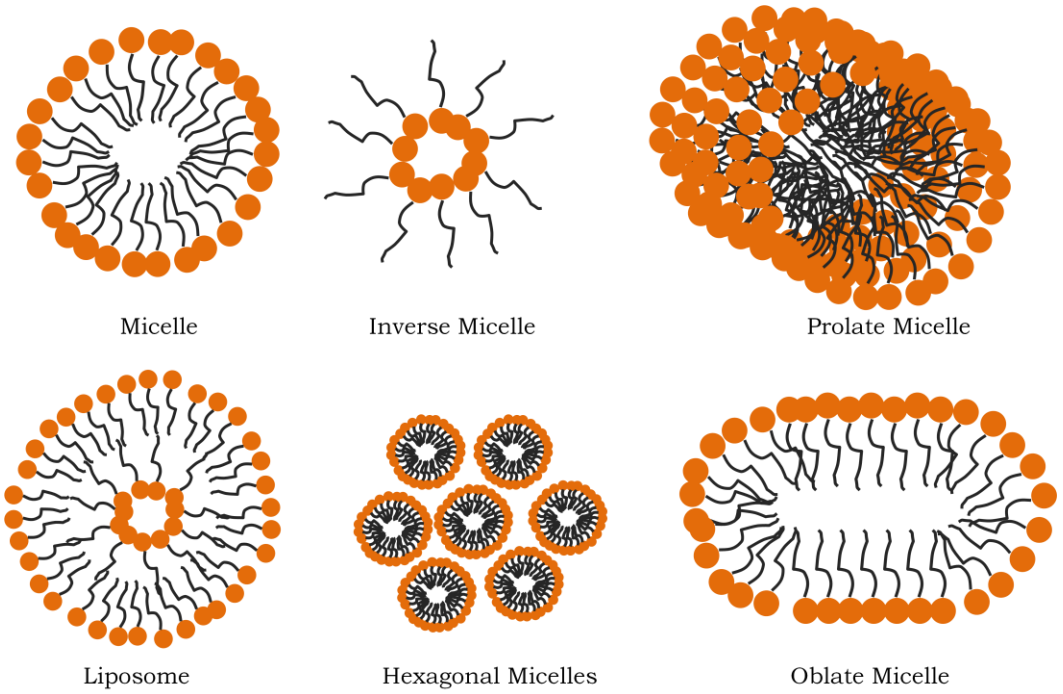


Figure 3. Forms of lipid-based nanoparticles

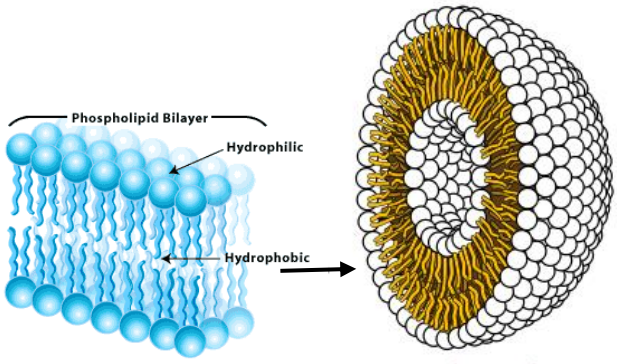
Lipid-based nanoparticles can be between 20-1000 nm in size depending on the method of preparation and the resultant form. In addition to the preparation methods, there are also a variety of techniques for drug loading into lipid nanoparticles. Lipid nanoparticles can be modified for specific purposes such as PEGylated (polyethyleneglycol modified) to provide a hydrophilic shell, charged through the use of ionizable groups, or reacted with molecules that have a specific affinity towards certain tissues or targeted to a specific tissue due to charge or size.

1.3. Liposomes

Liposomes are a special category of lipid nanoparticles and their field of use in biotechnology is determined by physicochemical characteristics such as composition, size, loading and stability in addition to their interaction with

cells. For drug delivery purposes liposomes can be formulated in a suspension, as an aerosol or in a semi-solid form such as cream, gel or dry powder (Jung *et al.*, 2011, Chen and Bothun, 2011, Gibbons *et al.*, 2010).

Liposomes are spherical structures made of mostly phospholipids. They are formed spontaneously when the phospholipids are hydrated leading to a population of vesicles ranging in size from 10 nm to 10 μm in diameter. They were first described by Bangham in 1965 (Bangham *et al.*, 1965). Figure 4 is a schematic presentation of a unilamellar vesicle and the phospholipid bilayer.



(adapted from http://upload.wikimedia.org/wikipedia/en/8/8a/Liposome.png)

Figure 4. Scheme of a liposome structure

Phospholipids are amphiphilic molecules, consisting of a hydrophobic tail and a hydrophilic (polar) head. The hydrophobic tail is made up of two fatty acid chains (10-20 carbon length) and the hydrophilic head contains phosphoric acid bound to a water soluble molecule. Due to this amphiphilic nature of the phospholipids, structures like liposomes form spontaneously when they come in contact with water. Hydrophobic fatty acid chains gather around each other and avoid contact with water while polar head groups face the aqueous environment.

Although liposomes form spontaneously, there are ways of producing liposomes with different features by changing the steps of the preparation process. Size, lamellarity, stability and permeability of the vesicles are some of the features that can be adjusted for a particular purpose. For example, introduction of unsaturated lipids to the membrane leads to a more flexible and permeable membranes. Figure 5 is a presentation of how membrane stability is affected by the introduction of unsaturated lipids.

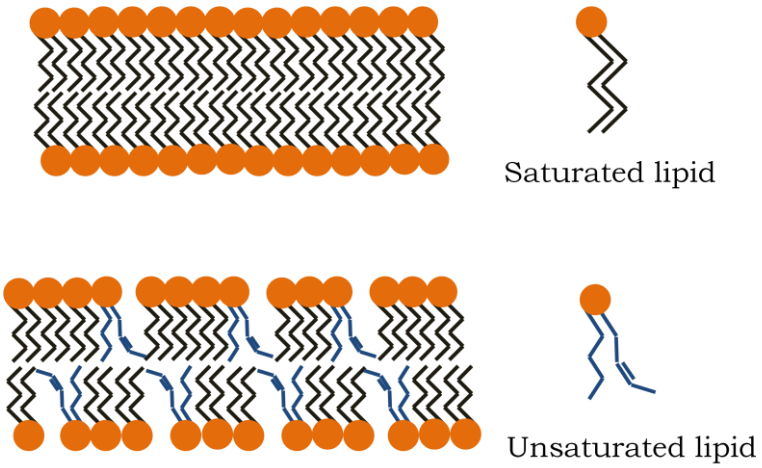


Figure 5. Effect of unsaturated lipids on membrane stability

Liposome can be designed to have neutral or charged membranes by choosing the right components. The major components of neutral liposomes are uncharged phosphatidyl cholines (PC), such as dipalmitoyl phosphatidyl glycerol (DPPG), and dipalmitoyl phosphatidyl choline (DPPC) or phosphatidyl ethanolamine (PE) (Figure 6). Negatively charged liposomes can be produced by addition of acidic phospholipids such as dipalmitoyl phosphatidic acid (DPPA) to the membrane (New, 1990).

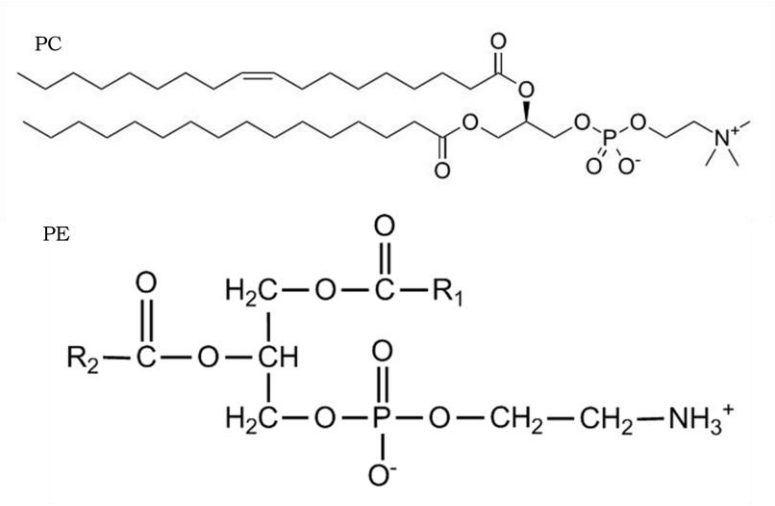


Figure 6. Structure of phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE)

There are components other than phospholipids in the lipid bilayer of liposomes. Cholesterol is the second major ingredient of a liposomal bilayer. Cholesterol provides rigidity and stability to the membrane. It does not form a bilayer by itself but is rather included in the bilayer structure in the cholesterol to PC molar ratios of up to 1:1 (50 %) (New, 1990). Cholesterol is also an amphipathic molecule and its alipathic chains are buried in the acyl chains of the phospholipids while the hydroxyl group is directed towards the aqueous environment. Figure 7 is a schematic presentation of how cholesterol is located in the lipid bilayer.

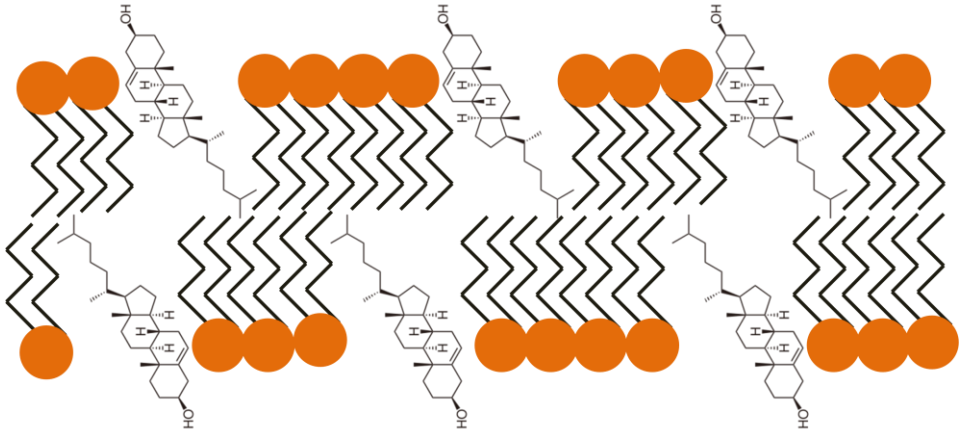


Figure 7. Cholesterol's position in the lipid bilayer of the liposome

Cholesterol's presence in the phospholipid membranes has a significant effect on the critical phase transition temperature (T_c), which is defined as the temperature at which the lipid membrane goes from a highly ordered gel (or solid) phase to a liquid-crystal phase. Above this temperature the mobility of the membrane components is high. Fluidity, permeability, fusion and aggregation are all affected by this transition temperature. With increasing concentration of cholesterol in the lipid membrane, the phase transition temperature also increases indicating that incorporation of cholesterol increases the stability, therefore the rigidity, of the membrane. Permeability and fluidity are also affected by the cholesterol content of the lipid bilayer. Cholesterol proportions over than 50% is difficult to achieve because the regular structure of the membrane and the intermolecular interactions are disrupted and stable liposomes cannot be obtained (New, 1990).

Liposomes can be assigned for drug-delivery purposes as controlled or sustained release systems in addition to medical diagnostics and gene therapy purposes. Moreover, liposomes can serve as a model for the cell membrane and is used in recognition and interaction studies. They are also useful in the investigation of the mode of action of active substances in pharmacology and medicine.

1.3.1. Classification of liposomes

Physical classification for liposomes is based on their size and lamellarity (number of bilayers).

- Multilamellar vesicles (MLV): They are generally larger than 500 nm in diameter and have multiple bilayers. They can be obtained spontaneously after the hydration of the dry phospholipid film and no extra step other than agitation is required at the hydration step.

- Multivesicular vesicles (MVV): They consist of several vesicles within one larger common vesicle. They are generally larger than 1000 nm.

- Large unilamellar vesicles (LUV): Liposomes of single bilayer larger than 1000 nm in diameter and have single bilayer known as large unilamellar vesicles. Ultrasonic homogenization process applied to multilamellar vesicles might yield unilamellar vesicles.

- Small unilamellar vesicles (SUV): Their diameters are generally in the range of 20 - 100 nm and they have a single bilayer. The diameter can be made smaller by increasing the power of ultrasonic homogenization process.

Figure 8 is a schematic presentation of different types of liposomes.

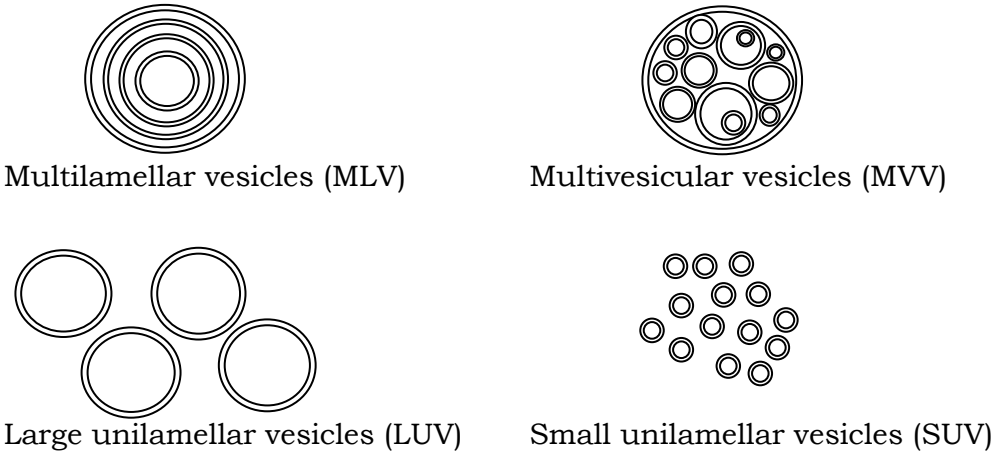


Figure 8. Different types of liposomes

Liposomes are prepared using different methods and approaches. Figure 9 is a scheme for liposome preparation process in general.

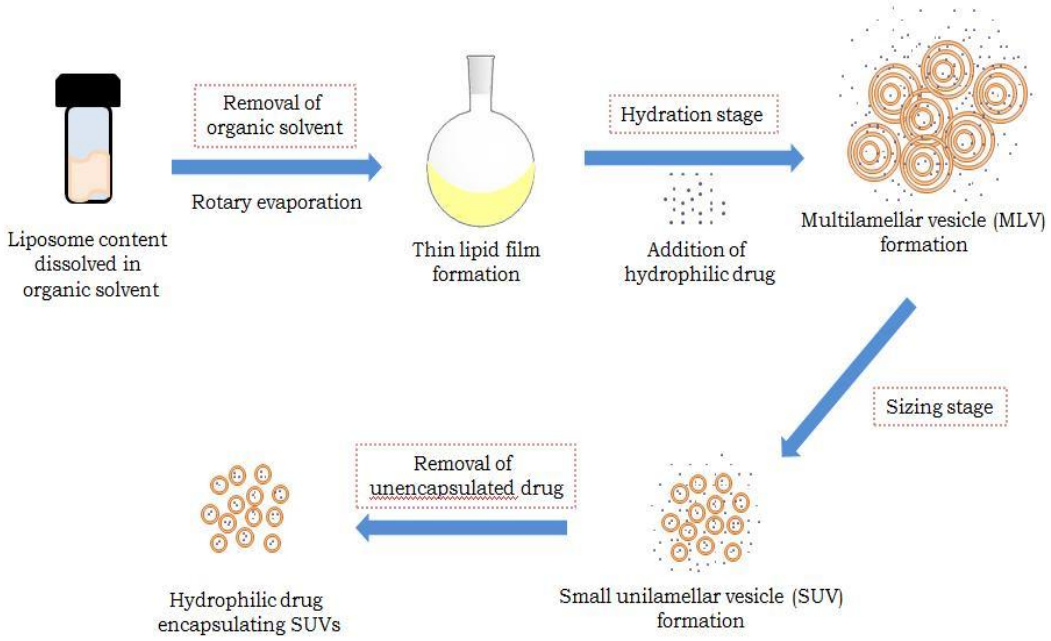


Figure 9. Liposome preparation process

Following are a variety of preparation techniques differing by changes in certain steps of liposome preparation (Verma *et al.*, 2009).

1.3.1.1. Variations in the hydration stage

Hydration stage is the first step of liposome preparation. The thin lipid film formed on the wall of flask by rotary evaporation is removed at this stage. Moreover, hydration is the step where aqueous drugs are encapsulated in liposomes.

1.3.1.1.1. Methods based on replacement of organic solvent(s) by aqueous media

During liposome preparation all lipid soluble membrane ingredients are dissolved in an organic solvent in order to obtain a homogenous membrane. In the removal of this organic solvent a rotary evaporator is the most common device used (Torchillin and Weissig, 1990). Organic solvent is evaporated and the phospholipid is dried on the walls of glass flask as a film. Then this is hydrated and further processed to form the liposome. Compounds to be loaded in the lipid membrane are added during the solution phase and those to be carried in the aqueous core are added in the hydration step.

1.3.1.1.1.1. Reverse phase evaporation

In this type of liposome preparation, the first step is removal of the organic solvent, generally with a rotary evaporator. Secondly, the lipids are then re-dissolved in the organic phase, an aqueous phase (might be carrying the drug) is added and the resulting two-phase system is sonicated until the mixture becomes a homogeneous suspension of oil-in-water. The organic solvent is gradually removed under vacuum and the aqueous suspension obtained at the end carries the reverse phase evaporation vesicles (REVs) (Szoka and Papahadjopoulos, 1978). This method leads to very high encapsulation efficiencies such as 65 %.

1.3.1.1.1.2. Use of water immiscible solvents

This technique involves injection of an organic solution into the aqueous phase through a needle in a very slow fashion while the organic phase is removed by evaporation. Large vesicles are formed as a result of this process. Ether injection is common for this method (Deamer and Bangham, 1976). This process yields liposomes with high entrapment efficiencies since removal of the solvent takes multiple runs resulting in a very concentrate population of liposomes (Meure *et al.*, 2008).

1.3.1.1.1.3. Use of water miscible solvents

Ethanol injection is a good example for this method where a lipid solution is prepared in ethanol and injected into an aqueous medium through a fine needle. The force of injection also achieves mixing resulting in an evenly

dispersed phospholipid solution (Batzri and Korn, 1973). With this technique especially small unilamellar vesicles (SUVs) are obtained.

1.3.1.1.2. Mechanical methods

These methods are applied to enhance the hydration process. Liposomes are formed spontaneously during this step and the size of liposomes differs depending on the method applied.

1.3.1.1.2.1. Vortexing or manual shaking of phospholipid dispersions

This is the most common and the simplest way of mechanical dispersion, and involves suspension of lipids which were dried on the walls of the glass flask with gentle manual agitation either with hand or with vortex. This procedure yields multilamellar vesicles (MLVs) (Bangham *et al.*, 1965).

1.3.1.1.2.2. Microfluidizer technique

Microfluidizer is a high pressure homogenizer which can be used in large scale production of liposomes in a continuous manner without need for any other procedure (Jahn *et al.*, 2007). In microfluidization the lipid suspension is forced through a filter under high pressure resulting in multilamellar vesicles (MLVs) of size demanded. These liposomes can be stored for a long time without aggregation or fusion.

1.3.1.1.2.3. High-shear homogenization

In this method liposome suspension is forced through a series of nozzles that break down the medium as it passes from chamber to chamber. Nozzle size can be adjusted according to the size needed. With this technique mainly large quantities of small unilamellar vesicles (SUVs) are produced.

1.3.1.1.3. Methods based on size reformation and fusion

These methods are applied to obtain larger vesicles than those obtained with conventional methods. This is possible by fusion of small vesicles with different methods explained in the following.

1.3.1.1.3.1. Spontaneous fusion of SUV in the gel phase

Fusion of SUVs can occur spontaneously in the gel form of liposomes if there is such a stage. Calcium ions or dramatic pH changes are also used for the reassembly of the vesicles (Hauser *et al.*, 1983). This method yields large unilamellar vesicles (LUVs) by fusion of SUVs.

1.3.1.1.3.2. Freezethawing

Repeated freezing and thawing process is applied to fuse SUVs with each others. After thawing the lipid solution is subjected to sonication. The method results in production of LUVs and MLVs.

1.3.1.1.3.3. Dried reconstituted / dehydration rehydration vesicles (DRVs)

This method is a freeze-drying method. In the simple freeze-drying method, a lipid solution in organic solvent is freeze-dried and then it is rehydrated with an aqueous medium to lead to MLVs. In dehydration-rehydration method, on the other hand, an aqueous suspension of empty SUVs is freeze-dried instead of a solution of lipid in an organic solvent. This lyophilized SUV powder is then rehydrated with an aqueous solution of the bioactive agent to be entrapped within the core.

1.3.1.1.3.4. pH-induced vesiculation

This method includes formation of small unilamellar vesicles from multilamellar vesicles without use of any mechanical force such as high pressure or sonication. Reassembly of MLVs into SUVs is induced by changes in pH. The change in pH of the environment leads to an increase in the surface charge density of the lipid vesicles. If this exceeds a threshold (1-2 $\mu C/cm^2$), spontaneous vesiculation takes place (New, 1990).

1.3.1.2. Variations in the sizing stage

Liposomes are formed spontaneously; however, extra steps might be required to obtain vesicles of specific size. Following are different methods applied to obtain vesicles of size needed.

1.3.1.2.1. High pressure extrusion

In this technique, phospholipid dispersion is extruded through an orifice with a predetermined diameter under high pressure, i.e. French press (Hamilton *et al.,* 1980). This process yields a homogeneous population of SUVs. These liposomes are far more stable than the ones produced by other methods. This enables the extruded liposomes used for drug or bioactive agent delivery purposes since the liposomes obtained have high stability.

1.3.1.2.2. Low pressure extrusion

This method is a gentler one compared to the French press mentioned above. Extrusion is performed through a membrane filter with a defined pore size with low pressure application (Olson *et al.,* 1979). Membranes are generally two types: tortuous path and nucleation track (straight). The former consists of fibers crossing one another while the latter is basically made of thin continuous sheets of polymer having straight channels precise pore sizes. As a result of extrusion with the former membrane, a population of liposomes with almost the same diameter is obtained. However, extrusion with the latter membrane results in liposomes with a size distribution in diameter (Rong, 2008). Because nucleation membranes offer low resistance to material passing through the membrane, liposomes can pass through even if they are larger than the pores.

1.3.1.2.3. Ultrasonic treatment

This is the most widely used method in producing small size liposomes. Ultrasonication applies a high level of energy to the lipid suspension resulting in reduction in the diameter of the liposomes produced. Starting

material is large and multilamellar vesicles. Consequent liposome size is directly related with the time and the power of the ultrasonication (Woodbury, *et al.,* 2006).

1.3.1.3. Variations in the removal of unencapsulated compound

It has been explained that liposomes are formed spontaneously by hydration of the thin lipid films and various methods were mentioned to enhance the hydration step. It was also stated that hydration is the step where aqueous drug encapsulation is preformed. Later several methods are applied to regulate the size of the vesicles obtained.

After sizing stage, additional steps are required to remove the unencapsulated drug present in the liposome suspension. Following are several methods that are commonly applied for removal of unencapsulated drug from the liposomes.

1.3.1.3.1. Dialysis

In liposome purification, unencapsulated compounds can be removed with dialysis. Solution to be dialyzed is placed into a dialysis bag whose pores are small enough to hold the desired materials inside and large enough to allow the passage of impurities through to the surrounding medium. Refreshment of the surrounding medium continuously or repeatedly increases the efficiency of the dialysis. This technique, however, is sometimes inefficient, time consuming and runs the risk of not purifying the solution properly.

1.3.1.3.2. Centrifugation

Centrifugation aims to sediment liposomes from a suspension. Moreover, MLVs and SUVs can be separated from each other with use of proper centrifugation conditions (speed and duration). The supernatant carries the unencapsulated compounds and the smallest liposomes. This method carries the risk of fusion of the liposomes.

1.3.1.3.3. Gel permeation chromatography

This technique involves separation based on the size of the samples. Separation takes place within a column of porous beads via size exclusion approach. Therefore, larger particles, mainly the liposomes, are collected in the earlier fractions (more likely in the void) and the unentrapped compounds in later fractions. This technique is effective but time consuming and leads to diluted products.

1.3.2. Liposomes in drug delivery

For certain drug delivery applications liposomes are among the best vehicles. They are preferred widely due to their being nontoxic, completely biodegradable, biocompatible and nonimmunogenic (Langer, 1990; Venkateswarlu *et al.*, 2011). Since liposomes are formed from amphiphilic molecules, it is possible to embed hydrophobic molecules into the bilayer while encapsulating hydrophilic molecules into the aqueous core. Due to their similarity to cell membranes, liposomes can avoid macrophages, fuse with cells and are metabolized without leaving any toxic metabolites behind. Moreover, it is easy to encapsulate a drug in liposomes. In other words,

encapsulation of a drug into liposome core requires no extra effort because it can be performed in the hydration step.

In addition to their ability to incorporate both hydrophilic and hydrophobic compounds, there are other advantages of liposomes. Liposomes have high versatility in terms of size, form, lamellarity, fluidity, charge, all of which can be regulated with parameters of different preparation methods. This flexibility makes it possible to encapsulate not only small molecules but also macromolecules inside their core or into the hydrophobic bilayer. Moreover, liposomes can be used in targeted delivery of drugs to a specific site in the body as well as in sustained and controlled delivery. Liposomes can undergo endocytosis and phagocytosis and this enables them to deliver the drugs intracellularly. Liposomes can be used to deliver DNA inside the cells, which make them very important as non-viral transfection systems especially in gene therapy (Jiang *et al.*, 2011).

Liposomal delivery systems should have long-term stability for shelf life, proper release rate, and narrow size distributions (Mozafari, 2005). Targeting of liposomes can be achieved through incorporation of antibodies or markers into the lipid bilayer and this helps accumulation of the liposomes, and therefore the drug, at a specific target tissue in the body. This enhances the therapeutic response and reduces the incidence of systemic toxicity of the drug. For systemic injection applications, liposomes are required to have higher durability so that they do not diffuse out of the circulatory system or get phagocytosed. For this purpose, polyethyleneglycol-modified liposomes (PEG-liposomes), which are also called “long-circulating liposomes” or “stealth liposomes” are produced.

As can be seen from the examples presented liposomes are a common and well accepted way of delivering drugs. There are a number of FDA approved liposomal drug delivery systems, such as Doxil, Caelyx, DaunoXome, Ambisome, Amphotec, Abelect designed to administer Doxorubicin, Daunorubicin citrate, and Amphotericin B (Lian and Ho, 2001). Table 2 is a list of commercial liposomal systems used in drug delivery.

Table 2. Commercial liposome based drug delivery systems

Product	Active Agent	Disease	Company	Ref
Doxil™ Caelyx™	Doxorubicin	Kaposi's sarcoma	SEQUUS, USA	Working *et al.*, 1994
DaunoXome™	Daunorubicin	Kaposi's sarcoma, breast and lung cancer	NeXstar, USA	Forssen *et al.*, 1996
Amphotec™	Amphotericin-B	Fungal infections, Leishmaniasis	SEQUUS, USA	Hiemenz *et al.*, 1996
Abelect	Amphotericin-B	Fungal infections	Elan Co., Ireland	Lian *et al.*, 2000
Fungizone®	Amphotericin-B	Fungal infections, Leishmaniasis	Bristol-squibb, Netherland	Wasan *et al.*, 1998
VENTUS™	Prostaglandin-E_1	Systemic inflammatory diseases	The liposome company, USA	Lasic *et al.*, 1998
Topex-Br	Terbutaline sulphate	Asthma	Ozone, USA	Chung *et al.*, 1989
Depocyt	Cytarabine	Cancer therapy	Skye Pharm, USA	Patil *et al.*, 2005
Novasome®	Smallpox vaccine	Smallpox	Novavax, USA	Patil *et al.*, 2005
Avian retrovirus vaccine	Killed avian retrovirus	Chicken pox	Vineland lab, USA	Gregoriadis *et al.*, 1998
Epaxal-Berna Vaccine	Inactivated hepatitis-A Virions	Hepatitis A	Swiss serum & vaccine institute, Switzerland	Gluck, 1995
Doxil®	Doxorubicin HCl	Refractory ovarian cancer	ALZA, USA	Forssen and Ross, 1994
Evacet™	Doxorubicin	Metastatic breast cancer	The liposome company, USA	Vyas *et al.*, 2002
VincaXome	Vincristine	Solid tumors	NeXstar, USA	Vyas *et al.*, 2002
Mikasome®	Amikacin	Bacterial infection	NeXstar, USA	Vyas *et al.*, 2002
Autragen™	Tretinoin	Kaposi's sarcoma	Aronex Pharm, USA	Vyas *et al.*, 2002
Shigella Flexneri 2A Vaccine	Shigella flexneri 2A	Shigella Flexneri 2A infections	Novavax, USA	Vyas *et al.*, 2002
Nyotran™	Nystatin	Systemic fungal infections	Aronex Pharm, USA	Vyas *et al.*, 2002

Liposomes are also known to be used in passive targeting of cancer drugs due to their small size. Liposomes smaller than 200 nm were reported to accumulate in the vicinity of the cancer tissues due to Enhanced Permeability and Retention (EPR) effect (Matsumura and Maeda, 1986), which basically is accumulation of nanoparticles in the solid tumors because of the increased permeability of the capillaries and poor lymphatic drainage at the tumor site.

There are several studies on liposomal drug delivery systems reported to be successful in cancer treatment. In one study, Cheng *et al.* (2011) succeeded tumor suppression in colon cancer models with liposomal honokiol combined with Cisplatin. In another study, Mikhaylov *et al.* (2011) experimented targeting magnetic nanoparticle clusters encapsulated into liposomes to tumor site under the influence of an external magnet. Wang *et al.* (2011) used multivesicular liposomes (MVLs) to encapsulate LTX-101, a cationic peptide antagonist of gonadotropin-releasing hormone (GnRH), and observed the sustained anticancer effect of the system (MLVs encapsulating LTX-101) in rats for prostate cancer.

1.3.2.1. Controlled and responsive delivery from liposomes

Drug delivery systems are designed to achieve high local doses of the drug at the target site. Responsive or intelligent delivery achieves the release of a drug in response to specific stimuli. These systems can achieve the release of the drug in a continuous manner or in response to environmental stimuli such as changes in temperature (Bikram and West, 2008, Zhang *et al.*, 2011), pH (Zheng *et al.*, 2011), or light (Cirli and Hasirci, 2004, Shamay *et al.*, 2011). These “intelligent” delivery systems use the destabilization of liposomes by a change in conformation, charge or physical state of one or more of its constituents.

As was stated in the earlier section, liposomes can be passively targeted to a tumor area for cancer treatment. They can also be designed to deliver their

content in a responsive or controlled manner and the resultant targeted-responsive delivery system would both deliver to a specific site and achieve release there thus create a significant drug dose at the target site.

1.3.2.1.1. pH responsive liposomes

It is known that some sites in the body have different pH values such as the gastrointestinal tract, lysosomes, vagina and blood vessels. It was reported that tumor areas also have lower pH values than the physiological pH of 7.4 (Drummond *et al.*, 2000). This fact lead to the development of systems responsive to changes in pH (Garg and Kokkoli, 2011, Soares *et al.*, 2011, Tomoyasu *et al.*, 2011).

pH-sensitive liposomes were initially designed to be destabilized at acidic environments such as tumor sites. The most widely studied pH-sensitive liposomes are made of derivatives of phosphatidyl ethanolamine (PE) in combination with mildly acidic amphiphiles. The PE head group has a primary amino group which is protonated at low-pH conditions. The design involves destabilization of lipid membrane upon protonation of PE at low pH conditions. When the membrane packing is disrupted, the content is released. However, in vivo studies with PE derivatives are not promising due to their instability in the serum and rapid clearance (Wang, 2005).

Plasmalogen is a naturally occurring pH-responsive lipid (Gerasimov and Boomer, 1999, Rui *et al.*, 1998). One of the hydrocarbon chains in plasmalogen is attached to the head group by an enol ether linkage, which is broken at low pH, leading to disruption of membrane packing.

There are also synthetic pH-responsive systems containing acid-cleavable groups. These systems are mainly made of derivatives of naturally available lipids such as modified PE and plasmalogens. Synthetic orthoester derivatives have been developed as acid-cleavable lipids. However, the major problem of these synthetic systems is their low stability (Wang, 2005).

pH-responsive systems might be seen to be promising; however, they have several drawbacks. First of all it is difficult to synthesize or purify the natural lipid-based pH-sensitive molecules in large quantities. The low stability of these systems in physiological environments is another problem. In addition, changes in pH in some parts of the body can occur in response to numerous, and sometimes unexpected stimuli. This may lead to loss of the control on drug release in the body.

1.3.2.1.2. Thermoresponsive liposomes

Release from temperature sensitive liposomes depends on the leakage of the content upon destabilization of the membrane at temperatures above the phase transition temperature of their membrane components (Zhang *et al.*, 2011). These liposomes are generally stable up to 37ºC but their membrane integrity is disrupted above 40ºC (Wang, 2005). In order for these systems to be successful, there has to be external, local heating or an interior temperature increase at the disease area (target site). These liposomes are prepared with lipids which have a phase transition temperature close but above the physiological temperature. Thermoresponsive liposomes can also be obtained with incorporation of thermosensitive polymers as well.

There are several successful applications of temperature sensitive liposomal systems (Kono *et al.*, 2011, Djanashvili, *et al.*, 2011). However, there are also drawbacks such as the temperature range that the system can work is very limited since only slight changes in temperature can be experimented in organisms. This brings out the requirement of an external heat source to trigger the release from the liposomes. Heating the organism is not practical because extreme temperatures cannot be tolerated in organisms and it might be difficult to be successful for the cases of distant metastases (Bikram and West, 2008). Uncontrolled leakage of encapsulated drugs from these systems is another problem. Regulation of temperature sensitive

systems is difficult and further studies are required to control the release and increase the efficiency of the delivery from these systems.

1.3.2.1.3. Photoresponsive liposomes

Photosensitive delivery from liposomes is based on disruption of membrane integrity upon exposure to a specific light source (Bisby *et al.*, 2000). In order to achieve this, light sensitive molecules (molecules that change their conformation or chemistry upon exposure to UV or visible light) such as retinoids (Gursel and Hasirci, 1995), azobenzene-based moieties (Hamada *et al.*, 2009) or photochromic lipid Bis-Azo PC (Bisby *et al.*, 2000) are incorporated into the lipid bilayer. The mechanisms involved in photoresponsiveness include photopolymerization of lipid tails (Wang, 2005) that result in phase separations, azo benzene isomerization (Bisby *et al.*, 2000), sensitized photo-oxidation cleavage of the lipid tail of plasmalogen and diplasmalogen (Gerasimov and Boomer, 1999, Shum *et al.*, 2001) and a photocleavable DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) derivative NOVC-DOPE (Zhang and Smith, 1999).

In this study, photoresponsivess was achieved by using Suprofen, a non-steroidal anti-inflammatory drug (NSAID). Suprofen, when exposed to the radiation, is converted to its decarboxylated form which can transfer electrons to a suitable acceptor (Figure 10). As a result of this, a number of transient species such as free radicals, superoxide ions, singlet oxygens, hydroxyl radicals and photodegradation products are generated (Castelli *et al.*, 1999). These transient species and photodegradation products initiate an osmotic shock that leads to cell lysis (Guidi *et al.*, 2005). Suprofen is derived from 2-arylpropionic acid which is a strong photosensitizer (Condorelli *et al.*, 1996) due to its ability to absorb the radiation that penetrates the skin (wavelengths of longer than 310 nm) (Klefah *et al.*, 2009). When Suprofen is introduced into the bilayer structure and exposed

to UV (UV-A or UV-B), it becomes decarboxylated and this leads to the destabilization of liposome bilayer resulting in the release of its contents.

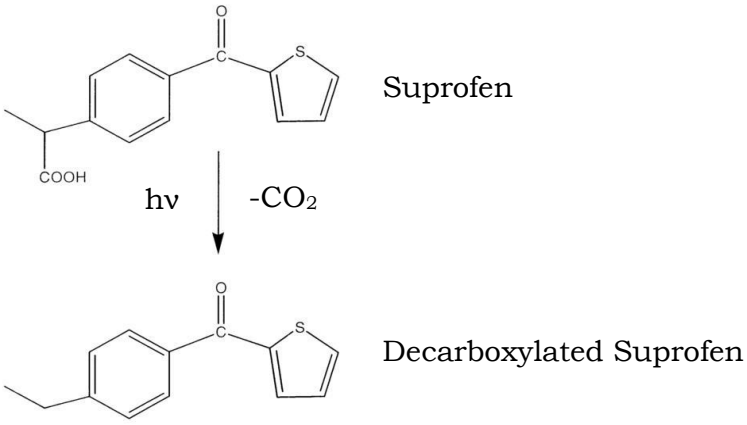


Figure 10. Decarboxylation of Suprofen

In addition, UV decarboxylation reaction of SPF is a photosensitization reaction and it also results in the production of singlet oxygen molecules. These molecules are highly reactive, react with any biomolecule in their vicinity and trigger destructive reactions which could lead to apoptosis of the cells (Wilson and Petterson, 2008).

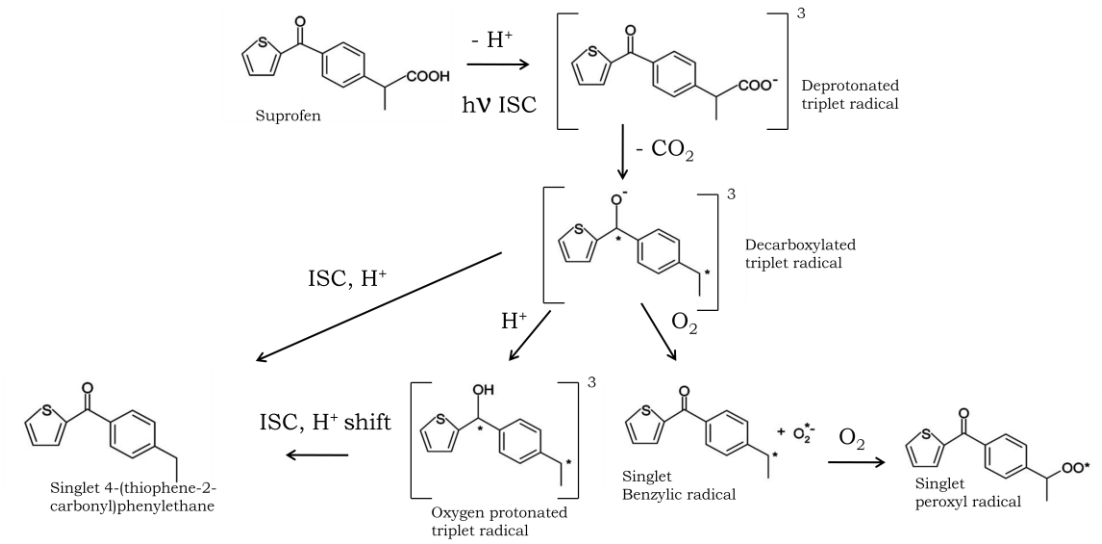


Figure 11. Photosensitization reaction of Suprofen (ISC: intersystem crossing, transition between two electronic states)

SPF in the bilayer has a dual effect on its environment upon UV exposure. It leads to destabilization of liposome membrane and the release of the anticancer drug encapsulated in the liposome. It also produces highly reactive singlet oxygen molecules, triggering series of reactions which lead to apoptosis of the cells. Therefore, SPF incorporated photoresponsive liposomes are promising vehicles for cancer therapy.

1.4. Cancer therapy with Cisplatin

Cisplatin (*cis*-diaminedichloroplatinum (II), CDDP) was chosen as the anti-cancer drug for this study to be released from UV responsive liposomes. Cisplatin is commonly used in the treatment of various cancers such as metastatic testicular tumors, advanced bladder cancer (Patinol®), lung carcinoma (Michalke, 2010), osteosarcoma (Qi *et al.*, 2011), ovarian cancer (Qi *et al.*, 2011), lymphoma (Miguel and Bestetti, 2011), and germ cell tumors (Hendricks *et al.*, 2011). Cisplatin was first introduced to clinical

practice in 1971 and it also was the first Pt-containing drug used in cancer treatment (Rosenberg, 1999).

Cisplatin (Figure 12) interacts with the free nitrogen atoms of the bases of DNA and forms a DNA-Cisplatin adduct which leads to bending of the DNA structure by 35–40º (Takahara *et al.*, 1995, Takahara *et al.*, 1996, Silverman *et al.*, 2002). This complex formation leads to inhibition of the DNA polymerase (Gelasco and Lippart, 1998), which then induces apoptosis of the cancer cells (Siddik, 2003, Pil and Lippard, 1997, Boulikas and Vougiouka, 2003). The preferred target moiety on DNA is recognized as the guanine residue(s) where mostly intra strand adducts are formed between the Pt complex and DNA.

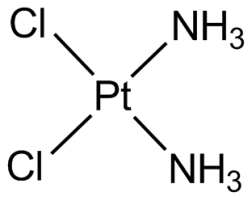


Figure 12. Cisplatin structure

Cisplatin use in clinical applications is limited due to considerable side effects of the drug such as acute toxicity, nephrotoxicity, neurotoxicity, ototoxicity, etc. (Platinol®). Anticancer drugs are among the most harmful drugs when introduced systemically. Thus, liposomal delivery of cancer drugs is an effective way of limiting the damage at the non-target sites due to masking of the molecule within the liposomes.

1.5. Approach in this study

This study aimed to develop intelligent, photoresponsive liposomes that would release of their content upon exposure to UV. The main approach was to incorporate a light sensitive molecule into the liposomal bilayer, then destabilize the membrane by exposure to UV (Figure 13). This would result in an "on demand" release of the bioactive content. Suprofen, a nonstereoidal, anti-inflammatory drug, also a light sensitive molecule, was selected to achieve the destabilization of the membrane in this study. Lipid vesicles were prepared in different ratios of PC:CHOL:SPF and characterized in terms of encapsulation efficiency, release rate and responsiveness to UV. Preliminary studies were carried out with calcein (CAL), a fluorescent dye, due to the ease of determination and the *in vitro* studies were carried out with the cancer drug Cisplatin.

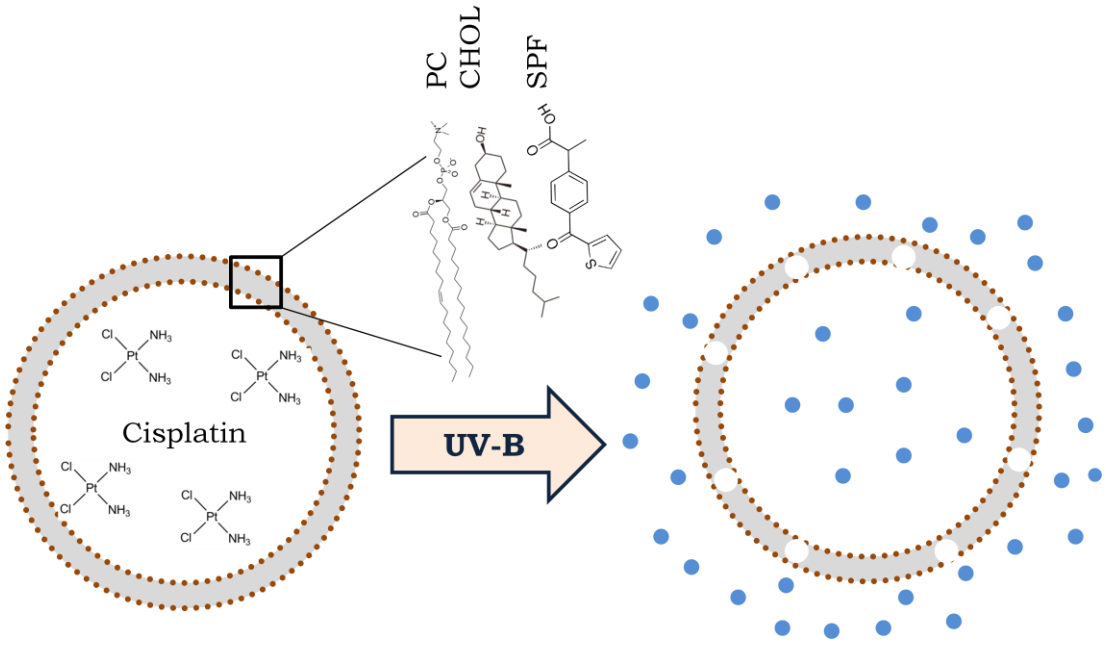


Figure 13. Design of the photosensitive liposomes used in this study and the mechanism behind the release

This study involves development of a photoresponsive drug delivery system by incorporating Suprofen into liposome bilayer. There have been studies to test the photoresponsiveness of Suprofen in response to different light sources (Cirli, Hasirci, 2004) or to determine the intermediate molecules in photosensitizing reactions (Klefah, *et al.*, 2009). In this study, the photoresponsive drug delivery system was tested on Saos-2 cells. The effect of responsive release of the cancer drug upon UV exposure and the effect of Suprofen decarboxylation process was tested in vitro.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Lecithin (L-α-phosphatidylcholine from egg yolk) was bought from Fluka (USA).

Cholesterol (3β-hydroxy-5-cholestene), calcein (fluorescein-bis (methyl-iminodiacetic acid)), Suprofen (α-methyl-p-[2-thenoyl]phenylacetic acid), chloroform (trichloromethane) and Cisplatin (*cis*-Platinum(II) diamine dichloride) were purchased from Sigma-Aldrich (USA).

Sephadex G 50-80 was obtained from Sigma-Aldrich (USA) and was swollen in excess phosphate buffer (0.5 M, pH 7.4) before use.

Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Merck (Germany).

Dialysis bag (Snake Skin pleated dialysis tubing, 10000 MWCO) was purchased from Thermo Scientific (USA).

The human osteosarcoma cell line (Saos-2) was obtained from the American Type Culture Collection (ATCC HTB-85™).

Fetal bovine serum (FBS), RPMI (Roswell Park Memorial Institute) 1640 medium, DMEM Low Glucose Medium, Amphotericin B and Penicillin/Streptomycin were purchased from HyClone (USA).

Linco-spectin was purchased from Pfizer (USA). Vancomycin was bought from Vial Hospira (USA).

CellTiter 96® Non-Radioactive Cell Proliferation Assay (*MTS*) was purchased from Promega (Germany).

2.2. Methods

The methods applied in this study includes preparation of the liposomes, characterization of the liposomes and in vitro studies performed with liposomes loaded with anti-cancer drug and Saos-2 cells.

2.2.1. Preparation of liposomes

The preparation stage includes preparation of photoresponsive and standard liposomes.

2.2.1.1. Preparation of standard liposomes

Liposomes were prepared by the thin lipid film hydration technique (Bangham *et al.*, 1965). Standard liposome components phosphatidylcholine (PC) (23.7 mg) and cholesterol (CHOL) (1.7, 3.4, and 5.1 mg) were dissolved in chloroform and mixed (7:1, 7:2, and 7:3 molar ratios). Chloroform was then removed by a rotary evaporator (Bibby Sterilin RE100, UK) at 42^0C and rotation speed setting of 8 to obtain a thin lipid film on the walls of a round bottom flask. The temperature of the water bath was not allowed to exceed the boiling temperature of the solvent (e.g. chloroform). Any residual chloroform in the film was removed by flushing

with nitrogen gas. Liposomes form spontaneously when the thin lipid film is hydrated. This is the step at which drug encapsulation is performed. At the hydration step typically 0.5 mL of drug solution or PB or calcein solution was introduced and agitated for 5 min on a vortex (Heidolph Reax Top, Germany). Multilamellar large vesicles are obtained as a result of the hydration step. The size and the lamellarity of the vesicles were reduced by homogenization using a probe sonicator (Cole Parmer Ins. Co. 4710 series, USA) at 20 Watts for 10 min with 30 s off and 30 s on intervals. Liposome solution was kept on ice during this step to absorb the heat generated during sonication. Resulting solution was applied onto a gel permeation chromatograph (GPC) system consisting of Sephadex G 50-80 to separate the drug containing liposomes from the unencapsulated drug. Eluates were collected as 1 mL fractions during 90 s for each fraction.

A typical liposome solution was prepared using 3 different ratios of the ingredients in order to achieve the highest encapsulation efficiency. PC:CHOL solutions with 7:1, 7:2, and 7:3 molar ratios were prepared with 15.4 mM PC and 2.2 mM, 4.4 mM, and 6.6 mM CHOL concentrations, respectively (Table 3).

2.2.1.2. Preparation of photoresponsive liposomes

Photoresponsive liposomes were also prepared by the same thin lipid film hydration technique (sec 2.2.1.1). The liposome components PC, CHOL and Suprofen (SPF) were dissolved in chloroform which was then removed by rotary evaporation as in the standard liposome preparation. Same steps of the standard liposome preparation procedure were followed. Photoresponsive liposomes were also prepared in 3 different ratios in order to determine the highest encapsulation value. PC:CHOL:SPF solutions of 7:1:3, 7:2:3, and 7:3:3 molar ratios were prepared with 15.4 mM PC, 6.6 mM SPF, and 2.2 mM, 4.4 mM, and 6.6 mM CHOL concentrations,

respectively. Table 3 presents the concentrations of all the ingredients for standard and photoactive liposomes.

Table 3. Compositions of various liposome formulations

	Composition (M)	PC (mM)	CHOL (mM)	SPF (mM)
Standard liposomes PC:CHOL	7:1	15.4	2.2	-
	7:2	15.4	4.4	-
	7:3	15.4	6.6	-
Photoresponsive liposomes PC:CHOL:SPF	7:1:3	15.4	2.2	6.6
	7:2:3	15.4	4.4	6.6
	7:3:3	15.4	6.6	6.6

2.2.2. Characterization of liposomes

After the preparation process, liposome suspension obtained was further processed for characterization. The liposomes prepared were first separated from unencapsulated drug and then examined in terms of size distribution, zeta potential and encapsulation efficiency. In situ release studies were also performed in order to determine the release behavior of the liposomal delivery system.

2.2.2.1. Gel permeation chromatography

In preparation of liposomes, thin lipid film was hydrated with the aqueous solution containing the drug. Hydration was enhanced with agitation with vortex and smaller vesicles were obtained with ultrasonication. This

liposome suspension includes both the liposomes and the unencapsulated drug. For separation process, gel permeation chromatography (GPC) was applied. The column used for this process was 15 cm in length and 1 cm in diameter. The column was filled with Sephadex G 50-80. Elates were collected manually as 1 mL fractions in 1.5 mL eppendorf tubes during 90 s for each fraction.

The eluates collected from gel permeation chromatography were analyzed with a UV-Vis spectrophotometer (Shimadzu UV-1201) at 410 nm for their UV absorbance and scatter. Only the liposome containing fractions are detected at this step due to the light scattering by the liposomes.

Preliminary drug entrapment studies were performed with calcein (CAL), and its detections to calculate encapsulation efficiency and rate of release were done with spectrofluorimetry of calcein (Shimadzu RF-5000) at excitation wavelength of 494 nm and emission wavelength of 517 nm. Resulting profile is expected to consist of two different peaks, one indicating the presence of liposome-encapsulated calcein, and the other referring to the free (unencapsulated) calcein.

2.2.2.2. Size distribution and zeta potential of liposomes

The eluates collected from GPC were analyzed with spectrophotometry and the eluates containing the liposome suspension were detected. These eluates were measured with Malvern Mastersizer 2000 (METU Central Laboratory) for their particle size determination. The same eluates were also analyzed with Malvern Nano ZS90 (METU Central Laboratory) for determination of the zeta potential, which is the overall charge that the vesicles acquire.

2.2.2.3. Encapsulation efficiency

Encapsulation efficiency is defined as the ratio of drug encapsulated in the liposomal fraction to total amount of the input drug. CAL was used as a model dye in the encapsulation efficiency calculations due to the ease of determination. Liposome suspension eluted from gel permeation chromatography (GPC) column was mixed with chloroform in a 1:1 ratio. Two phases form, upper one being the aqueous phase and the lower one being the organic phase. Organic phase includes the lipid components dissolved in chloroform and the aqueous part contains the calcein and the phosphate buffer (PB). The amount of calcein in upper phase is measured with spectrofluorimetry and corresponding concentration was calculated using the calibration curve (Appendix A). The concentration value obtained was then used in the calculation of the amount of encapsulated drug. Below is the equation used in calculating the encapsulation efficiency of liposomes.

$$EE\ (\%) = \frac{\text{CAL in liposomes (mg)}}{\text{total CAL in the input (mg)}} \times 100 \quad \text{.....(1)}$$

2.2.2.4. Photoresponsive in situ release

In situ release studies were conducted with calcein-loaded liposomes. Calcein (0.01 mL, 2 mM) was added at the hydration step at a final concentration of 0.04 mM. After spectrophotometric and spectrofluorimetric characterization, half of the liposome solution (1 mL) was exposed to UV-B (312 nm, 99 μWatt at a distance of 11 cm) for 45 minutes in a glass dish while the other half (1 mL) was kept in dark at room temperature. Then the two solutions (1 mL each) were placed in seperate dialysis bags (10,000 MWCO) and immersed into 40 mL phosphate buffer saline (PBS, 10 mM, pH 7.4). Spectrofluorimetric measurements were performed for calcein (λ_{ex} =

494 nm, λ_{em} = 517 nm) from the surrounding medium at predetermined time points. Release profiles (amount of calcein released versus time) were plotted.

2.2.3. In vitro studies

In vitro studies included the tests performed for determination of any adverse effects of standard and photoresponsive liposomes as well as UV exposure on Saos-2 cells. In addition to that, Cisplatin release from UV responsive liposomes was also examined with Saos-2 cells.

2.2.3.1. Cell type and culture conditions

Osteosarcoma cells (Human sarcoma osteogenic cell line, Saos-2, passage numbers between 17 and 21) was used in the *in vitro* studies to study the effectiveness of the liposome-encapsulated drugs. Cells were grown in RPMI-1640 medium (Hyclone) containing 10% of fetal bovine serum (FBS) in addition to antibiotics (Table 4).

Table 4. Cell culture medium antibiotics

Antibiotic	Concentration in Medium
Penicillin / Streptomycin	100 u·mL^{-1} / 100 µg·mL^{-1}
Vancomycin	100 µg·mL^{-1}
Amphotericin B	1 µg·mL^{-1}
Linco-Spectin	200 µg·mL^{-1}

The cells were maintained in a carbon dioxide incubator at 37^0C with a humidified environment of 5% CO_2. Cells were grown in tissue culture polystyrene (TCPS) flasks until they reached at least 70% confluency.

For passaging, cells were detached by trypsinization. After discarding the cell culture medium, the attached cells were washed with PBS and then incubated with Trypsin-EDTA (diluted to 0.05% in PBS) at 37^0C for 5 minutes. Detached cells were collected with RPMI cell culture medium including 10% serum and used for further seeding procedures.

2.2.3.2. Determination of Saos-2 cell numbers with MTS assay

Cell numbers were detemined with Cell Titer 96® Non-Radioactive Cell Proliferation Assay (MTS). Cells in the tissue culture plate wells were washed twice with PBS (10 mM, pH 7.4) to remove any residual medium. Later, they were incubated with DMEM low glucose medium containing 10% MTS which also contains 10% FBS and 1% antibiotics (Pen/Strep, Vancomycin) for 2 h at 37^0C in the 5% CO_2 environment. MTS solution is composed of a tetrazolium salt and an electron transfer reagent (phenazine methosulfate, PMS or phenazine ethosulfate, PES). The tetrazolium salt is reduced to a colored formazan molecule by the enzyme dehydrogenase which is found in metabolically active (living) cells (Figure 14). The amount of formazan is then determined spectrophotometrically at 490 nm using an ELISA microplate reader (Molecular Devices Inc., USA). The absorbance due to the formazan is converted to cell numbers by using a calibration curve (Appendix B).

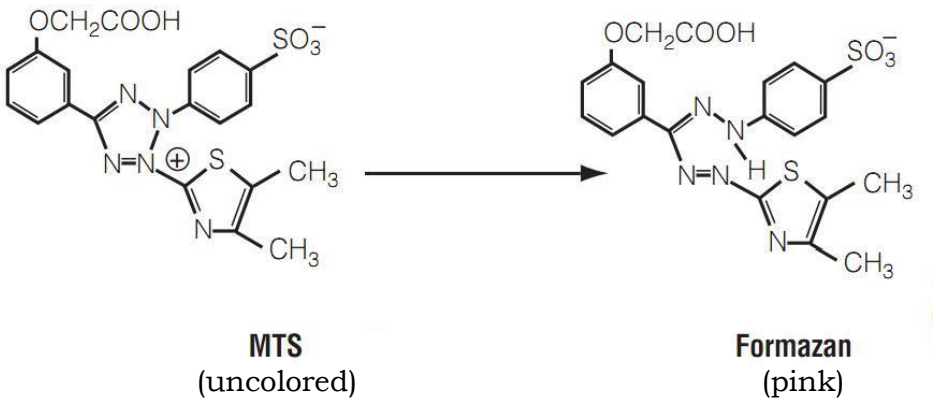


Figure 14. Reduction of MTS to Formazan

2.2.3.3. Effect of free Cisplatin on Saos-2 cell viability

The effect of free Cisplatin on Saos-2 cell viability was studied. Cells were seeded in 96-well plates ($2x10^4$ cells / well) and incubated in RPMI 1640 medium containing 10% FBS and antibiotics listed in Table 4 for at least 4 h to achieve cell attachment on the plate surface. Later 20% of the medium was withdrawn and replaced with equal volume of the free Cisplatin of varying concentrations (final concentrations in the cell medium were 0.25, 0.50, 0.75, 1.00, 1.25, 2.50, and 5.00 μg/mL). Control group cells received only the Cisplatin-free medium. MTS test was performed at predetermined time points (Day 1, Day 3, and Day 7) in order to study the effect of free Cisplatin on Saos-2 cells.

2.2.3.4. Effect of standard liposomes on Saos-2 cell viability

In order to detect any adverse effects that could arise from the presence of liposomes (PC:CHOL) in the cell culture, various amounts of liposomes were

added to the cell culture and proliferation was studied. Saos-2 cells were seeded in 96-well plates ($1.5x10^4$ cells / well) and incubated in RPMI 1640 cell medium containing 10% FBS and antibiotics listed in Table 4 for at least 4 h to achieve cell attachment on TCPS surface. Predetermined amounts of culture medium was withdrawn and replaced with liposome suspension (prepared as mentioned in Section 2.2.1.1.) or PB in control groups. Liposome suspension was added to culture medium to constitute a certain volume fraction i.e. 10%, 20%, and 30%. MTS test was performed at predetermined time points (Day 1, Day 3, and Day 7) to determine the proliferation of cells in the presence of standard liposomes of different volumes.

2.2.3.5. Effect of photoresponsive liposomes on Saos-2 cell viability

Photoresponsive liposomes contained Suprofen, which is also a non-steroid anti-inflammatory drug. These studies were conducted to examine how presence of Suprofen in the cell culture affects Saos-2 cell viability. In brief, predetermined amount of photoresponsive liposomes (PC:CHOL:SPF), (prepared as given in Section 2.2.1.2.) were added to the cell culture and cell viability was studied. Saos-2 cells were seeded in 96-well plates ($1.5x10^4$ cells / well) and incubated in RPMI 1640 culture medium containing 10% FBS and antibiotics listed in Table 4 for at least 4 h to achieve cell attachment on TCPS surface. 20% of cell culture medium was withdrawn and replaced with photoresponsive liposome suspension (which has been exposed to UV-B for 45 min as stated in Section 2.2.2.4.). One of the control groups included unexposed liposome suspension (20% of the culture medium) and the other contained only the culture medium (TCPS control). MTS test was performed at predetermined time points (Day 1, Day 3, and Day 7) to determine cell proliferation in the presence of photoresponsive liposomes.

2.2.3.6. Effect of UV-B exposure on Saos-2 cell viability

This study was performed to determine how exposure to UV-B affects the viability of Saos-2 cells. Cells were seeded in 96-well plates ($2x10^4$ cells / well) and incubated in RPMI 1640 medium containing 10% FBS and antibiotics listed in Table 4 for at least 4 h to achieve cell attachment on TCPS surface. Later cells were exposed to UV-B (312 nm, 99 µWatts at a distance of 11 cm) for 45 min. Control cells were not exposed to UV and included only the culture medium. UV exposed and unexposed cells were counted with MTS test as was reported in Section 2.2.3.2.

2.2.3.7. Effect of Cisplatin release from photoresponsive liposomes on Saos-2 viability

In vitro drug release studies were performed using Cisplatin as the bioactive agent. Photoresponsive liposomes were prepared as mentioned in section 2.2.1.2. At the hydration step, Cisplatin (500 µg/mL so that it would have a final concentration of 10 µg/mL with 2% encapsulation efficiency of liposomes) was encapsulated in the liposomes and the resultant liposomes were separated from unentrapped Cisplatin by gel permeation chromatography as explained in Section 2.2.2.1. The effect of Cisplatin release was tested with two studies. In the first study, photoresponsive liposomes were exposed to UV-B (312 nm, 99 µWatts at a distance of 11 cm) for 45 min before they were introduced into cell culture. In the second study, photoresponsive liposomes were first introduced into the cell culture, later the culture containing the cells and the photoresponsive liposomes were exposed to UV-B (312 nm, 99 µWatts at a distance of 11 cm for 45 min). In both cases cells were seeded in 96-well plates ($2x10^4$ cells / well) and incubated in RPMI 1640 medium containing 10% FBS and antibiotics

listed in Table 4 for at least 4 h to achieve cell attachment on TCPS surface. 20% of the culture medium was withdrawn and replaced with liposome suspension. One of the 3 control groups included unexposed photoresponsive liposomes at the same volume of exposed ones (20% of the culture medium). The second one included free Cisplatin (10 μg/mL) at the volume of 20% of the culture medium. The third control included only the culture medium. The number of Saos-2 cells was determined with MTS test on predetermined time points (Day 1, Day 3, and Day 7).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Characterization of liposomes

Several tests were conducted on the liposomes to make sure that the liposomes had the desired properties. Analyses performed were particle size determination, zeta potential analysis, encapsulation efficiency determination and in situ release studies.

3.1.1. Preparation of liposomes

GPC was used to separate the liposomes from unentrapped bioactive agents. Sephadex G 50-80 packed glass column constituted the major component of the gel permeation chromatography set up. The eluates were collected as 1 mL per tube. All the tube contents were tested with a spectrophotometer for their absorbance at λ = 410 nm for the detection of liposomes. The same eluates were also tested for their fluorescence intensity at λ_{ex} = 494 nm and λ_{em} = 517 nm using Shimadzu RF-5000 spectrofluorimeter. The data from both were plotted with 2 different y axes, one for absorbance, the other for fluorescence while the eluate number served as axis x (Figure 15).

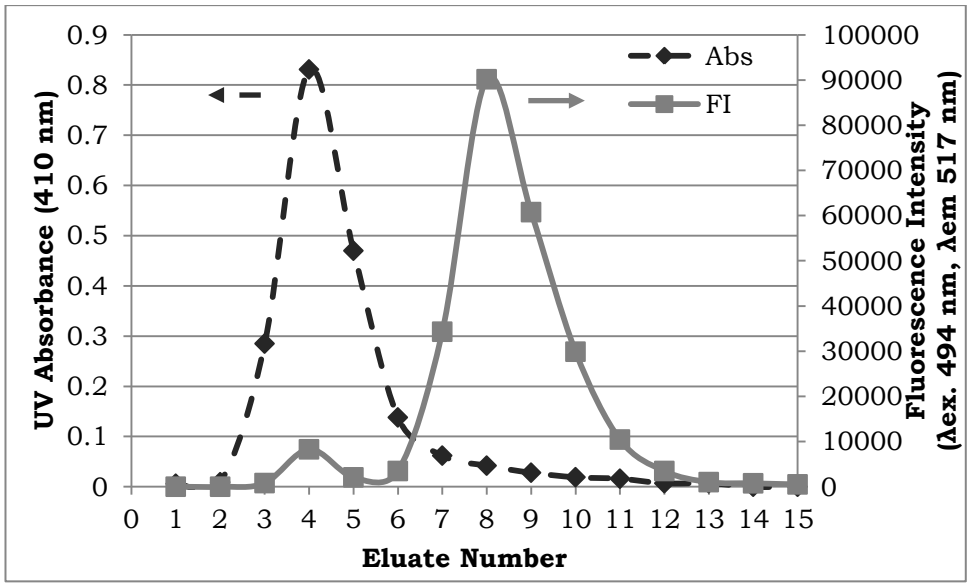


Figure 15. GPC of calcein loaded liposomes

The UV spectrometry results for liposome detection showed that liposomes were located in eluates number 3-6. This is also where the void is. The spectrofluorimetric measurement profile for calcein consists of two distinct peaks; one indicating the presence of liposome-encapsulated calcein (No 3-5), and the other referring to the free (unencapsulated) calcein (No 6-12). The first peak of fluorescence intensity overlaps with the peak of absorbance at 410 nm. These two spectroscopic measurements show that a small fraction of calcein is entrapped in the liposomes in eluates no 3-5 where the rest is unentrapped as shown in the second fluorescence peaks.

3.1.2. Size distribution and zeta potential of liposomes

The liposome type planned to be produced for this study was small unilamellar vesicles (SUVs) and the method to prepare the SUVs was selected to obtain SUVs.

Particle size analysis was carried out using Mastersizer 2000 and the size of liposomes in eluate no 3-6 results for each liposome formulation are presented in Table 5.

Table 5. Particle size analysis of different liposome formulations

Standard liposomes (PC:CHOL)	Particle size (nm)	Photoresponsive liposomes (PC:CHOL:SPF)	Particle size (nm)
7:3	106	**7:3:3**	114
7:2	103	**7:2:3**	112
7:1	82	**7:1:3**	111

According to the particle size analysis results, all the liposomes prepared in different formulations were in the range of SUV as a monodisperse population. Although, particle sizes of liposomes in different formulations are close to each other, there are slight differences. For example, sizes of the particles tend to increase as the cholesterol content increases. This indicates that increasing cholesterol content leads to more loose and a bit larger vesicles. Moreover, photoresponsive liposomes namely, SPF containing liposomes, have higher particle size compared to standard ones, SPF free liposomes. This shows that as the membrane gets crowded, larger vesicles are obtained.

Particle size analysis of 1-week-old liposomes yielded larger vesicles compared to freshly prepared counterparts (data not shown). This indicates the fusion of the vesicles and disintegration of the liposome membrane in 1 week duration.

Zeta potential analysis was performed with Malvern Nano ZS90. Liposome eluates (No 3-6) collected from GPC were analyzed for determination of zeta

potential value of the vesicles. Figure 16 is the result of zeta potential analysis.

Figure 16. Zeta potential analysis for standard liposomes (PC:CHOL 7:2)

Zeta potential analysis shows that the liposomes prepared have a negative zeta potential. The only peak presented in Figure 16 indicates the monodisperse population of the liposomes constituting the negative charge. Although the liposome constituents were chosen to obtain neutral vesicles, the liposomes have a negative zeta potential of -31 mV indicating that they have a net negative charge. This, however, is advantageous for the liposomes because their half life in the circulation would be longer due to avoiding the macrophages (Fraley *et al.*, 1981).

3.1.3. Encapsulation efficiency

Encapsulation efficiency (EE) is the measure of the fraction of input agent found in the liposomal product. This is important because when the EE is high, the amount of liposome that has to be added is low. Thus, it is a matter of economics and efficiency. This can be altered by varying certain number of steps during preparation.

3.1.3.1. Effect of ultrasonication duration

Ultrasonication is the step where MLVs, formed after hydration, are converted into SUVs. The power used during sonication and the duration of this process are expected to affect the size and the characteristics of the liposomes prepared.

In this study the power of the ultrasonic homogenization process was kept constant but the sonication durations were varied to test its effect on encapsulation efficiency. The longer duration of the sonication yields smaller vesicles.

Based on this, the surface to volume ratio would be largest in the smallest ones and this would lead them to entrap the lowest amount of aqueous solute, calcein in this case, in the core of the liposomes. This would lead to lowest EE (Table 6)

The encapsulation efficiencies and corresponding sizes for liposomes prepared with different sonication durations are given in Table 6.

Table 6. Calcein encapsulation efficiencies in liposomes (PC:CHOL 7:2) prepared with different sonication durations

Sonication duration (min)	Encapsulation efficiency (%)	Particle size (nm)
10	1.88 ± 0.33	103
15	0.82 ± 0.31	93
20	0.55 ± 0.15	82

Table 6 shows that as the duration of ultrasonic homogenization increases the encapsulation efficiency decreases. This situation is the same for size of the vesicles, longer the duration smaller the vesicles.

In order to obtain small unilamellar vesicles with high encapsulation efficiency 10 min ultrasonication was selected. Liposomes used in the further studies were prepared with 10 min (30 s on and 30 s off) ultrasonications.

3.1.3.2. Effect of cholesterol content

Different formulations of liposomes were prepared with 10 min ultrasonication and tested to study the effect of cholesterol on encapsulation efficiency. Table 7 is a summary of encapsulation efficiencies calculated for every liposome formulation.

Table 7. Calcein encapsulation efficiencies of various liposome formulations

Standard liposomes (PC:CHOL)	EE (%)	Photoresponsive liposomes (PC:CHOL:SPF)	EE (%)
7:3	0.90 ± 0.24	**7:3:3**	0.58 ± 0.15
7:2	1.88 ± 0.03	**7:2:3**	1.89 ± 0.04
7:1	1.26 ± 0.18	**7:1:3**	0.92 ± 0.10

In the standard liposomes (not sensitive to UV) it is observed that as the cholesterol content is increased from 7:1 to 7:2, the encapsulation efficiency is also increased significantly from 1.26 to 1.88 (Table 7). Upon further increase of cholesterol to 7:3 the encapsulation efficiency unexpectedly decreased. This was probably due to an increase in membrane rigidity caused by a high CHOL fraction.

Introduction of SPF to the bilayer composition followed basically the same trend. In this case, however, the EE was lower than those of standard (SPF-free) liposomes. In both cases the 7:2 or 7:2:3 formulations showed similar and the highest EE (Table 7).

It is reported that there is a competition between encapsulation efficiency of hydrophobic molecules and cholesterol content of liposome membrane. Zhang et al. (2005) reported that increasing cholesterol content (from 5 mol% to 37 mol%) resulted in a drammatic decrease in encapsulation efficiency of hydrophobic drug paclitaxel from 99.3% to 6.2%, respectively. However, there are cases where increasing cholesterol content has opposite effect on drug loading. Bhatia et al. (2004) reported that 30% cholesterol addition increased the entrapment efficiency of hydrophobic drug tamoxifen in PC liposomes from 45.2 to 57.5%.

The situation is different in water soluble molecule encapsulation. There is no competition between hydrophilic molecules and the membrane components. However, encapsulation efficiency of hydrophilic drugs is affected by the rigidity of the bilayer which is affected by CHOL content.

The highest encapsulation efficiencies were found to be 1.88±0.03% and 1.89±0.04% for 7:2 (PC:CHOL) and 7:2:3 (PC:CHOL:SPF) liposomes, respectively. Release and *in vitro* studies were, therefore, performed using those liposomes with the highest EE values.

3.1.4. In situ release kinetics

Release studies were designed to show the effect of UV-B on photoresponsive liposomes. The experiments were performed with calcein encapsulated in photoactive and standard liposomes and immersed in PBS in a dialysis bag. The amount of calcein released into the medium was determined with spectrofluorimetry at λ_{ex} = 417 nm and λ_{em} = 517 nm and percent calcein content released was calculated. Figure 17 is a presentation of in situ release profile plotted as percent calcein release vs time (days).

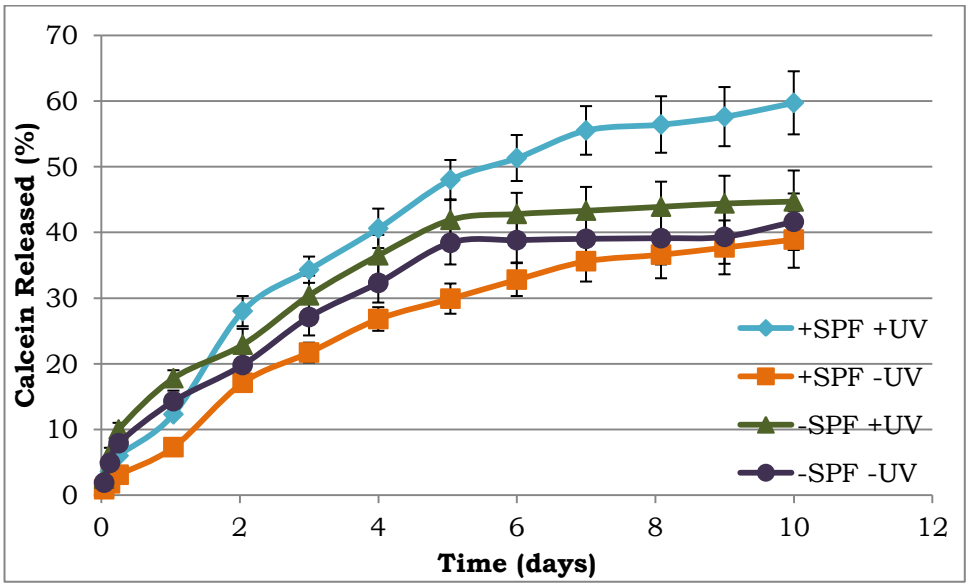


Figure 17. Influence of SPF presence and UV exposure on the release kinetics of calcein (+SPF: photosensitive, -SPF: standard, +UV: UV exposed, -UV: unexposed). n=3.

Release studies revealed that 25 to 40% of the calcein content was released in 4 days in almost a linear manner, then the release rate decreased. In 10 days the extent of release changed between 40 and 60%. It is seen that the highest release is observed with the SPF containing samples upon UV exposure. The lowest release rate was expected to be obtained with SPF free UV unexposed liposomes. However, lowest rates were observed with the unexposed photosensitive liposomes. This shows that SPF incorporation into the bilayer lead to a more rigid structure resulting in less release. As the SPF free liposomes (UV exposed and unexposed ones) were compared, no significant difference was observed. This shows that UV exposure itself does not have a major role in membrane destabilization. The significance of UV exposure is obtained with SPF incorporated liposomes.

Another observation was that as the SPF containing liposomes continued releasing for the whole 10-day-duration while the SPF-free liposomes almost stopped on day 5 at around 40%. The lowest rate was observed with unexposed SPF loaded sample indicating that it became less permeable probably due to higher rigidity of the membrane.

Release rate coefficients were calculated for photoresponsive and standard liposomes according to Higuchi equation (M_t/M_∞ vs $t^{1/2}$) (Table 8).

Table 8. Release rate coefficients according to Higuchi

Composition	k (UV exposed)	k (UV unexposed)
PC:CHOL (7:2)	3.58 ± 0.31	3.12 ± 0.40
PC:CHOL:SPF (7:2:3)	3.85 ± 0.26	2.44 ± 0.18

A linear correlation was obtained with the percent of drug released vs the square root of time as in the Higuchi equation (Vemuri and Rhodes, 1995). Statistical tests support that there is a significant difference between the release rate coefficients of UV exposed and unexposed photosensitive

liposomes (T-test, p=0.05). The data show that UV exposure leads to a higher release in comparison to no exposure (Release increases from 35% up to 60% with the effect of UV exposure). It is also observed that upon UV exposure the photosensitive liposomes released faster indicating that the introduction of SPF into the membrane composition made the liposome photosensitive. Among the unexposed samples the SPF liposomes released slower probably because of the SPF causing increased rigidity as was implied in the EE studies (Section 3.1.4.2.).

3.2. Cell culture studies

Effects of the standard liposomes, photoresponsive liposomes, free Cisplatin, Cisplatin loaded photoresponsive liposomes as well as UV-B exposure on cell viabilities were examined by using Saos-2 cells.

3.2.1. Effect of standard liposomes on cell viability

Standard liposomes were added onto Saos-2 cells to determine whether liposomes themselves have any toxic effect. To test this, standard liposomes were added into the cell culture medium to constitute up to 30% of the medium. Figure 18 presents the cell viabilities of Saos-2 cells in the culture medium containing standard liposomes at different concentrations and at different incubation periods. Controls had PBS instead of liposomes.

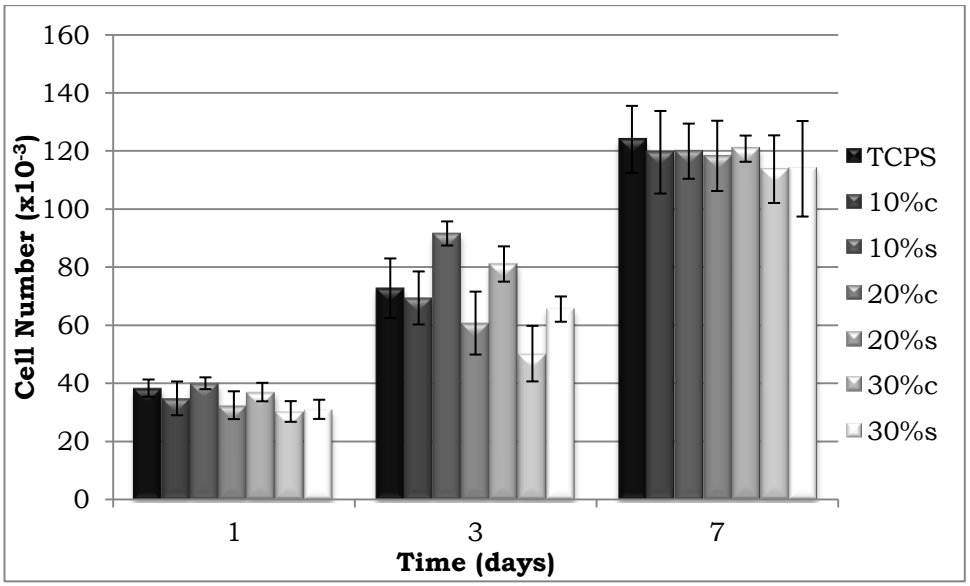


Figure 18. Influence of presence of standard liposomes on Saos-2 cell viability (PC:CHOL 7:2) (Percent of the cell culture medium constituted of liposomes, c: control group (without liposome), s: sample group (with liposome)). Cell number 1.5x10^4, n=3.

Figure 18 shows that the presence of standard liposomes up to 30% of the medium volume had no adverse effect on Saos-2 cell viability. Controls where the medium was substituted with PBS, showed a decrease in comparison to their liposome containing counterparts. This effect was distinct on Days 1 and 3 but quite leveled off by Day 7. However, these were not valid for the 30% sample which always showed the lowest viability although it was not highly different than the others. The TCPS control was always comparable to the 10% and 20% samples. This indicates that the concentration of the medium is quite critical for cell viability and when diluted by 30% then the medium became unsuitable for cell growth. Below that the cells could tolerate it.

3.2.2. Effect of photoresponsive liposomes on cell viability

Construction of photoresponsive liposomes were the main goal of this whole study. One expectation was that SPF, without the anticancer drug could be effective. Saos-2 cells were used to determine the effect of SPF in the liposome bilayer on cell viability. Cisplatin-free photoresponsive liposomes were exposed to UV-B to activate the decarboxylation of SPF. Figure 19 presents the cell viability results for Saos-2 cells in the presence of UV exposed photoresponsive liposomes at a volume of 20% of the culture medium. Unexposed cells included unexposed photoresponsive liposomes at a volume of 20% of the culture medium while the TCPS group included only the culture medium.

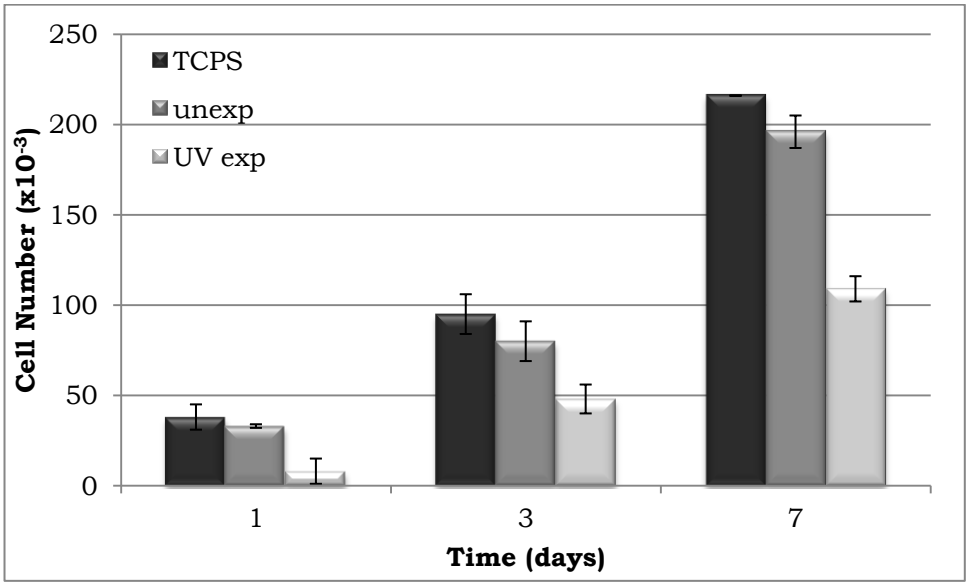


Figure 19. Saos-2 cell viability upon exposure of photoresponsive liposomes (PC:CHOL:SPF 7:2:3) to UV. Cell number $1.5x10^4$, n=3.

The test was made by exposing the liposome suspension to UV-B (312 nm, 99 μWatt at a distance of 11 cm) for 45 min and then adding the suspension into the culture medium (at a volume of 20% of the medium).

It is clearly seen that UV exposed photoresponsive liposomes (SPF containing liposomes) have decreased cell proliferation while unexposed liposomes and TCPS control cells had comparable values. It was previously stated that when suprofen is exposed to UV, it undergoes decarboxylation reaction bringing out highly reactive singlet oxygen molecules (Figure 11). These molecules are responsible for cells undergoing apoptosis.

Thus, the main cause for decreased cell viability is suprofen and its metabolites that arise in decarboxylation of SPF upon UV exposure.

3.2.3. Effect of free Cisplatin on cell viability

Free Cisplatin (without any carriers) was applied on Saos-2 cells to determine the appropriate lethal dose to be used in designing photoresponsive liposomes. Cisplatin was added into the culture medium at different concentrations (0.25-5 μg/mL) at the volume of 20% of the culture medium. Cell viability was determined on Days 1, 3 and 7 with MTS assay. Figure 20 is a presentation of viability of Saos-2 cells grown in culture medium containing Cisplatin at different concentrations.

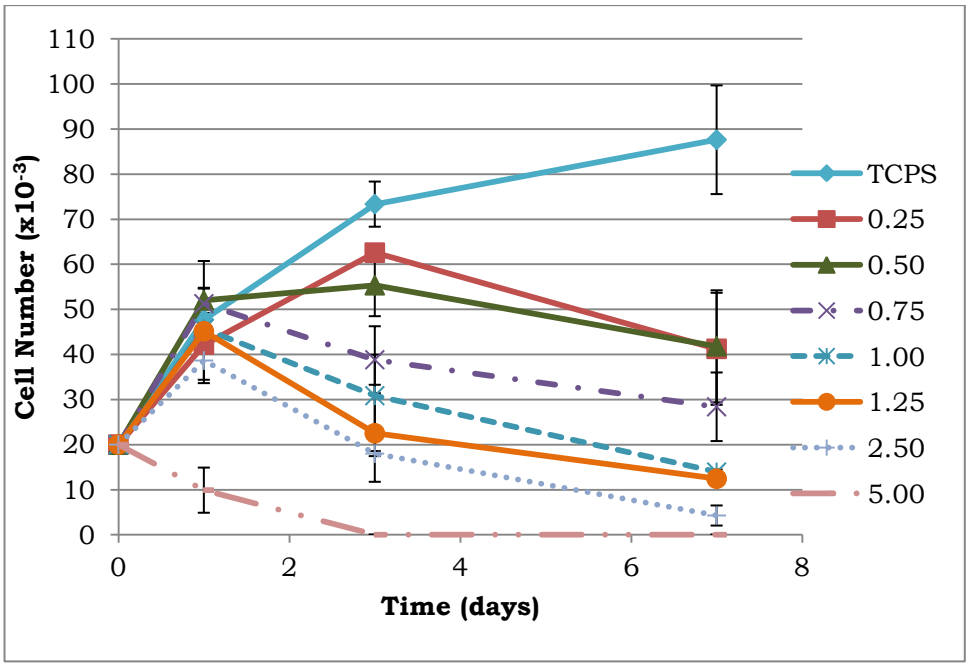


Figure 20. The effect of Cisplatin concentration on Saos-2 cell viability (concentrations are in µg/mL). Cell number 2x10⁴, n=3.

On Day 1, it is observed that Cisplatin presence did not have too significant effect on cell viability except 5 µg/mL. The lethal effect of Cisplatin was better observed on Days 3 and 7. It is clearly seen that Cisplatin of 5 µg/mL killed all the cells on Days 3 and 7 while the other doses caused continuing decrease in cell numbers as the time progressed. This is expected because as the cell division is prevented more and more there was a drop in cell numbers. No cell viability was detected on Days 3 and 7 at concentration of 5 µg/mL.

Depending on these results, highest concentration of Cisplatin was chosen in order to see the effect of release from photoactive liposomes. So, the dose of Cisplatin to be used in *in vitro* release studies was determined to be 5 µg/mL (or a bit higher i.e. 10 µg/mL in order to see the effects better) as a result of this test.

3.2.4. Effect of UV-B exposure on cell viability

The responsiveness of the drug delivery system designed in this study was planned to be achieved upon UV exposure. It is planed that Cisplatin loaded liposomes will be administered through the circulatory system and are expected to release their content wherever the tissues are exposed to UV. The influence of UV exposure used in these tests is close to that of gained from the sun. So it was expected that exposure of Saos-2 cells to UV would have no adverse effect on cell proliferation. UV exposure processes for the photoresponsiveness of the liposomes were always performed with the same light source under the same conditions namely, from the same distance, for the same time duration and in the same container. Figure 21 presents the effect of this UV exposure on Saos-2 cell viability.

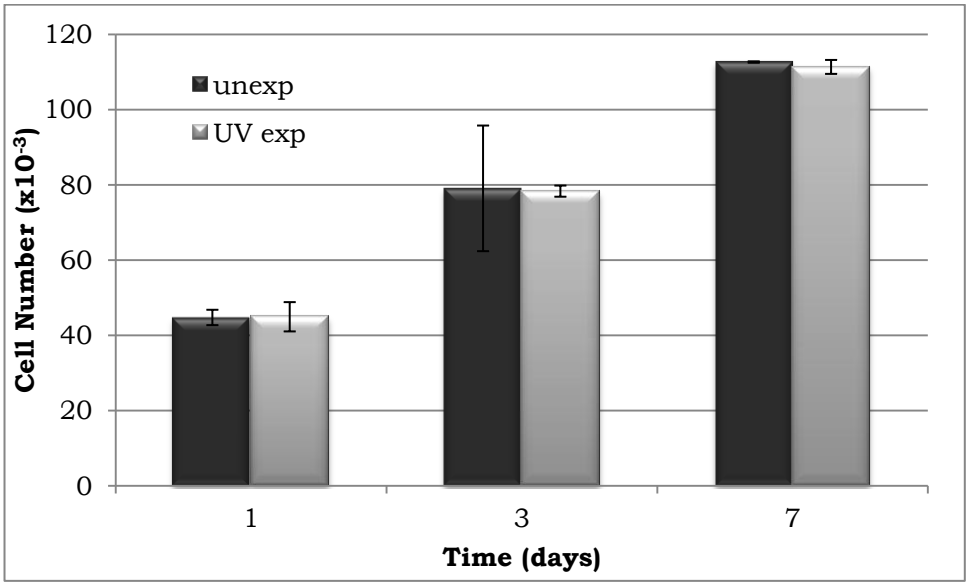


Figure 21. Saos-2 cell viability profile upon exposure to UV-B (312 nm, distance 11 cm, duration 45 min). Cell number $2x10^4$. n=3

According to the plot, no significant difference was observed between the UV exposed cells and the unexposed cells. Both groups of cells showed the same proliferation trend during the 7 day period. This, therefore, shows that the UV dose applied in this study to obtain the responsiveness has no harm on cell proliferation. In other words, any effect observed in cell viability is not due to exposure to UV.

3.2.5. Effect of Cisplatin release from photoresponsive liposomes on cell viability

Cisplatin loaded SPF incorporated liposomes were added onto Saos-2 cells to observe the effect of photoresponsive release from Cisplatin loaded liposomes. Here two types of tests were run. In the first one, liposomes were first exposed to UV and then added to the cell culture medium (Figure 22). In the second one, liposomes were first added to the cell culture medium and then the cells and the liposomes were exposed to UV together (Figure 23). One control group was unexposed cells and liposomes, another was TCPS control containing only the cells in the culture medium, and in the third control group free Cisplatin (10 μg/mL) was added to the culture medium at a volume of 20% of the culture medium.

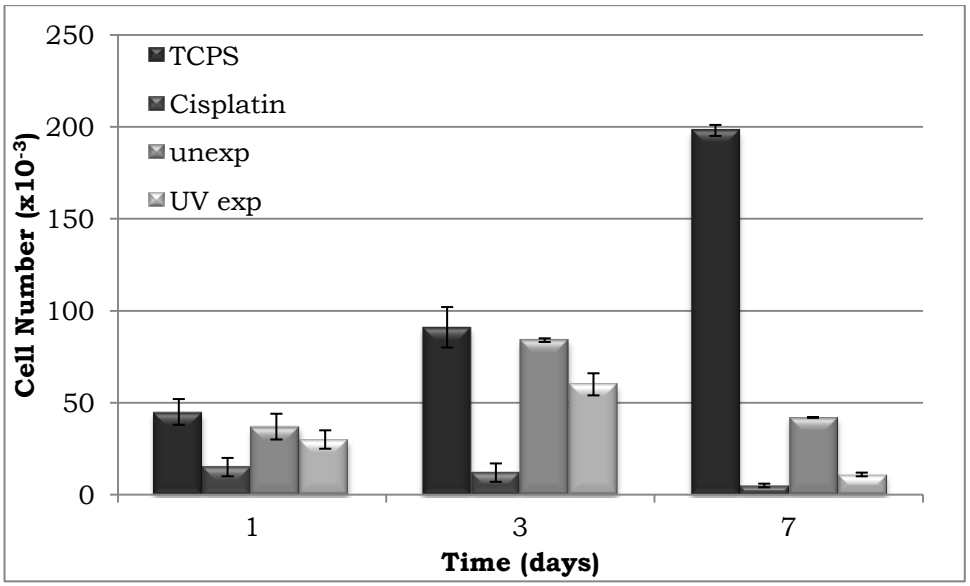


Figure 22. Saos-2 cell viability upon addition of Cisplatin loaded photoresponsive liposomes (PC:CHOL:SPF 7:2:3). Controls are free Cisplatin and untreated cells on TCPS. Liposomes were exposed to UV before addition to cell culture medium. Cell number $2x10^4$, n=3.

Cell viability profile gives indirect information about the Cisplatin release from photoactive liposomes. As the Cisplatin is released from the liposomes, it leads the cells to apoptosis; therefore, cell viability decreases. With the unexposed control, even though Cisplatin is in the medium encapsulated in liposomes, only a slight decrease in viability is observed, which proves the UV exposure effect on the system constructed. Another observation is that free Cisplatin added cells went to apoptosis immediately on Day 1 while the fatal effect of Cisplatin in photoresponsive liposomes was observed in longer time. This also proves that even without photoresponsiveness, liposomes are effective protectors of the environment from the effects of Cisplatin.

The significant decrease in the exposed samples is a clear proof that UV exposure releases the Cisplatin and our liposomal construct functions well as an effective responsive system.

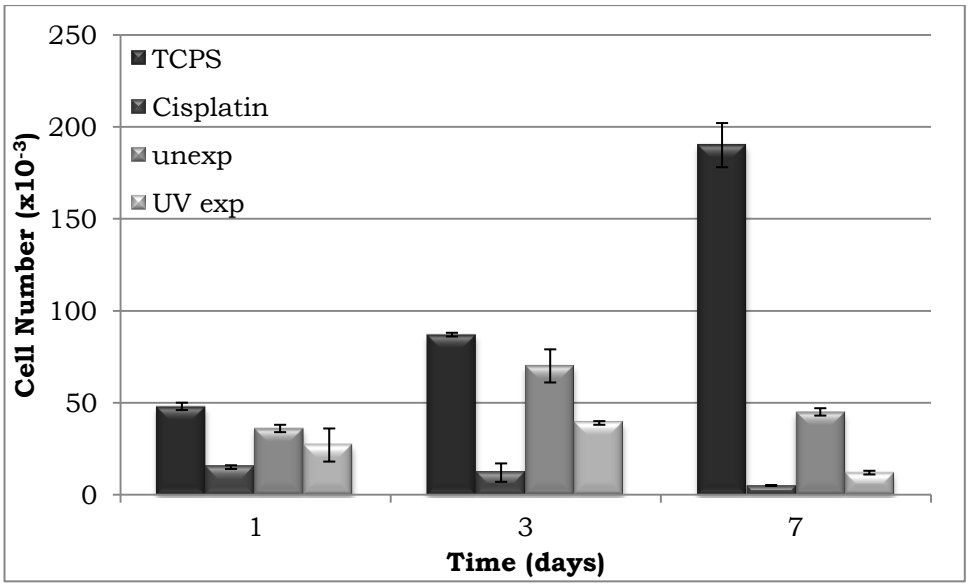


Figure 23. Saos-2 cell viability upon addition of Cisplatin loaded photoactive liposomes (PC:CHOL:SPF 7:2:3). Liposomes were first added in the culture medium and then the whole cell culture was exposed to UV. Cell number $2x10^4$, n=3.

A similar trend in cell viability was observed in the second test (Figure 23). Cell viability was lower at the group of cells containing UV exposed liposomes compared to unexposed ones indicating the photoresponsive Cisplatin release from SPF incorporated liposomes. The fatal effect of Cisplatin in photoresponsive liposomes was again observed in time instead of immediately on Day 1, as observed on the free Cisplatin added cells. The second test (Figure 23) proves that the drug delivery system, developed through this study, can be introduced to an organism and local release of the drug can be achieved upon external UV exposure at a specific area.

CHAPTER 4

CONCLUSION

Through this study a photoresponsive drug delivery system encapsulating anticancer agent Cisplatin was successfully developed using Suprofen (SPF) incorporated liposomes. Photoresponsive release was achieved through liposomes upon UV exposure. The dual activity of SPF, destabilization of the lipid membrane upon UV exposure and apoptosis of cells through photosensitization, was also confirmed in the *in vitro* studies which is a first in the literature.

Nevertheless, there are several points that can be further improved. For instance, the encapsulation efficiency of the liposomes was found to be low (~2%). The type of the liposomal system could be altered by applying a different preparation method in order to yield liposomes with higher encapsulation efficiency. Moreover, the duration of UV exposure should be reduced because 45 min is a considerably long time. Although, the intensity of the UV light source used in the study was shown to be harmless on Saos-2 cells, it is still not safe to be exposed to a UV source for that long time. The system can be developed with a different molecule that can lead to destabilization of the membrane in a shorter time of UV exposure.

In addition, in vivo experiments should be performed to study the effectiveness of photoresponsive delivery. Modifications of the chemical composition should be made to prolong the half life of the liposomes in the systemic circulation.

REFERENCES

Alexis F., Pridgen E., Molnar L. K., Farokhzad O. C., Factors affecting the clearance and biodistribution of polymeric nanoparticles, *Mol. Pharm.* 5:505–515, 2008.

Arias J. L., Clares B., Morales M. E., Gallardo V., Ruiz M. A., Lipid-based drug delivery systems for cancer treatment, *Curr. Drug Targets.* 12:1151-1165, 2011.

Bangham A. D., Standish M. M., Watkins J. C., Diffusion of univalent ions across the lamellae of swollen phospholipids, *J. Mol. Biol.*, 13:238-252, 1965.

Basel M. T., Shrestha T. B., Troyer D. L., Bossmann S. H., Protease-Sensitive, Polymer-Caged Liposomes: A method for making highly targeted liposomes using triggered release, *ACS Nano*. 5:2162–2175, 2011.

Basmanav F. B., Kose G. T., Hasirci V., Sequential growth factor delivery from complexed microspheres for bone tissue engineering. *Biomaterials.* 29:4195-4204, 2008.

Batzri S., Korn E. D., Single bilayer liposomes prepared without sonication, *(BBA) – Biomembranes.* 298:1015-1019, 1973.

Bhatia A., Kumar R., Katare O. P., Tamoxifen in topical liposomes: development, characterization and in-vitro evaluation, *J. Pharm. Pharm. Sci.* 7:252-259, 2004.

Bikram M., West J. L., Thermo-responsive systems for controlled drug delivery, *Expert Opin. Drug Del.* 5:1077-1091, 2008.

Bisby R. H., Mead C., Morgan C. C., Wavelength-programmed solute release from photosensitive liposomes, *Biochem. Biophys. Res. Commun.* 276, 169–173, 2000.

Boulikas T., Vougiouka M., Cisplatin and platinum drugs at the molecular level, *Onc. Rep.* 10:1663-1682, 2003.

Bourne D.W., Pharmacokinetics. Eds. Banker G. S., Rhodes C. T., In *Modern pharmaceutics*. 4th ed., Marcel Dekker Inc, New York, 2002.

Brewer E., Coleman J., Lowman A., Emerging technologies of polymeric nanoparticles in cancer drug delivery, *J. Nanomater.* 2011:1-10, 2011.

Castelli F., De Guidi G., Giuffrida S., Miano P., Sortino S., Molecular mechanism of photosensitization XIII. A combined differential scan calorimetry and DNA photosensitization study in non-steroidal antiinflammatory drug-DNA interaction, *Int. J. Pharm.* 184:21-33, 1999.

Chen Y., Bothun G. D., Cationic gel-phase liposomes with “decorated” anionic SPIO nanoparticles: morphology, colloidal, and bilayer properties, *Langmuir*. 27:8645–8652, 2011.

Cheng N., Xia T., Han Y., He Q. J., Zhao R., Ma J. R., Synergistic antitumor effects of liposomal honokiol combined with Cisplatin in colon cancer models, *Oncol. Letters*. 957-962, 2011

Choudhury S. R., Nair K. K., Kumar R., Gogoi R., Srivastava C., Gopal M, Subhramanyam B. S., Devakumar C., Goswami A., Nanosulfur: a potent fungicide against food pathogen, *Aspergillus Niger.* 1276:154-157, 2010.

Chung K. F., Barens P. J., Drug treatment of asthma, *Drug of today,* 25:721-732, 1989.

Cirli O O, Hasirci V. UV-induced drug release from photoactive REV sensitized by suprofen. *J. Cont. Release.* 96:85-96, 2004.

Condorelli G., Costanzo L. L., De Guidi G., Giuffrida S., Miano P., Sortino S., Velardita A., Photosensitization induced by non steroidal anti-inflammatory drugs: an overview of molecular mechanisms in biological systems, *EPA Newsletters*. 58:60-77, 1996.

Dash S., Murthy P. N., Nath L., Chowdhury P., Kinetic modeling on drug release from controlled drug delivery systems, *Acta Pol. Pharm.* 67:217-223, 2010.

Deamer D., Bangham A. D., Large volume liposomes by an ether vaporization method, *Biochimica et Biophysica Acta (BBA) – Biomembranes.* 443:629-634, 1976.

Demirbag B., Kardesler S., Buyuksungur A., Kucukturhan A., Eke G., Hasirci N., Hasirci V., Nanotechnology in biomaterials: nanoparticulates as drug delivery systems, in Global Prospects in Nanobiotechnology, (Article in Press).

Djanashvili K., ten Hagen T. L., Blangé R., Schipper D., Peters J. A., Koning G. A., Development of a liposomal delivery system for temperature-triggered release of a tumor targeting agent, Ln(III)-DOTA-phenylboronate, *Bioorg. Med. Chem.* 19:1123-1130, 2011.

Domb J. A., Kumar N., Sheskin T., Bentolila A., Slager J., Teomim D., Biodegradable polymers as drug carrier systems, In *Polymeric Biomaterials,* Marcel Dekker Inc. 91-98, 2002.

Drummond D. C., Zignani M., Leroux J.-C., Current status of pH-sensitive liposomes in drug delivery, *Prog. Lipid Res.* 39:409–460, 2000.

Forssen E. A., Male-Brune R., Alder-Moore J. P., Lee M. J. A., Schmit P. G., Krasieva T. B., Shimizu S., Fluorescence imaging studies for the disposition of daunorubicin liposomes (DaunoXome) within tumor tissue, *Cancer Res.* 56:2066-2075, 1996.

Forssen E. A., Ross M. E., Daunoxome treatment of solid tumors: preclinical and clinical investigations, *J. Liposomes Res.* 4:481-512, 1994.

Fraley R., Straubinger R. M., Rule G., Springer E. L, Papahadjopoulos D., Liposome-mediated delivery of deoxyribonucleic acid to cells: enhanced

efficiency of delivery related to lipid composition and incubation conditions, *Biochemistry*. 20:6978-6987, 1981.

Garg A., Kokkoli E., pH-sensitive PEGylated liposomes functionalized with a fibronectin-mimetic peptide show enhanced intracellular delivery to colon cancer cells, *Curr. Pharm. Biotechnol.* 12:1135-1143, 2011.

Gelasco A., Lippart S. J., NMR solution structure of a DNA dodecamer duplex containing a cis-diammineplatinum(II) d(GpG) intrastrand cross-link, the major adduct of the anticancer drug Cisplatin, *Biochemistry*. 37:9230–9239, 1998.

Gibbons A., McElvaney N. G., Cryan S-A., A dry powder formulation of liposome-encapsulated recombinant secretory leukocyte protease inhibitor (rSLPI) for inhalation: preparation and characterisation, *AAPS Pharm. Sci. Tech.* 11:1411-1421, 2010.

Gluck R., Liposomal Hepatitis A vaccine and liposomal multiantigen combination vaccines, *J. Liposome Res.,* 5:467-469,1995

Gregoriadis G., McCormack B., Perrie Y., Saffie R., DNA vaccination: a role for liposomes, Eds. Lasic D. D., Papahadjopoulos D., In *Medical applications of liposomes*, Elsevier, Amsterdam, 61-65, 1998

De Guidi G., Ragusa S., Cambria M. T., Belvedere A., Catalfo A., Cambria A., Photosensitizing effect of some nonsteroidal antiinflammatory drugs on natural and artificial membranes: dependence on phospholipid composition, *Chem. Res. Toxicol.* 18:204–212, 2005.

Gursel I., Hasirci V., Influence of membrane components on the stability and drug release properties of reverse phase evaporation vesicles (REVs): light sensitive all-trans retinal, negatively charged phospholipid dicetylphosphate and cholesterol, *J. Microencap.* 12:661-669, 1995.

Hadjiioannou T. P., Christian G. D., Koupparis M. A., Quantitative calculations in pharmaceutical practice and research, VCH Publishers Inc., New York, 1993.

Hamada T., Ishii K.-I., Sugimoto R., Nagasaki T., Takagi M., Photochemical control on morphologies of a cell-sized synthetic vesicle, *Micro-NanoMechatronic. Human Sci.* 161-165, 2009.

Hamilton R L Jr, Goerke J, Guo L S S, Williams M M C, Havel R J. Unilamellar liposomes made with the french pressure cell: a simple preparative and semi-quantitative technique. *J. Lipid Research* 2:981-992, 1980.

Hauser H, Gains N, Mueller M, Vesiculation of unsonicated phospholipid dispersions containing phosphatidic acid by pH adjustment: physicochemical properties of the resulting unilamellar vesicles, *Biochemistry*. 22:4775–4781, 1983.

Hendricks M., Davidson A., Pillay K., Desai F., Millar A., Carboplatin-based chemotherapy and surgery: a cost effective treatment strategy for malignant extracranial germ cell tumors in the developing world, *Pediatr Blood Cancer*. 57:172–174, 2011.

Hiemenz J. W., Walsh T. J., Lipid formulations of amphotericin B: recent progress and future directions, *Clin. Infect. Diseases.* 22:133-144, 1996

Hixson A. W., Crowell J. H., Dependence of reaction velocity upon surface and agitation: I-theoretical consideration, *Ind. Eng. Chem.* 23:923-931 1931.

Huang S., Li J., Han L., Liu S., Ma H., Huang R., Jiang C., Dual targeting effect of angiopep-2-modified, DNA-loaded nanoparticles for glioma, *Biomaterials*. 32:6832-6838, 2011.

Hughes G. A., Nanostructure-mediated drug delivery. *Nanomed.-Nanotechnol.* 1:22–30, 2005.

Jahn A., Vreeland W. N., DeVoe D. L., Locascio L. E., Gaitan M., Microfluidic directed formation of liposomes of controlled size, *Langmuir.* 23:6289-6293, 2007.

Jain R. A., Rhodes C. T., Railkar A. M., Malick A. W., Shah N. H., Controlled delivery of drugs from a novel injectable *in situ* formed biodegradable PLGA microsphere system, *J. Microencapsul.* 17:343-362, 2000.

Jessirie D., Hilton J. K., Amanda V. E., Nanotechnology as a new tool for fingermark detection: a review, *Curr. Nanosci.* 7:153-159, 2011.

Jiang N., Zhang X., Zheng X., Chen D., Zhang Y., Siu L. K. S., Xin H-B., Li R., Zhao H., Riordan N., Ichim T. E., Quan D., Jevnikar A. M., Chen G., Min W., Targeted gene silencing of TLR4 using liposomal nanoparticles for preventing liver ischemia reperfusion injury, *Am. J. Transplant.* 11:1835–1844, 2011.

Klefah A. K., Eriksson M., Eriksson L. A., Photodegradation mechanism of nonsteroidal anti-inflammatory drugs containing thiophene moieties: suprofen and tiaprofenic acid, *J. Phys. Chem. B.* 113:11306–11313, 2009.

Kono K., Nakashima S., Kokuryo D., Aoki I., Shimomoto H., Aoshima S., Maruyama K., Yuba E., Kojima C., Harada A., Ishizaka Y., Multi-functional liposomes having temperature-triggered release and magnetic resonance imaging for tumor-specific chemotherapy, *Biomaterials*. 32:1387-1395, 2011.

Kumari A., Yadav S. K., Yadav S. C., Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B: Biointerfaces.* 75:1-18, 2010.

Langer R., New methods of drug delivery, *Science*. 249:1527-1532, 1990.

Lasic D. D., Papahadjopoulos D., Liposomes in medicine, In *Medical applications of liposomes* Eds. Lasic D. D., Papahadjopoulos D., Elsevier, Amsterdam, 9-12, 1998

Li Z., Guan J., Thermosensitive hydrogels for drug delivery, *Exp. Opin. Drug Del.* 8:991-1007, 2011

Lian T., Ho R. J. Y., Trends and developments in liposome drug delivery systems, *J. Pharma. Sci.* 90:667-680, 2001.

Liong M., Lu J., Kovochich M., Xia T., Ruehm S. G., Nel A. E., Tamanoi F., Zink J. I., Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. *J. Am. Chem. Soc.* 2:889-896, 2008.

Maderuelo C., Zarzuelo A., Lanao J. M., Critical factors in the release of drugs from sustained release hydrophilic matrices, *J. Control. Release*. 154: 2-19, 2011.

Matsumura Y., Maeda H., A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, *Cancer Res.* 46:6387–6392, 1986.

Meure L. A., Foster N. R., Dehghani F., Conventional and dense gas techniques for the production of liposomes: a review, *AAPS Pharm. Sci. Tech.* 9:798-809, 2008.

Michalke B., Platinum speciation used for elucidating activation or inhibition of Pt-containing anti-cancer drugs, *J. Trace Elem. Med. Bio.* 24: 69–77, 2010.

Miguel C. E., Bestetti R. B., Primary cardiac lymphoma, *Int. J. Cardiol.* 149:358-363, 2011.

Mikhaylov G., Mikac U., Magaeva A. A., Itin V. I., Naiden E. P., Psakhye I., Babes L., Reinheckel T., Peters C., Zeiser R., Bogyo M., Turk V., Psakhye S. G., Turk B., Vasiljeva O., Ferri-liposomes as an MRI-visible drug-delivery system for targeting tumours and their microenvironment, *Nature Nanotechnol.* 6:594–602, 2011.

Montazer M., Pakdel E., Behzadnia A., Novel feature of nano-titanium dioxide on textiles: Antifelting and antibacterial wool, *J. App. Polymer Sci.* 121:3407–3413, 2011.

Mozafari M. R., Liposomes: an overviw of manufacturing techniques, *Cell. Mol. Biol.* 10:711–719, 2005.

Mudshinge S. R., Deore A. B., Patil S., Bhalgat C. M., Nanoparticles: emerging carriers for drug delivery, *Saudi Pharm. J.*, 19:129-141, 2011.

Nair L. S., Laurencin C. T., Polymers as biomaterials for tissue engineering and controlled drug delivery, *Adv. Biochem Eng. Biotechnol.* 102:47-90 2006.

New R. R. C., Preparation of liposomes, In *Liposomes: a practical approach*, Ed. New R. R. C., Oxford University Press, London, 33-104, 1990

Olson F., Hunt C. A., Szoka F. C., Vail W. J., Papahadjopoulos D., Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys Acta* 557:9–23, 1979.

Patil S. G., Gattani S. G., Gaud R. S., Surana S. J., Dewani S. P., Mahajan H. S., *The Pharma Review.* 18:53-58, 2005.

Pierigè F., Serafini S., Rossi L., Magnani M., Cell-based drug delivery, *Adv. Drug Del. Rev.* 60:286–295, 2008.

Pil P., Lippard S. J., Cisplatin and related drugs, In *Encyclopedia of Cancer*, Ed. J. R. Bertino, Academic Press: San Diego, 392-410, 1997

Pillai O., Panchagnula R., Polymers in drug delivery, *Curr. Opi. Chem. Biol.* 5:447-451, 2001.

Preiss M. R., Bothun G. D., Stimuli-responsive liposome-nanoparticle assemblies, *Exp. Opin. Drug Del.* 8:1025-1040, 2011.

Qi W-X., Shen Z., He A-N., Yao Y., Evaluation of pirarubicin–Cisplatin chemotherapy in the treatment for refractory and recurrent high-grade osteosarcoma: experience of a single institute, *Med. Oncol.* 2011 (Article in Press).

Qi X., Song X., Liu P., Yi T., Li S., Xie C, Zheng Y., Bai Y., Sun C., Wei Y., Zhao X., Antitumor effects of PLGA nanoparticles encapsulating the human PNAS-4 gene combined with Cisplatin in ovarian cancer, *Oncol. Reports*. 3:703-710, 2011

Rijcken C. J., Soga O., Hennink W. E., van Nostrum C. F., Triggered destabilisation of polymeric micelles and vesicles by changing polymers polarity: An attractive tool for drug delivery, *J. Control. Release.* 120:131-148, 2007.

Rong Liu, Water insoluble drug formulation, *CRC Press 2nd Ed.* 2008

Rosenberg B., The start platinum complexes for the treatment of cancer: why the search goes on, In *Cisplatin: chemistry and biochemistry of a leading anticancer drug*, Eds. Lippert B., Helvetica Chimica Acta, Zurich, 3–30, 1999.

Rui Y., Wang S., Low P. S., Thompson D. H., Diplasmenylcholine−folate iiposomes: an efficient vehicle for intracellular drug delivery, *J. Am. Chem. Soc.* 120:11213–11218, 1998.

Shamay Y., Adar L., Ashkenasy G., David A., Light induced drug delivery into cancer cells, *Biomaterials*. 32:1377-1386, 2011.

Shum P., Kim J-M., Thompson D. H., Phototriggering of liposomal drug delivery systems, *Adv. Drug Deliv. Rev.* 54:273–284, 2001.

Siddik Z. H., Cisplatin: mode of cytotoxic action and molecular basis of resistance, *Oncogene*. 22:7265–7279, 2003.

Silverman A. P., Bu W., Cohen S. M., Lippard S. J.. 2.4-A crystal structure of the asymmetric platinum complex [Pt(ammine)(cyclohexylamine)](2+) bound to a dodecamer DNA complex, *J. Biol. Chem.* 277:49743–49749, 2002.

Soares D. C. F., de Oliveira M. C., de Barros A. L. B., Cardoso V. N., Ramaldes G. A., Liposomes radiolabeled with 159Gd: In vitro antitumoral activity, biodistribution study and scintigraphic image in Ehrlich tumor bearing mice, *Eur. J. Pharm. Sci.* 43:290-296, 2011.

Sonal G., Reema G., Javed A., Shweta D., Exploring novel approaches to vaginal drug delivery, *Recent Patents on Drug Del. Form.* 5:82-94, 2011

Szoka F. Jr., Papahadjopoulos D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci 9*:4194–4198, 1978.

Torchillin V., Weissig V., *Liposomes: A Practical Approach*, 1st Ed, Oxford University Press, 1990.

Takahara P. M., Frederick C. A., Lippard S. J., Crystal structure of the anticancer drug cisplatin bound to duplex DNA, *J. Am. Chem. Soc.* 118:12309–12312, 1996.

Takahara P. M., Rosenzweig A. C., Frederick C. A., Lippard S. J., Crystal-structure of double-stranded DNA containing the major adduct of the anticancer drug Cisplatin. *Nature*. 377:649–652, 1995.

Torchilin V., Multifunctional and stimuli-sensitive pharmaceutical nanocarriers, *Eur. J. Pharm. Biopharm.* 71:431-444, 2009.

Tu C., Ng T. S., Sohi H. K., Palko H. A., House A., Jacobs R. E., Louie A. Y., Receptor-targeted iron oxide nanoparticles for molecular MR imaging of inflamed atherosclerotic plaques, *Biomaterials*. 32:7209-7216, 2011.

Vashist S. K., Zheng D., Pastorin G., Al-Rubeaan K., Luong J. H. T., Sheu F. S., Delivery of drugs and biomolecules using carbon nanotubes, *Carbon*. 49:4077-4097, 2011.

Vemuri S., Rhodes C. T., Development and validatıon of a drug release rate method for a water soluble drug in a liposome preparation, *Drug Dev. Ind. Pharm.* 21:1353-1364, 1995.

Venkateswarlu I., Jayashankar R. V., Ramesh Y., Viswanatha R. M., Pravallika C. H., Suneetha K. C., A review on liposomes, *Res. J. Pharm. Biol. Chem. Sci.* 2:739-751, 2011

Verma A. M. L., Misra U., Mishra N., Bhardwaj P., Liposomes as Carrier Systems. *InPharm Communique* 2:20-24, 2009.

Vyas S. P., Khar R. K., Gastroretentive systems, In *Controlled drug delivery: concepts and advances*, Eds. Vyas S. P., Khar R. K., New Delhi, 219-243, 2002

Wang G., Liposomes as drug delivery vehicles, In *Drug Delivery: Principles and Applications*, Eds. Wang B., Siahaan T., Soltero R., John Wiley & Sons Inc., New York, 411-434, 2005

Wang M., Thanou M., Targeting nanoparticles to cancer, *Pharm. Res.* 62:90–99, 2010.

Wang T., Gao L., Quan D., Multivesicular liposome (MVL) sustained delivery of a novel synthetic cationic GnRH antagonist for prostate cancer treatment, *J. Pharm. Pharmacol.* 63:904–910, 2011.

Wasan M. W., Lopez-Berestein G., The development of liposomal amphotericin B: an historical perspective, In *Medical applications of liposomes*, Eds. Lasic D. D., Papahadjopoulos D., Elsevier, Amsterdam, 165-169,1998.

Wilson B. C., Patterson M. S., The physics, biophysics and technology of photodynamic therapy, *Phys. Med. Biol.* 53:R61-R109, 2008

Wolff K., Hilleringmann U., Inverter circuits on glass substrates based on ZnO-nanoparticle thin-film transistors, *Solid-State Electronic.*, Article in Press

Woodbury D. J., Richardson E. S., Grigg A. W., Welling R. D., Knudson B. H., Reducing liposome size with ultrasound: bimodal size distributions. *J. Lipos. Res.* 16:57-80, 2006.

Working P. K., Newman M. S., Hunag S. K., Pharmacokinetics, Biodistribution and Therapeutic Efficacy of Doxorubicin Encapsulated in Stealth® Liposomes (Doxil®), *J. Liposomes Res.,* 4:667-687, 1994.

Xu Z. P., Zeng Q. H., Lu G. Q., Yu A. B., Inorganic nanoparticles as carriers for efficient cellular delivery, *Chem. Eng. Sci.* 61:1027-1040, 2006.

Yilgor P., Tuzlakoglu K., Reis R. L., Hasirci N., Hasirci V., Incorporation of a sequential BMP-2 /BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering, *Biomaterials.* 30:3551-3559, 2009.

Zhang J. A., Anyarambhatla G., Ma L., Ugwu S., Xuan T., Sardone T., Ahmad, I., Development and characterization of a novel Cremophor (R) EL free liposome- based paclitaxel (LEP-ETU) formulation, *Eur. J. Pharm. Biopharm.* 59:177-187, 2005.

Zhang Z. Y., Smith B., Synthesis and characterization of NVOC-DOPE, a caged photoactivatable derivative of dioleoylphosphatidylethanolamine, *Bioconjug. Chem.* 10:1150–1152, 1999.

Zhang H., Wang Z. Y., Gong W., Li Z. P., Mei X. G., Lv W. L., Development and characteristics of temperature-sensitive liposomes for vinorelbine bitartrate, *Int. J. Pharm.* 414:56-62, 2011.

Zheng H., Gao C., Peng B., Shu M., Che S., pH-responsive drug delivery system based on coordination bonding in a mesostructured surfactant/silica hybrid, *J. Phys. Chem. C.* 115:7230-7237, 2011.

APPENDIX A

CALIBRATION CURVE FOR CALCEIN

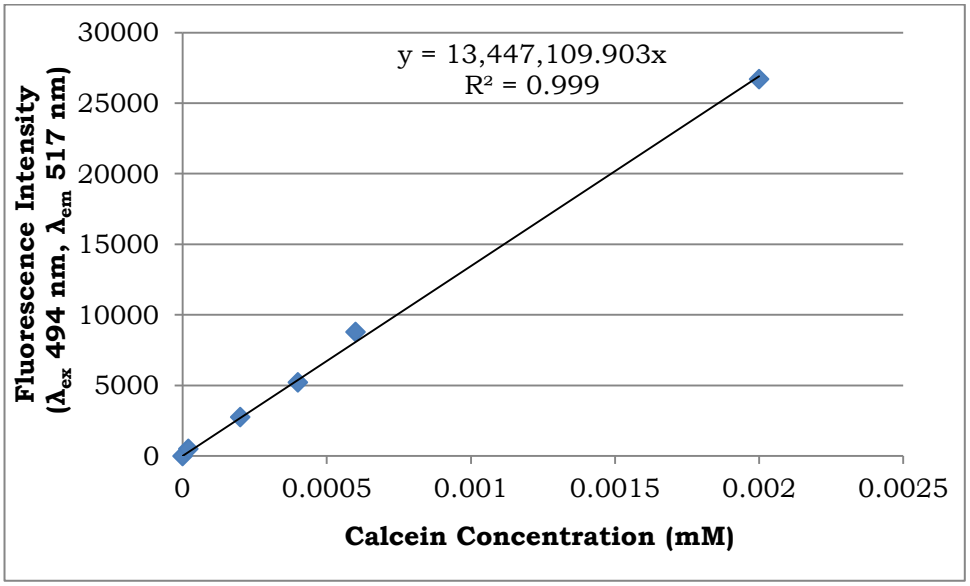


Figure A.1. Calcein calibration curve prepared for encapsulation efficiency and release studies

APPENDIX B

CALIBRATION CURVE FOR MTS ASSAY

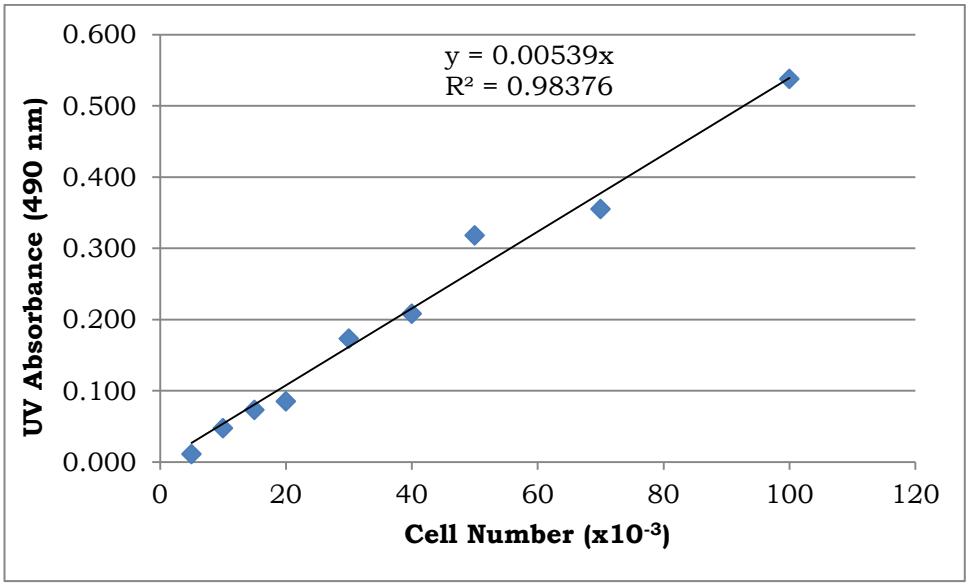


Figure B.1. Calibration curve for determining Saos-2 cell number with MTS Assay