DEVELOPMENT OF ULTRASOUND TRIGGERED DRUG DELIVERY SYSTEMS FOR CANCER TREATMENT

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ABSTRACT

DEVELOPMENT OF ULTRASOUND TRIGGERED DRUG DELIVERY SYSTEMS FOR CANCER TREATMENT

Doxorubicin (DOX) is one of the most commonly used hydrophilic anticancer drug in cancer treatment. However, when it is used in free form, it can attack not only cancer cells but also healthy cells. So as to prevent entering of DOX to the healthy cells, the encapsulation method is employed. Liposomes are suitable for encapsulation of DOX but the most important problems with the use of liposome are hand-foot syndrome and stomatitis. Encapsulation method is not enough because of these reasons, thus delivery of DOX to the desired site by targeted therapy has gained interest in recent years.

In this study, DOX was encapsulated into liposomes and the DOX loaded liposomes (LipoDOX) was attached to microbubbles (MBs). MBs as ultrasound contrast agents are widely used in medical imaging. Use of MBs in combination of DOX loaded liposomes facilitates the uptake of the drug because ultrasound cavitation results in opening of transient pores in cell membrane via a process named sonoporation. Herein, MB-LipoDOX complex was engineered to optimize the size of the complex as well as the loaded DOX content. For this purpose, determination of incubation temperature and time for DOX loading into liposome and optimization of liposome formulation for maximum DOX loading were studied. Ratios of Lipid/Cholesterol/PEGylated lipid, PEG chain length and PEG molar ratio in liposome were determined. Also, determination of Strept Avidin (StAv) to Biotin ratio in LipoDOX and the amount of LipoDOX in LipoDOX-MB complex were studied. For characterization, Dynamic Light Scattering (DLS) method, Fluorescence Spectrometry method and Coulter Counter device were used. Lipoosme size was found to be associated with the pore size of polycarbonate membrane (200nm) resulting in liposomes at around 190±5 nm in size . When the PEGylated lipid with PEG chain of 2000 was used in liposome structure, particle size distribution is more monodispersed than the others. The maximum amount of DOX loaded liposomes was obtained at 32% Cholesterol, 5% DSPE-PEG_{2000}, after 90 min. incubation at 65ºC incubation. Optimum StAv to Biotin ratio in LipoDOX was determined as 1.0. The optimum molar ratio of Biotinylated lipids in LipoDOX was determined as 0.05% and the optimum molar ratio of Biotinylated lipids in MBs was determined as 8%.

ÖZET

KANSER TEDAVİSİ İÇİN ULTRASON TETİKLİ İLAÇ TAŞIMA SİSTEMLERİ GELİŞTİRİLMESİ

Doksorubisin (DOX), kanser tedavisinde en yaygın kullanılan hidrofilik antikanser ilacıdır. Ancak serbest formda kullanıldığında, sadece kanser hücrelerine değil aynı zamanda sağlıklı hücrelere de zarar verir. DOX'un sağlıklı hücrelere zarar vermesini önlemek için, kapsülleme yöntemi kullanılır. Lipozomlar, DOX'un kapsüllenmesi için uygundur, ancak el-ayak sendromu ve ağız içi yaralarına sebep olmaktadır. Bu sebeple DOX'un hedeflenmiş tedavi yöntemi ile istenen bölgeye verilmesi son yıllarda oldukça ilgi kazanmıştır.

Bu çalışmada, DOX lipozomların içine enkapsüle edilmiş ve DOX yüklü lipozomlar (LipoDOX) mikro-kabarcıklara (MB) bağlanmıştır. Ultrason kontrast maddeleri olarak MB'lar, tıbbi görüntülemede yaygın olarak kullanılmaktadır. MB'lar ultrason yardımıyla ilacın hedef hücre içine alımını kolaylaştırır. Çünkü ultrasonun yarattığı kavitasyon, sonoporasyona yani hücre zarındaki geçici gözeneklerin açılmasına, hücre geçirgenliğinin artmasına neden olur. MB-LipoDOX kompleksi, boyutunun yanı sıra, içeriğindeki DOX miktarının optimize edilmesi için tasarlanmıştır. Bu amaçla, optimum LipoDOX inkübasyon sıcaklığı, süresi ve liposozom formülasyonu incelenmiştir. Lipozomdaki Lipid / Kolesterol / PEGile lipit, PEG zincir uzunluğu ve PEG molar oranı belirlenmiştir. Ayrıca, LipoDOX'taki StAv/Biotin oranı belirlenmiş ve LipoDOX-MB kompleksi içindeki optimum LipoDOX miktarı incelenmiştir. Çalışmalarda, karakterizasyon için Dinamik Işık Saçılımı (DLS) yöntemi, Floresan Spektrometresi yöntemi ve Coulter Counter cihazı kullanılmıştır. Lipozom büyüklüğü 200 nm olarak belirlenmiştir. Lipozom yapısında PEG_{2000} kullanıldığında, partikül büyüklüğü dağılımının diğerlerinden daha monodispers olduğu gözlenmiştir. Maksimum DOX yüklü lipozomlar, lipozom formulasyonunda %32 Kolesterol ve %5 DSPE-PEG_{2000} kullanıldığında ve DOX ile lipozomlar 65^{o}C'de 90 dakika inkübe edildiğinde elde edilmiştir. LipoDOX'taki StAv/Biotin oranı 1.0 olarak; LipoDOX'taki Biotinli lipid mol oranı %0,05 ve MB'daki Biotinli lipid mol oranı %8 olarak belirlenmiştir.

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CHAPTER 1

INTRODUCTION

There are many nanomaterials used as nanocarriers. Nanocarriers can be used to provide targeted delivery of drugs, more circulation time of drugs and more efficiency of drugs on the tumor site, improve the stability of therapeutic agents against enzymatic degradation and oral bioavailability, sustain drug/gene effect in target tissues. These nanocarriers composed by diverse materials and thus presenting different characteristics. There are 3 main groups include nanocarriers and these are polymeric, lipid, metal and inorganic nanocarriers. The most commonly used nanocarrier is liposomes. Liposomes are small, simple, macroscopic, artificial vesicles of spherical shape with one or multiple concentric bilayers that can compose of cholesterol and natural nontoxic phospholipids. They have hydrophilic and hydrophobic groups in their structure. Thus, they have capable of encapsulating hydrophilic and hydrophobic drugs through these hydrophilic head groups and hydrophobic tails concurrently. Liposomes have different sizes as by their structure, preparation methods and composition. Their sizes change between micrometer and nanometer (Pandey, Rani, & Agarwal, 2016).

There are many advantages of liposomes used in drug delivery systems as nanocarrier. Liposomes are biocompatible, and they reduce doxorubicin side effects, provide reduction in toxicity of the encapsulated agent, allow loading of both hydrophilic and targeted therapy, and enhance the activities of drugs against intracellular pathogens. Apart from these advantages, liposomes have improved pharmacokinetic effects, high drug loading capacity, physicochemical stability, thermodynamic stability and manageable size control (Akbarzadeh, Rezaei-Sadabady, Davaran, Joo, & Zarghami, 2013; Scholtz, 2010).

Liposomes are suitable for encapsulation of anticancer drugs but there are some disadvantages of liposomes. They have problems of very high production cost because of expensive equipment needed to increase manufacturing, short half-life and stability. There are leakage and fusion of encapsulated drugs, oxidation of bilayer and sterilization problems. These problems may be overcome. However, the most important problems are hand-foot syndrome and stomatitis. They cannot be overcome (Akbarzadeh, Rezaei-

Sadabady, Davaran, Joo, & Zarghami, 2013; Scholtz, 2010). Encapsulation method is insufficient because of these reasons, targeted therapy is used. There are many anticancer drugs such as doxorubicin, cisplatin, tacrolimus, epirubicin, paclitaxel, curcumin, verapamil used for a targeted therapy method.

There are many studies about combine anticancer drugs with an externally applied "trigger". Thanks to this, actions of the drug in the targeted region can be controlled by light, magnetic field, neutron beam or mechanical energy. These applications improve the anticancer drug delivery and ultrasound irradiation are most commonly used in such this therapy method. Ultrasound is the most widely used medical imaging technique worldwide. There are many advantages using of ultrasound waves for drug delivery system. Thanks to ultrasound wave, drugs can reach to deep into the body, tissue or cancerous cells. Beyond these, ultrasound waves damage cell membranes, increase permeability of the cell membrane and easy to pass of the drug into the cell. For this technology, some ultrasound contrast agents are used. One of the most important and widely used in medical imaging is microbubbles (Hernot, & Klibanov, 2008). Using an ultrasound agent is important for imaging of tumors. By using microbubble, tumor site can be visualized and observe change in tumor after the drug delivery (Schutt, Klein, Mattrey, & Riess, 2003).

Microbubbles are tiny, non-toxic, mechanically oscillate, hydrophobic gas-filled microspheres encapsulated by a biocompatible shell and size of the microbubbles is typically between 0.5 to 10 μm (approximately equal to the size of a red blood cell). Microbubbles are widely used as contrast agents in medical imaging and carriers for targeted drug delivery. They have a biocompatible shell which sizes are range from 1 to 200 nm by the composition of the particle. There are three types of shell structure and these are lipid, protein and polymers. They have the thickness of 3 nm, 15-20 nm, and 100-200 nm, respectively. These shell structures show differences by their compliance, stability and drug loading capacity under ultrasound. Lipid shell-microbubble has high compliance and high echogenicity under ultrasound but their stability and drug loading capacity less than polymer shell-microbubble. However, polymer shell-microubbles have low compliance and low echogenicity under ultrasound. Echogenicity is important. When the echogenicity is low, the imaging quality is low (Cavalli, Bisazza, & Lembo, 2013; Hernot, & Klibanov, 2008; Sirsi, & Borden, 2009).

Doxorubicin loaded liposomes (LipoDOX) and microbubble combine with each other by using streptavidin-biotin conjugation. Streptavidin is a protein and biotin is a

water-soluble vitamin. They make a very strong bond with each other (Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010). When the ultrasound is applied to LipoDOX-Microbubble complex, volume of the microbubble increases and then decreases. This compression and expression processes continue like this. And then microbubbles are destroyed and cavitation occurs. Cavitation creates transient pores on the cell membrane and uptake of drugs by the targeted tissue becomes easy under sonoporation effect. LipoDOX is released to the direction of the targeted cells (Lu, Zhao, Ge, Jin, & Du, 2013).

This thesis contains five chapters. In chapter one, general introduction for ultrasound triggered drug delivery systems, liposomes used as nanocarriers, microbubbles used as ultrasound contrast agents and the mechanism of anticancer drug (DOX) loaded liposomes-microbubble complex in cell are introduced. In chapter two, a literature survey on doxorubicin, liposomes, microbubbles and targeted therapy are presented. In chapter three, the chemicals used in this study, sample preparation procedures and characterization methods for these samples are explained in details. In chapter four, results of all experiments are presented and discussed. Finally, the conclusions are in chapter five.

CHAPTER 2

LITERATURE SURVEY

2.1. Cancer

Cancer is one of the most important health problems worldwide. The death rate is high and it is the second cause of death after cardiovascular system diseases. Every year, there are millions of people that are diagnosed with cancer and more than half of these patients die from this disease (Ma, & Yu, 2006).

The terms of “cancer” and “tumor” are often confused. It is assumed that the two terms have the same meaning. However, these differ each other. Tumor is different than cancer. Every cancer is a tumor, but not every tumor may be cancer. A tumor, also known as a neoplasm, is an abnormal mass of tissue that may be solid or fluid-filled and does not necessarily pose a health threat. There are three types of tumors. These are benign, premalignant and malignant tumors. Benign tumors are non-cancerous, not harmful to human health and do not spread to the body and do not pose any danger. They remain in their current form. They do not melt the site of contamination. After they are removed from the body, they do not regenerate. Even though they are not cancerous, some may press against nerves or blood vessels and cause pain or other negative effects. Benign tumors include adenomas, fibroids or fibromas, hemangiomas and lipomas. Premalignant tumors are not cancerous yet, but appears to improve cancer characteristics. Premalignant tumors include actinic keratosis, cervical dysplasia, metaplasia of the lung and leukoplakia. Malignant tumors are cancerous. They can grow, spread, get worse and potentially result in death. The malignant tumor multiply at a very fast rate and grow quickly. They can spread to new tissues and organs and this event is named as metastasis. Forms of malignant cancer include carcinoma, sarcoma, leukemia, lymphoma, and melanoma (Nordqvist, 2017). The major types of malignant tumor (cancer) and the regions in which they occur are in Table 2.1. (Cooper, & Hausman, 2007)

The first definitions of cancer are encountered in Egyptian papyrus, Babylon cuneiform script tablets and ancient Indian writings. In the Greek medical records of ancient times and Galen's studies, it is told that many types of cancer are encountered

even though it is not known that what kind of tumors are. Moreover, in the medical book written by the Chinese emperor Huang-Di, who was born in BC 2698, the description of the tumors is made and the treatment methods are explained. The first definitions of cancer are encountered in Egyptian papyrus, Babylon cuneiform script tablets and ancient Indian writings. In the Greek medical records of ancient times and Galen's studies, it is told that many types of cancer are encountered even though it is unknown what kind of tumors are. Moreover, in the medical book written by the Chinese emperor Huang-Di, who was born in BC 2698, the description of the tumors is made as general and the treatment methods are explained.

Table 2.1. The major types of malignant tumor and their regions in the body (Source: Cooper, & Hausman, 2007)

Type of Malignant Tumor	**The Region in the Body**
Carcinoma	Stomach, breast, prostate, pancreas, lung, liver, colon, intestine, kidneys and bladder
Sarcoma	Bone, fat, cartilage, muscle, blood vessels, tendons and joints or other connective tissues and mostly of the arms or legs
Myeloma	Cells of bone marrow, the soft tissue inside bones or other blood-forming tissue
Leukemia	Bone marrow and blood
Lymphoma	Cells of the immune system, white blood cells of the lymphatic system
Melanoma	Pigment in skin

Description of cancer was done for the first time by the Hippocrates (460-377 BC) as "karkinos" or "karkinoma", which grows on the body surface and are usually ulcerated, red, hot, painful swellings. Galen (2nd century AD) called this disease as Cancer. The reason is that he likes these looks to crabs. Traditionally, another meaning of the word "cancer" in English is the crab. Crabs insert themselves with their own needle when they are trapped in somewhere. Cancer disease is similar to this situation. This is a disease in which a person's cells kill their own body.

In Greek medicine, abnormal pathological growth called "praeter naturam" is called tumor. Galen has classified the tumors into three groups: those matched to the nature (development of uterus for pregnancy), transient (hypertrophy), and against nature (malign tumors) (Cooper, & Hausman, 2007).

Cancer is malignant tumor or neoplasm that are caused by uncontrolled proliferation of cells in different organs and tissues. There are more than 100 types of cancer that begin to multiply uncontrolled by causing many risk factors of the human body, primarily by damaging the tissues that they live in, and then spreading to other organs to the deadly size. They have different names by the organ or the cell line that they are from (Gültekin, & Boztaş, 2014). The most common cancer types among these are lung, stomach, liver, breast, prostate, colon/rectum, bladder, thyroid, skin (melanoma), lymphomas, uterus, kidney, pancreas, leukemia, and cervix. Especially, breast, lung, and cervix are the three cancer types have the highest mortality rate in women. For men, lung, liver and stomach cancers have the highest mortality. Globally while the most common type of cancer is breast cancer, lung cancer is still the most deadly type of cancer (Ma, & Yu, 2006).

There are many differences between cancer cells and normal or healthy cells. Some major differences between normal cells and cancer cells are cell growth, cell repair, reproduction and cell death, morphology, communication of cells with each other, ability to metastasize to other regions of the body, appearance under the microscope, the rate of growth, maturation, evading the immune system, cell mechanism, blood supply, adhesion and invasion, evading growth suppressors, mortality/immortality, ability to hide, genomic instability and signal recognition (Jameson et al., 2015; Min, Wright, & Shay, 2017).

Cells are divided for various reasons, such as creating new tissue or replacing old and damaged cells. In a colony of healthy cells, it is certain when cells divide and when to stop dividing. However, the cancer cells do not listen to the signals given to them and are constantly divided in an uncontrolled manner. Instead of being stimulated by an external signal, cancer cells develop something that they can mediate between themselves and divide. This is called metabolic autonomy (DeBerardinis, Hatzivassiliou, & Thompson, 2008). Immortality of cancer cells is another characteristic of them. When the cancer cells become old or damaged, they disappear. However, cancer cells continue to grow and divide, even if they are damaged (Rycaj, & Tang, 2015). The characteristics of normal cells and cancer cells are summarized in Table 2.2 (DeBerardinis, Hatzivassiliou,

& Thompson, 2008; Jameson et al., 2015; Min, Wright, & Shay, 2017; Rycaj, & Tang, 2015).

Table 2.2. The characteristics of normal and cancer cells (Source: Jameson et al., 2015; Min, Wright, & Shay, 2017)

Characteristics	Normal Cell	Cancer Cell
Shape	Regular	Irregular
Nucleus	Proportionated size	Larger size and darker
Growth	Controlled, systematic	Out of control
Maturation	Mature	Doesn't mature
Communication	Communicates	Doesn't communicate
Visibility	Visible to immune cells	Invisible to immune cells
Blood Supply	Angiogenesis during repair	Tumor angiogenesis
Oxygen	Requires oxygen	Doesn't like/require oxygen
Glucose	Requires some glucose	Loves, craves glucose
Energy Efficiency	Very high (95%)	Very low (5%)
Amount of ATP	38 units of ATP	2 units of ATP
Cell Environment	Alkaline (pH usually above 7)	Acidic (pH less than 7)
Nutrient Preference	Fat, ketone, glucose	Glucose

There are eight hallmarks for cancer biology. These are sustaining proliferative signaling, resisting cell death, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, reprogramming energy metabolism and evading immune response (Fouad, & Aanei, 2017).

2.2. Treatment Methods of Cancer

Cancer is treated with different ways. These are surgical therapy, chemotherapy, radiotherapy, immunotherapy, hormone therapy, laser treatment, stem cell transplant and targeted therapy. Surgery is a procedure in which a surgeon removes cancer from your

body. Radiation therapy is a type of cancer treatment that uses high doses of radiation to kill cancer cells and shrink tumors. Immunotherapy is a type of treatment that helps patient immune system fight cancer. Hormone therapy is a treatment that slows or stops the growth of breast and prostate cancers that use hormones to grow. Targeted therapy is a type of cancer treatment that targets the changes in cancer cells that help them grow, divide, and spread. Laser therapy is used to shrink or destroy tumors or precancerous growths via high-intensity light (Baskar, Dai, Wenlong, Yeo, & Yeoh, 2014; Schiller, & Combs, 2018). Chemotherapy is the use of anticancer drugs designed to slow or stop the growth of rapidly dividing cancer cells in the body. Chemotherapy is most commonly used in cancer treatment methods. This treatment method is time consuming compared to other methods (Cheson, 2017). There are many types of anticancer drugs used in chemotherapy. These drugs are in Table 2.3.

Table 2.3. The anticancer drugs used in chemotherapy (Source: Qian-Liu et al., 2012)

Anticancer Drugs	
Hydrophilic	**Hydrophobic**
✓ Doxorubicin hydrochloride	✓ Paclitaxel
✓ Cisplatin	✓ Doxorubicin
✓ Tacrolimus	✓ Curcumin
✓ Oxaliplatin	✓ Docetaxel
✓ Epirubicin	✓ Irinotecan
✓ Carboplatin	✓ Etoposide
✓ Indocyanine green	✓ Verapamil
✓ 5-Fluoroucacil	✓ Digoxin
✓ Gemcitabine	✓ Rapamycin

These drugs may be used as a primary treatment to destroy cancer cells, before another treatment to shrink a tumor, after another treatment to destroy any remaining cancer cells and to relieve the symptoms of advanced cancer. There are two types of chemotherapy: systematic chemotherapy and regional chemotherapy. In systematic chemotherapy, the drug travel through the bloodstream to reach cells throughout the body. In regional chemotherapy, the drugs are directed to a specific area of the body. There are many types of chemotherapy delivery methods. Some of them are orally (by mouth as a

pill or liquid), intravenously (by infusion into a vein), topically (as a cream on the skin), injection and direct placement (via a lumbar puncture or device placed under the scalp) (Di Saia, & Creasman, 2012).

2.3. Doxorubicin Hydrochloride (DOX-HCl)

Doxorubicin (DOX) is the most commonly used amphiphatic anticancer drug in cancer treatment. Its commercial name is Adriamycin or Rubex. It has both phenolic group and sugar amino group. There is a water insoluble aglycone (adriamycinone: $C_{21}H_{18}O_9$) in phenolic group of DOX and DOX shows acidic property because of adriamycinone. There is water soluble region (daunosamine: $C_6H_{13}NO_3$) in sugar amino group of DOX and DOX shows alkaline property because of daunosamine (Abraham et al., 2005). These water insoluble aglycone and water-soluble regions are in Figure 2.1.

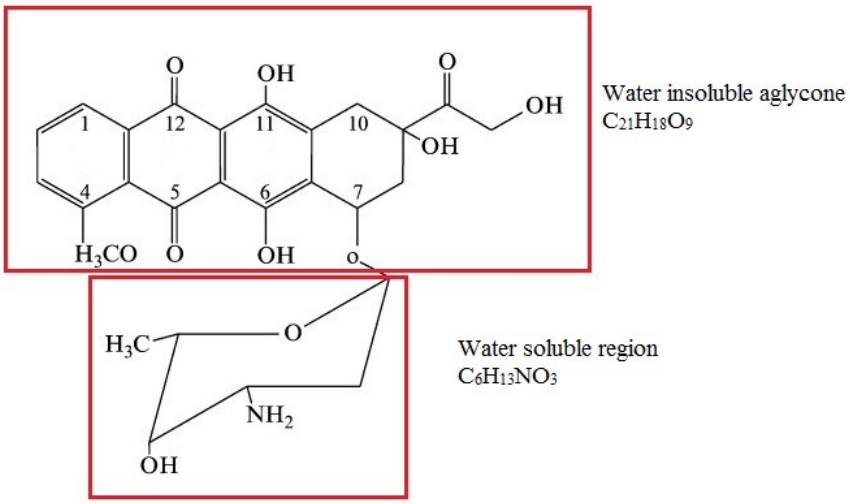


Figure 2.1. The chemical structure of DOX (Source: Abraham et al., 2005)

DOX has three different pK_a values. These are at the amino group, at the phenolic group at C_{11} and at the phenolic group at C_6 and their values are pK_1=8.15, pK_2=10.16 and pK_3=13.2, respectively. The appearance of DOX differs at different pH value. DOX is orange-colored at pH 7, violet at pH 11 and blue at pH 13. It has a melting point of 229-231°C.

DOX is commonly used as hydrochloride form of DOX (DOX-HCl) in drug delivery and DOX-HCl is hydrophilic anticancer drug. DOX is classified as an

"anthracycline antibiotic" (Fonseca, van Winden, & Crommelin, 1996). It has a shows excellent antineoplastic activity against many human neoplasms like acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), non-Hodgkin's disease, multiple myeloma, neuroblastoma, thyomas, Wilms' tumor, lung carcinomas and sarcomas (İlbasmış-Tamer, 2016). Although it is used in cancer treatment, the therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to congestive heart failure and death and 2% of patients who receive total dose of 450-500 mg/m^2 DOX during their whole life are faced with cardiomyopathy. DOX slows or stops rapidly-growing of cancer cells by Topoisomerase Two inhibition. It also induces the formation of covalent topoisomerase–DNA complexes. Thus, in inhibition of the relegation portion of the ligation–relegation reaction in replicating DNA (Abraham et al., 2005). However, there are many side effects of free form of DOX. These are hand-foot syndrome, darkening of the nail beds, hair loss, eyes watering, vomiting, swelling of the feet or ankles, mouth sores (stomatitis). Beyond these, darkening of the skin, fast or irregular heartbeats, rapid weight gain, dark urine, chest pain, stomach pain, anxiety, tightness etc. are the side effects of doxorubicin (Fritmze, Hens, Kimpfler, Schubert, & Peschka-Süss, 2006). Also, when DOX is used in free form, it can attack not only cancer cells but also healthy cells. In healthy cells, there are continuous and well endothelial cells but in tumor cells, there are defective or leaky endothelial cells as shown in Figure 2.2.

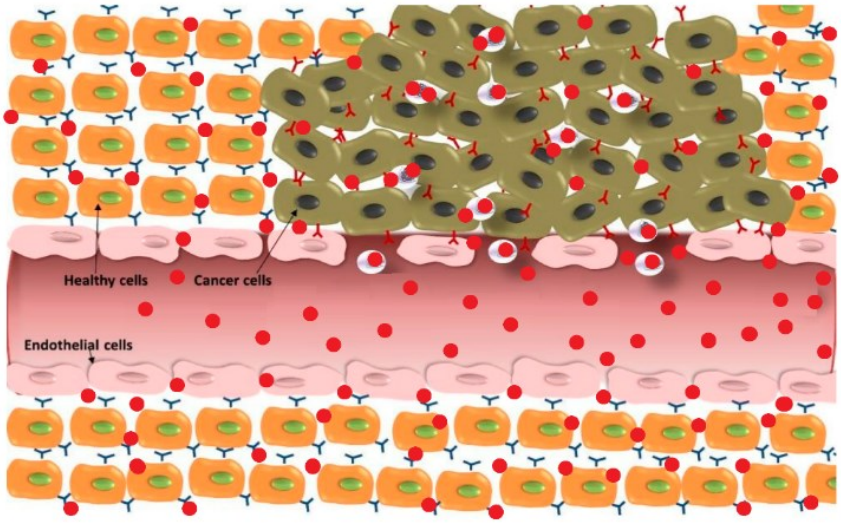


Figure 2.2. DOX enters the both healthy and tumor cells (Source: Nam et al., 2018)

The healthy cells are demonstrated with orange-colored shape and the tumor cells are shown with green shape. Red dots are DOX molecules. DOX can enter the both healthy and tumor cells easily because of being a small molecules. So as to prevent entering of DOX to the healthy cells and to prevent from side effects of free form of DOX, the encapsulation method is used. When encapsulation method is used, encapsulated drug enters only to the tumor site, cannot enter to the healthy site because of its size. Even, encapsulation method provides more circulation time of doxorubicin and more efficiency of doxorubicin on the tumor site (Nam et al., 2018).

2.4. Encapsulation of DOX-HCl

There are many nanocarriers for encapsulation. These nanocarriers composed by diverse materials and thus presenting different characteristics. There are 3 main groups include nanocarriers and these are polymeric, lipid, metal and inorganic nanocarriers. The whole nanocarriers and their shape are in Figure 2.3.

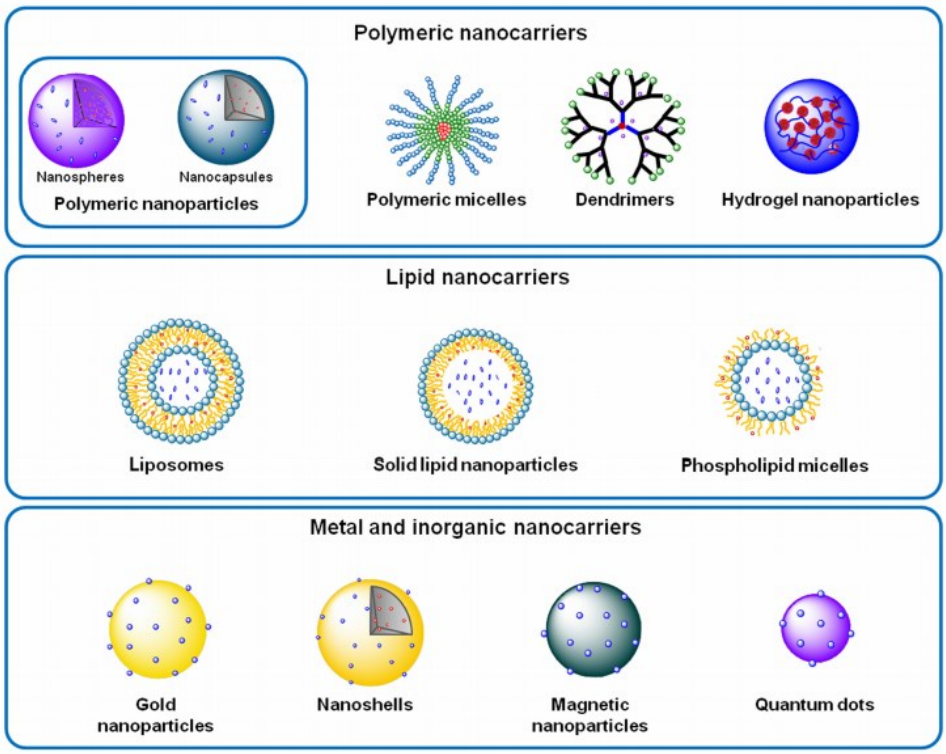


Figure 2.3. Nanocarriers and their shape (Source: Canniot, 2014)

Among them, polymeric nanoparticles, polymeric micelles, dendrimers, quantum dots and liposomes are most commonly used in encapsulation of anticancer drugs (Canniot, 2014).

Polymeric nanoparticles are stable systems which can entrap and adsorb both hydrophilic and hydrophobic molecules. They protect the trapped drug against degradation. They can be easily moved inside the cell because of their nano-sized. There are two types of polymeric nanoparticles. These are nanospheres and nanocapsules. The drug disperses homogenously in nanospheres. However, homogenously dispersing is not observed in nanocapsules; the drug is in the center and there are a polymer wall that surrounds a core containing the drug (Canniot, 2014).

Polymeric micelles are self-assembled spherical nanocarriers that composed of hydrophobic core and a hydrophilic surface and their size changes from 10 to 100 nm of range. They are suitable for hydrophobic anticancer drugs. Polymeric micelles have low toxicity, more circulation time in the body, more accumulation in tumor sites, providing of bioavailability of hydrophobic molecules, protection of the drug from in vivo degradation (Canniot, 2014).

Dendrimers are artificial, can encapsulate hydrophobic drug molecules, hyperbranched spherical nanocarriers with tree-like which have a core in its middle and have branched and functionalized monomers around this core. They are synthesized from branched monomer units in a stepwise manner and so their molecular properties such as size, shape, dimension, density, polarity, flexibility, and solubility can be controlled. They have low viscosity and hyperbranched molecular topology. Their size is in the macromolecular level. The release profile of the loaded agents is controlled through the depolymerization of dendrimers. Dendrimers protect imaging agents, decreasing its toxicity and enhancing specificities (Canniot, 2014; Yang, Cheng, Xu, Wang, & Wen, 2009).

Quantum dots are small, fluorescent semiconductor, hydrophobic, only 4-5 nanometers in size (core size of 2 nm in diameter) nanocrystals. Generally, they are used as hybrid with lipid nanoparticles (Tian, Al-Jamal, & Kostarelos, 2011).

In general, nanocarriers can be used to;

- ✓ provide targeted delivery of drugs,
- ✓ provide more circulation time of drugs,
- ✓ improve the stability of therapeutic agents against enzymatic degradation,
- ✓ improve oral bioavailability,

- ✓ provide more efficiency of drugs on the tumor site,
- ✓ sustain drug/gene effect in target tissues,
- ✓ solubilize drugs for intravascular delivery

Owing to their nanometer size ranges, there are many important advantages for drug delivery (Moghimi, Hunter, & Murray, 2001). Nanoparticles have higher intracellular uptake compared to microparticles. According to Desai et al. (1996) uptake efficiency of nanoparticles was 15-250 fold greater than larger size microparticles (Desai, Labhasetwar, Amidon, & Levy, 1996). They can easily reach to tissues via fine capillaries. They are taken up by the cells as efficiently through the pass the fenestration in the epithelial lining of these nanocarriers. Thus, therapeutic agents can be delivered to target sites in the body efficiently (Moghimi, Hunter, & Murray, 2001).

There are two encapsulation method for doxorubicin: active and passive encapsulation methods. Hydration of dried lipid film with doxorubicin solution and incorporation of negatively charged lipids into the liposomal composition are passive encapsulation methods. However, the encapsulation efficiency of drug is low in the passive encapsulation method. So as to improve the encapsulation efficiency of the drug, active encapsulation method is used. DOX is an uncharged molecules and can pass through the lipid bilayer. However, protonated form of doxorubicin is positively charged and cannot pass through the lipid bilayer. Using these properties of DOX, active loading is occurred. Ammonium sulfate gradient method is most commonly used for active encapsulation method. When the ammonium sulfate loaded liposome is mixed with DOX solution and temperature is applied to this mixture, uncharged DOX passes into the lipid bilayer. An equilibrium occurs between ammonium sulfate and uncharged DOX. Doxorubicin and sulfate form a crystal complex and they are charged positively. Thus, doxorubicin that enters to inside of liposome cannot exit from the lipid bilayer (Cheung, 1998). Doxorubicin loading mechanism into liposome and the equilibrium between DOX and ammonium sulfate are in Figure 2.4. Doxorubicin is demonstrated with D.

2.5. Liposomes

Liposomes are small, simple, macroscopic, artificial vesicles of spherical shape with one or multiple concentric bilayers that can compose of cholesterol and natural nontoxic phospholipids. A phospholipids compose of one hydrophilic (polar) head group

includes choline, phosphate, and glycerol and two hydrophobic (nonpolar) tail includes fatty acids.

Liposomes have capable of encapsulating hydrophilic and hydrophobic drugs through these hydrophilic head groups and hydrophobic tails at the same time (Akbarzadeh, Rezaei-Sadabady, Davaran, Joo, & Zarghami, 2013; Scholtz, 2010; Yang et al., 2011). The structures of a phospholipid and liposome as shown in Figure 2.5.

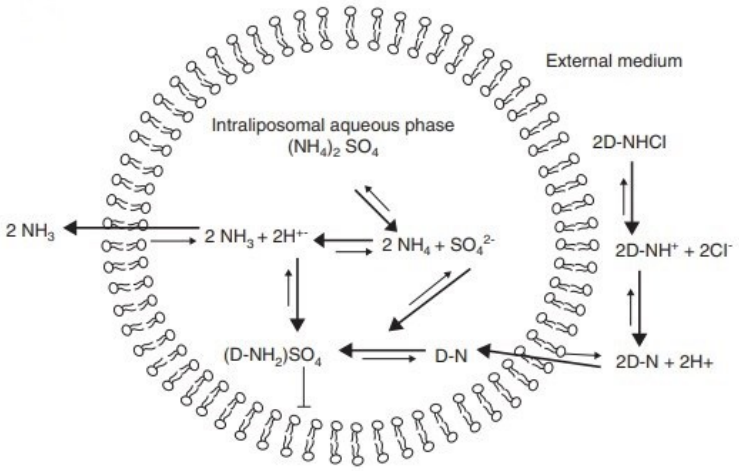


Figure 2.4. Doxorubicin loading mechanism into liposomes (Source: Gubernator, 2011)

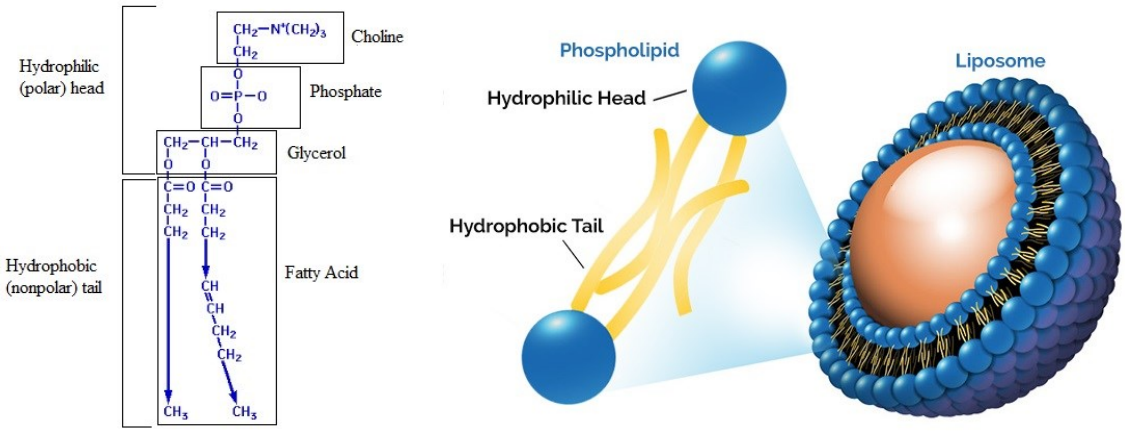


Figure 2.5. The structure of phospholipid and liposome (Source: McMurry, Castellion, Ballantine, Hoeger, & Peterson, 2010)

Liposomes are classified as by their structure, preparation methods and composition (Pandey, Rani, & Agarwal, 2016). Classification of liposomes based on structural parameters is demonstrated in Figure 2.6.

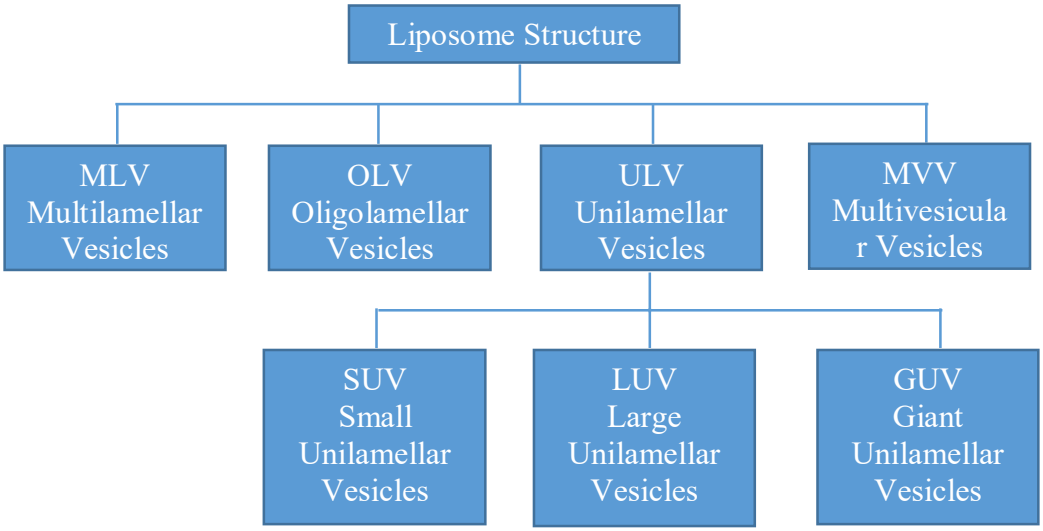


Figure 2.6. Classification of liposome on the basis of structural parameters

They can be collected as four main groups by structural parameters: Multi Lamellar Vesicles (MLV), Oligolamellar Vesicles (OLV), Unilamellar Vesicles (ULV) and Multivesicular Vesicles (MVV). Also, Unilamellar Vesicles can be classified as Small Unilamellar Vesicles (SUV), Large Unilamellar Vesicles (LUV) and Giant Unilamellar Vesicles (GUV). Multi lamellar vesicles are composed of about 5-25 number of concentric phospholipid bilayer membrane separated by aqueous phase and their size is more than 0.5 µm. Oligolamellar vesicles are composed of around 5 phospholipid bilayer membrane and their size is between 0.1-1.0 µm. Multivesicular vesicles are composed of multi compartmental structure and their size is more than 1.0 µm. Unilamellar vesicles are composed of one lipid bilayer. SUVs are composed of a single lipid bilayer. The size of SUV is between 20-100 nm. LUVs are also composed of a single lipid bilayer and size of these vesicles is more than 100 nm. GUVs are composed of a single lipid bilayer like as LUVs and SUVs and size of these vesicles is more than 1.0 µm (Pandey, Rani, & Agarwal, 2016; Patil, & Jadhav, 2014). In Figure 2.7 the shapes of these vesicles are demonstrated.

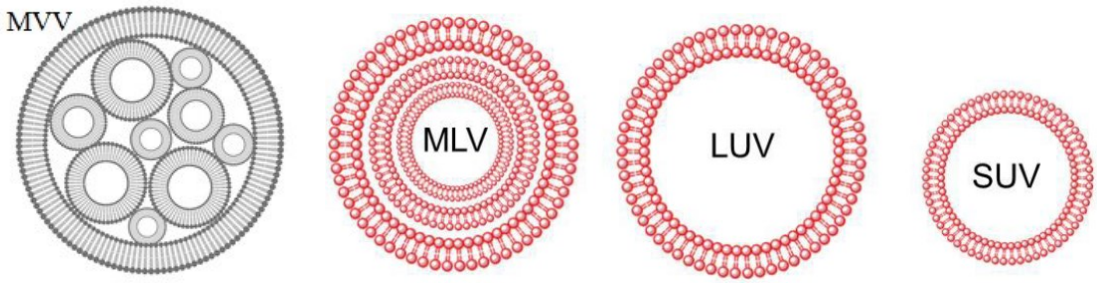


Figure 2.7. Classification of liposomes (Source: Pandey, Rani, & Agarwal, 2016)

To obtain these vesicles, there are many of the preparation methods in the literature. These formation methods and type of the obtained structure as depicted in Table 2.4 (Patil, & Jadhav, 2014).

Table 2.4. Liposome formation methods

Liposome Preparation Method	**Type of Liposome**
Micro Hydrodynamic Focusing (MHF)	Monodisperse SUVs and LUVs
Thin Film Hydration in Micro-tubes	Monodisperse MLVs and LUVs
Supercritical Fluids (SCFs) Method	Monodisperse LUVs
Size Reduction of MLVs and GUVs	Monodisperse SUVs and LUVs
Freeze Drying of Double Emulsions	Monodisperse SUVs
Membrane Contactor Method	Monodisperse LUVs
Hydration of Phospholipids Deposited on Nanostructured Material	Monodisperse SUVs and LUVs
Liposome Formation by Curvature Tuning	Monodisperse SUVs
Microfluidic Droplets	Monodisperse GUVs
Pulsed Jet Flow Microfluidics	Monodisperse GUVs
Modified Electroformation Method	Polydisperse GUVs
Biomimetic Reaction for Vesicular Self-assembly	Polydisperse GUVs

Micro Hydrodynamic Focusing (MHF) was proposed by Jahn and coworkers first. This technique provides exquisite control over SUVs and LUVs sizes. An aqueous buffer flows between two opposite walls of a rectangular channel, while a phospholipid solution which is in isopropyl alcohol flows between the aqueous layers along the axis of the

channel. Thanks to counter the diffusion of water and isopropyl alcohol, phospholipids are forced to self-assemble into bilayers (Jahn et al., 2004). The same technique was also used by Hong (Hong et al., 2010), Hood (Hood et al., 2013), Huang (Huang et al., 2010), Wi (Wi et al., 2012) and their coworkers.

Microfluidic Droplets method was proposed by Sugiura and coworkers at 2008. In this method, first, water droplets are generated in the oil and then they are encapsulated again in water. The phospholipid monolayers are arrayed at the oil water interfaces. The oil phase evaporates and so the monolayers merge so as to form bilayer of a giant vesicle (Sugiura et al., 2008). The same technique was also used by Davies (Davies et al., 2012) and Shum (Shum et al., 2008) and their coworkers.

Pulsed Jet Flow Microfluidics method was proposed by Funakoshi and coworkers. In this method, two macro-sized aqueous drops join in a phospholipid containing the oil phases. The oil film between the drops drains and then a planar bilayer is formed through periodic pulses of a fluid jet by using a microdispensor (Funakoshi et al., 2007).

Thin Film Hydration in Micro-Tubes method was proposed by Suzuki and coworkers at 2008. In this method, the phospholipids dissolved in the chloroform were dried in 200-530 nm of micro-tubes. The obtained thin film was hydrated perfusing an aqueous buffer through the tube (Suzuki et al., 2008).

Supercritical Fluids (SCFs) method was proposed by Castor and Chu. They proposed two methods. In these methods, the phospholipid in an organic co-solvent and SCF are contacted with an aqueous phase. The phospholipid, SCF and organic co-solvent mixture is injected into the aqueous phase through a nozzle. This event is the first method proposed by Castor and Chu. In the second method, when the mixture of phospholipid, SCF and co-solvent is mixed with aqueous phase firstly, decompressed by spraying through a nozzle and so liposomes are obtained (Castor, & Chu, 1998). This method was also used by Otake (Otake et al., 2001), Imura (Imura et al., 2003a, 2003b), Karn (Karn et al., 2013) and their coworkers.

Modified Electroformation method was proposed by Akashi and coworkers. This method yields GUVs only under certain constraints. GUVs can only be obtained if charged phospholipids are used in the presence of physiological ionic solutions (Akashi et al., 1996).

In Size Reduction of MLVs and GUVs method, MLV suspensions are subjected to extrusion or sonication so as to obtain mono-disperse liposomes. Yamaguchi and coworkers proposed that the size reduction of liposomes is possible by using sonication.

When the low ultrasound frequency is applied, larger amplitude oscillations occur and smaller liposomes are formed (Yamaguchi et al., 2009).

Freeze Drying of Double Emulsion method was proposed firstly by Wang and coworkers. When the lipids and water-soluble carrier materials dissolved in tert-butyl alcohol/water co-solvent systems, the cakes of an isotropic monophasic solution occur. And then water is added to the freeze-dried product, homogenous dispersion of MLVs are occurred spontaneously (Wang et al., 2006).

Membrane Contactor Method was developed by Charcosset and coworkers. This is a modified ethanol injection method. In the first study of Jaafar-Maalej and coworkers, a phospholipid solution in ethanol was extruded into an aqueous phase by using a membrane contactor and tubular Shirazu porous glass (SPG) membrane with 900 nm pore sizes (Jaafar-Maalej et al., 2011). After that Laouini and coworkers were studied on this method and they used polypropylene hollow fibers because of accessing to larger membrane areas and uniform flows of these fibers (Laouini et al., 2011).

The monodisperse liposomes were prepared by Yu and coworkers by using hydrophilic polymer polyvinyl pyrrolidone and soybean lecithin.
Phospholipids deposited on amphiphilic nanofibers. These nanofibers include hydrophilic polymer polyvinyl pyrrolidone and soybean lecithin. The deposited phospholipids were hydrated and liposomes were prepared. When the fibers were added to water, liposomes were formed spontaneously (Yu et al., 2011).

In Liposome Formation by Curvature-tuning method, Hauser and coworkers proposed that when the pH was changed rapidly, vesiculation occured spontaneously (Hauser et al., 1989). Genc and coworkers studied on the preparation of monodisperse SUVs by spontaneous vesiculation. Charged or zwitterionic lipids, mixed with lyso-palmitoylphosphatidylcholine were subjected to rapid pH change (Genc et al., 2009).

The major components of liposomes are natural nontoxic phospholipids and cholesterol. There are three different types of phospholipids. These are neutral phospholipids, negatively charged phospholipids and positively charged phospholipids. Neutral phospholipids are sphingomyelin, phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Negatively charged phospholipids are dipalmitoylphosphatidylcholine, dipalmitoylphosphatidyl acid (DPPA), distearoylphosphatidyl choline (DSPC), dioleolphosphatidyl choline (DOPC) etc. Positively charged phospholipids are 1, 2-dihexadecyl-N, N-dimethyl-N-trimethyl amine methyl ethanol amine etc. (Akbarzadeh, Rezaei-Sadabady, Davaran, Joo, & Zarghami,

2013; Yang et al., 2011). PC and PE are the most common phospholipid head groups found in cell membrane. Except for these phospholipids, there are different phospholipids which can be used in the preparation of liposomes such as phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI). These head groups can be attached to fatty acyl chains with different length. The fatty acids are named by the number of their carbon atoms. For example, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic and behenic are saturated fatty acids, and they have 12, 14, 16, 18, 20 and 22 carbon atoms, respectively. Beyond these, palmitoelic acid, oleic acid, vaccenic acid, linoleic acid, linolenic acid and arachidonic acid are unsaturated fatty acids and they have 16, 18, 18, 18, 18, 20 carbon atoms, respectively. The most commonly used phospholipids to prepare liposome are DMPC, DPPC DSPC and HSPC. The phase transition temperature (T_m) of these phospholipids changes with length of lipid chain (Cheung, 1998). The phase transition temperature is defined as the temperature required to convert a gel phase to a disordered liquid crystalline phase from the lipid physical state. DMPC has 14 carbon atoms and its phase transition temperature is 24°C. DPPC has 16 carbon atoms and its phase transition temperature is 41°C. DSPC has 18 carbon atoms and its phase transition temperature is 55°C.

The other main component of liposomes is cholesterol. Cholesterol is a sterol. Cholesterol has hydrophobic property and so it places to interior portion of lipid bilayers. It fills the gap created because of imperfect packing of phospholipid molecules thereby arranging itself among the phospholipid molecules with its hydroxyl group facing toward the water phase (Vemuri, & Rhodes, 1995). It stabilizes the bilayer of liposomes. Cholesterol provides membrane fluidity, elasticity, permeability, rigidity and stability to liposomes according to varying of the molar percentage of cholesterol. It decrease the fluidity of the liposomal membrane bilayer, reduce the permeability of water soluble molecules through the liposomal membrane, and improve the stability of the liposomal membrane in biologic fluids, such as blood and plasma. It prevents the phase transition of lipid bilayers. Thus, the leakage of an encapsulated drug is reduced and also the phase transition temperature of the liposome is affected. In the presence of cholesterol, it also prevents the interaction of liposomes with blood proteins and high density lipoprotein. These proteins destabilize liposomes and so encapsulation capacity of liposomes reduces (Kirby, & Gregoriadis, 1980; Damen, Regts, & Scherphof, 1981).

Apart from cholesterol, polyethylene glycol (PEG) is the other most commonly used component for liposome preparation. The reason for using PEG in liposome

structure, PEG increase the blood circulation times thanks to its stealth properties. PEG allows liposome to extend their circulation half-life and, consequently, increasing the accumulation of liposomes within tumors. PEG is a hydrophilic and flexible polymer thus it is conjugated to the surface of liposomes. PEG reduces the interaction of liposomes with plasma proteins through steric hindrance and reduces uptake of liposomes by the reticuloendothelial system (RES) (Bergström et al., 1994; Allen et al., 2002).

There are several factors that must be taken into consideration for the preparation of effective PEGylated liposome nanocarriers. One of them is PEG-lipid ratio in liposome formulation. PEG density on the liposomal surface affects liposome surface structure. When the PEG concentration was low (below 5 mol %), PEG has the mushroom-like conformation on liposomal surface. However, when the PEG concentration was high (above 5 mol %), PEG has the brush-like conformation on liposomal surface. The brush conformation of PEG provides repulsive force against proteins and other liposomes and prolong the circulation time of liposomes (Allen et al., 2002; Garbuzenko, Barenholz, & Priev, 2005; Gbadamosi, Hunter, & Moghimi, 2002). Mushroom and brush conformation of PEG as in Figure 2.8.

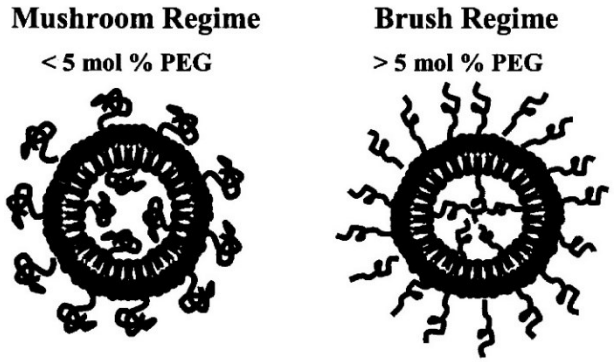


Figure 2.8. Mushroom and brush-like conformation of PEG (Source: Allen et al., 2002)

There are many advantages of liposomes used in drug delivery systems as nanocarriers. Liposomes are biocompatible, and they:

- ✓ reduce doxorubicin side effects
- ✓ provide reduction in toxicity of the encapsulated agent
- ✓ allow loading of both hydrophilic
- ✓ allow targeted therapy
- ✓ enhance activity of drugs against intracellular pathogens

Beyond these advantages, liposomes have improved pharmacokinetic effects, high drug loading capacity, physicochemical stability, thermodynamic stability and manageable size control (Akbarzadeh, Rezaei-Sadabady, Davaran, Joo, & Zarghami, 2013; Scholtz, 2010). Liposomes reduce anticancer agent side effects thanks to Enhanced Permeability and Retention (EPR) Effect. EPR effect was depicted in Figure 2.9. In the figure, green cells and orange-colored cells show tumor cells and healthy cells, respectively.

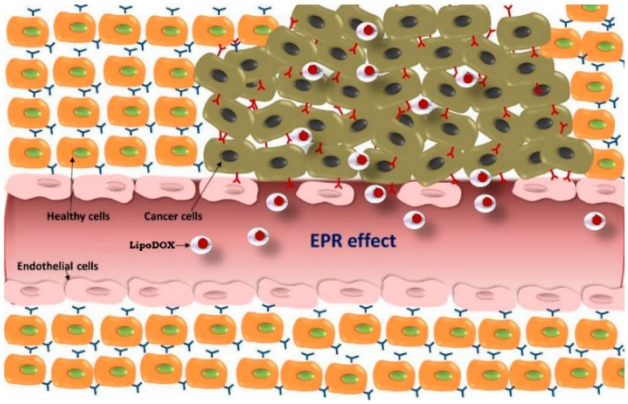


Figure 2.9. Schematic representation of EPR Effect (Source: Nam et al., 2018)

There are two types of targeting for liposomes: active targeting and passive targeting. In the healthy site, there are continuous and well endothelial cells and pore cutoff size in healthy tissues is between 4-25 nm. LUVs are too large to penetrate into healthy tissues due to their size is more than 100 nm. However, in the tumor site, there are defective or leaky endothelial cells and pore cutoff size in tumor cells is between 380-780 nm. When the drug is encapsulated by using liposome, this new structure cannot enter the healthy cells, only enter the tumor cells. This is passive targeting for liposomes, and known as Enhanced Permeability and Retention Effect (Nam et al., 2018).

In the active targeting for liposomes, the surface of the liposome is coated with the appropriate ligands to reach tumor tissues. Thus, ligand conjugated liposomes can accumulate at cancerous tissues (Cheung, 1998).

Liposomes are suitable for encapsulation of anticancer drugs but there are some disadvantages of liposomes. They have problems of very high production cost because of expensive equipment needed to increase manufacturing, short half-life and stability. There are leakage and fusion of encapsulated drugs, oxidation of bilayer and sterilization problems. These problems may be overcome. However, the most important and main

problems are hand-foot syndrome and stomatitis. They cannot be overcome (Akbarzadeh et al., 2013; Scholtz, 2010). Encapsulation method is insufficient because of these reasons, and so targeted therapy is used.

2.6. Targeted Therapy

Chemotherapy and targeted therapy are two effective methods for cancer therapy. There are many anticancer drugs such as doxorubicin, cisplatin, tacrolimus, epirubicin, paclitaxel, curcumin, verapamil used for both cancer therapy method. These chemotherapy drugs affect cells at specific stages of a cell's cycle and work by killing cells in the body that grow and divide quickly. Cancerous cells divide quickly, and the chemotherapy drugs work against them. However, the other cells in the body that divide quickly can also be affected by chemotherapy drugs, and these drugs cannot distinguish cancerous cells and healthy cells from each other. Thus, they also damage to healthy cells. In short, though chemotherapy drugs are particularly toxic to cancer cells, they also damage healthy cells. Hence, in last years, targeted therapy method is commonly used for the treatment of cancer (Lordick, & Hacker, 2014; Flaherty, 2006).

There are specific differences between normal and cancerous cells and so targeted therapy targets these differences within the cell and treat with drugs. One of the most fundamental difference between normal and cancerous cells is that the presence of mutations in the genes which are responsible for causing cell growth are found in cancer cells. These mutated genes produce the defective proteins. Thus, the defective proteins are prime candidates for targeted therapy. Thanks to these defective proteins, the anticancer agents block only the mutant form of the protein in cancerous cells but do not affect the activity of the healthy cells. Beyond this, drug efficiency is low because of the distribution of drugs in the blood stream. So high dose of drug is needed to kill the cancer cells. However, in targeted therapy, drug efficiency is high because of the direct delivery of the drug to the tumor site (Lordick, & Hacker, 2014; Flaherty, 2006; Joo, Visintin, & Mor, 2013).

2.7. Ultrasound Triggered Drug Delivery and Microbubbles

In last years, there are many studies about combine anticancer drugs with an externally applied “trigger”. Thanks to this approach, action of the drugs in the targeted region can be controlled by light, magnetic field, neutron beam or mechanical energy. These applications improve the anticancer drugs delivery and ultrasound irradiation are most commonly used in such this therapy method. There are many advantages using of ultrasound waves for drug delivery system. Thanks to ultrasound wave, drug can reach to deep into the body, tissue or cancerous cells. Beyond these, the ultrasound waves damage cell membrane, increase permeability of the cell membrane and easy to pass of the drug into the cell. For this technology, some ultrasound contrast agents are used. One of the most important and widely used in medical imaging is microbubbles (Hernot, & Klibanov, 2008). Using an ultrasound agent is very important for imaging of tumor. By using microbubble, tumor site can be visualized and observe change in tumor after the drug delivery. The image of microbubble under ultrasound as shown in Figure 2.10. When the ultrasound agent does not present in the tumor site, tumor can not be imaged. However, when the ultrasound agent present in the tumor site, tumor can be imaged easily (Schutt, Klein, Mattrey, & Riess, 2003).

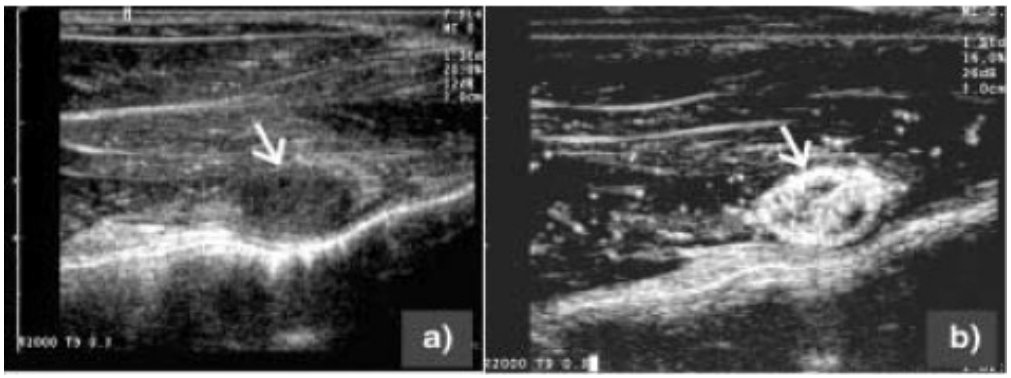


Figure 2.10. The image of microbubbles under ultasound (Source: Schutt, Klein, Mattrey, & Riess, 2003)

Microbubbles are tiny, non-toxic, mechanically oscillate, hydrophobic gas-filled microspheres encapsulated by a biocompatible shell and size of the microbubbles is typically between 0.5 to 10 μm (approximately equal to the size of a red blood cell). Microbubbles are widely used as contrast agents in medical imaging and carriers for

targeted drug delivery. They have a biocompatible shell which sizes are range from 1 to 200 nm by the composition of the particle. There are three types of shell structure and these are lipid, protein, and polymers. They have the thickness of 3 nm, 15-20 nm, and 100-200 nm, respectively. These shell structures show differences by their compliance, stability, and drug loading capacity under ultrasound. Lipid shell-microbubble has high compliance and high echogenicity under ultrasound but their stability and drug loading capacity less than polymer shell-microbubble. However, polymer shell-microbubbles have low compliance and low echogenicity under ultrasound. Echogenicity is important. When the echogenicity is low, imaging quality is low. Microbubble core-shell structure and different shell thickness are in Figure 2.11. Microbubbles are similar with biological membranes as structural and thus, they are very suitable structures for drug delivery system (Cavalli, Bisazza, & Lembo, 2013; Hernot, & Klibanov, 2008; Sirsi, & Borden, 2009).

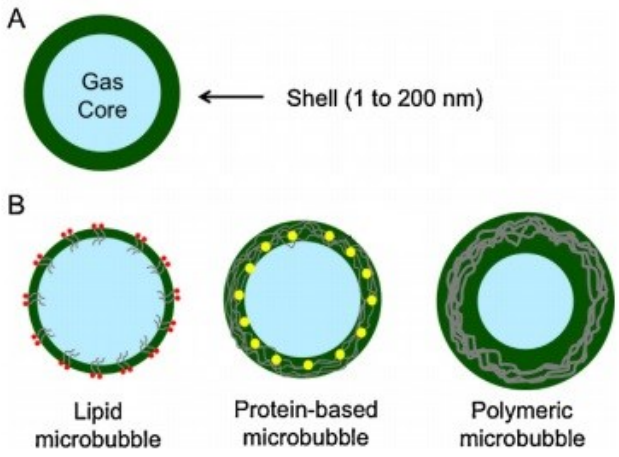


Figure 2.11. (A) Microbubble shell structure, (B) different shell structure by microbubble components (Source: Cavalli, Bisazza, & Lembo, 2013)

2.8. Microbubble and LipoDOX Coupling

LipoDOX and microbubble combine with each other by using streptavidin-biotin conjugation. Streptavidin is a protein and biotin is a water-soluble vitamin. They make a very strong bond with each other (Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010). LipoDOX and microbubble couple is demonstrated in Figure 2.12.

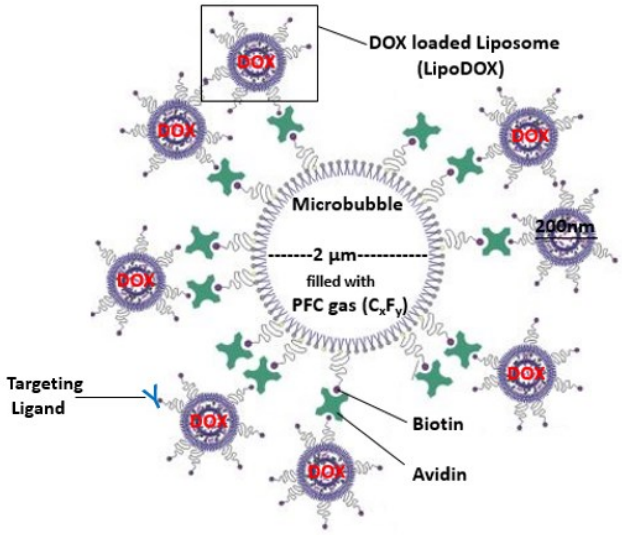


Figure 2.12. LipoDOX-MBs complex (Source: Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010)

When the ultrasound is applied to lipoDOX-microbubble complex, volume of the microbubble increases and then decreases. These compression and expression processes continue like this. And then microbubbles are destroyed and cavitation occurs. Cavitation creates transient pores on the cell membrane and uptake of drug by the targeted tissue becomes easy under sonoporation effect. LipoDOX are released to direction of the targeted cells (Lu, Zhao, Ge, Jin, & Du, 2013). Figure 2.13 shows ultrasound triggered drug delivery and cavitation.

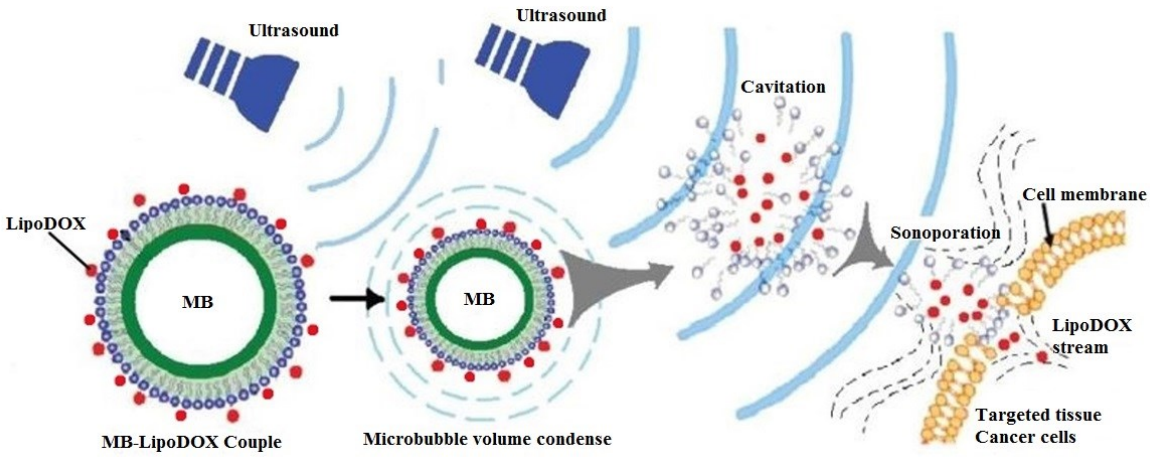


Figure 2.13. US triggered drug delivery (Source: Lu, Zhao, Ge, Jin, & Du, 2013)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-1000] (DSPE-PEG_{1000}), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-350] (DSPE-PEG_{350}), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}-Biotin) and 1,2-Distearoyl-sn-Glycero-3-Phosphocholine (DSPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol from lanolin (5-Cholesten-3β-ol), Polyethylene glycol-40 Stearate (PEG_{40}St), Doxorubicin Hydrochloride (DOX), Chloroform and Triton X-100 used in characterization were obtained from Sigma-Aldrich®, Inc. (St. Louis, MO, USA). Epidermal growth factor (EGF)-biotin conjugate (biotin-EGF) and Streptavidin-PE were from Life Technologies. The dialysis membrane RC tubing (MWCO: 10 kD, Spectra/Por® 6 Dialysis Membrane) was provided by GE Healthcare Life Sciences (Marlborough, MA, USA). Ammonium sulfate was used to prepare a buffer solution, and it was purchased from Merck (Kenilworth, NJ 07033 USA). Some materials used in the experiments and their chemical structures were shown in Table 3.1.

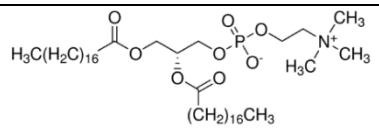
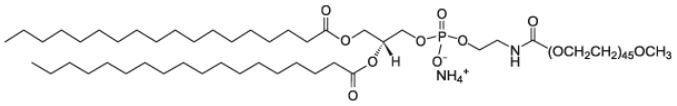
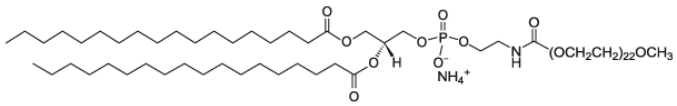
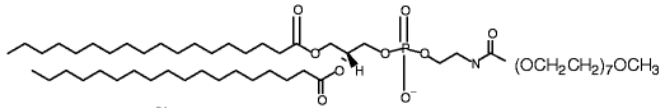
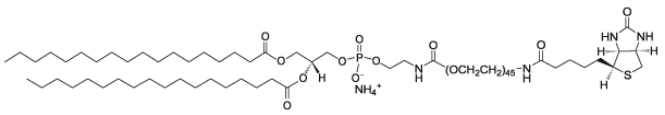
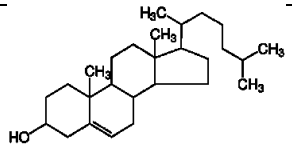
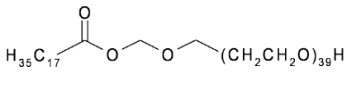
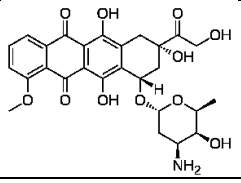
3.2. Methods

3.2.1. Liposome Preparation

In general, the liposomes were prepared in three steps. These are thin lipid film formation, hydration and extrusion steps shown in Figure 3.1. Thin lipid film was formed by mixing of DSPC/Cholesterol/DSPE-PEG_{2000}/DSPE-PEG_{2000}-Biotin at molar ratio of

57/38/4.95/0.05 Optimized formulation was as used to prepare DOX loaded liposome (LipoDOX)-Microbubble complex.

Table 3.1. Some of the materials used in the experiments, and their chemical structure

Materials	Chemical Structure
1,2-Distearoyl-sn-Glycero-3-Phosphocholine (DSPC)	
1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000})	
1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-1000] (DSPE-PEG_{1000})	
1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-350] (DSPE-PEG_{350})	
1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}-Biotin)	
Cholesterol from lanolin (5-Cholesten-3β-ol)	
Polyethylene glycol-40 Stearate ($PEG_{40}St$)	
Doxorubicin Hydrochloride (DOX)	

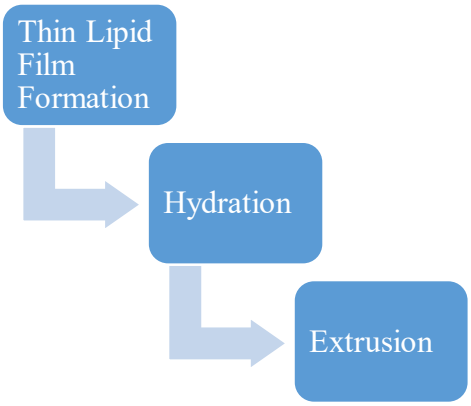


Figure 3.1. The formation steps of liposome

In general, the thin lipid film was formed by mixing of DSPC, Cholesterol, DSPE-PEG_{2000} at the compositions illustrated in Table 3. 1. In the formulations, PEGylated lipid compositions were kept constant as 5% (molar ratio) for all compositions in Table 3.2. In all studies except we examine the effect of lipid compositions, 57:38:05 was chosen as basic liposome composition.

Table 3.2. Composition of Lipid Combinations (Phosphocoline:Chol:PEGylated lipid)

Molar Ratios
95:00:05
80:15:05
75:20:05
67:28:05
57:38:05

To prepare thin lipid film, predetermined amounts of DSPC, Cholesterol, DSPE-PEG_{2000} and DSPE-PEG_{2000}-Biotin were weighed via precision balance into a 20 mL scintillation vial. Then, few of chloroform was added to this powder mixture to dissolve and make homogeneous mixture. The homogenous mixture was shaken on a mixer(Yellowline OS 10) about 1 hour at 250 rpm under the stream of nitrogen gas until most of the chloroform was removed. Then, lipid films were placed in a vacuum oven at

room temperature overnight to remove the remaining chloroform. Lipid films from a vacuum oven were stored at -24°C until they use. Thus, thin lipid film formation steps were completed as shown in Figure 3.2.

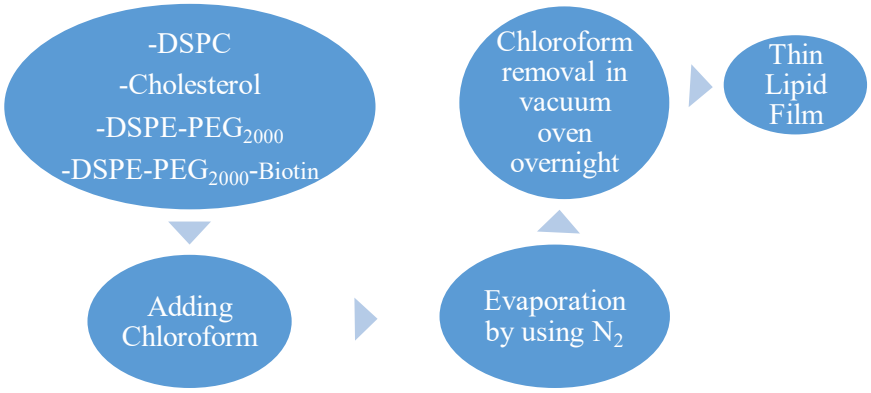


Figure 3.2. The formation steps of thin lipid film for liposome

The second step for liposome preparation was hydration of the thin lipid film as shown in Figure 3.2. Thin lipid films were hydrated in 1 mL 250 mM of Ammonium Sulfate [$(NH_4)_2SO_4$] Buffer (pH 5.4) at a temperature of 65°C via orbital shaking water bath (WiseClean) at 150 rpm for about 1 hour. At the end of the hydration process, Multi Lamellar Vesicles (MLV) were formed. MLV is one of the major types of liposomes, and it has several lamellar phase layers. Size of Multi Lamellar Vesicles is between 0.05-10 µm and its shape was shown in Figure 3.3.

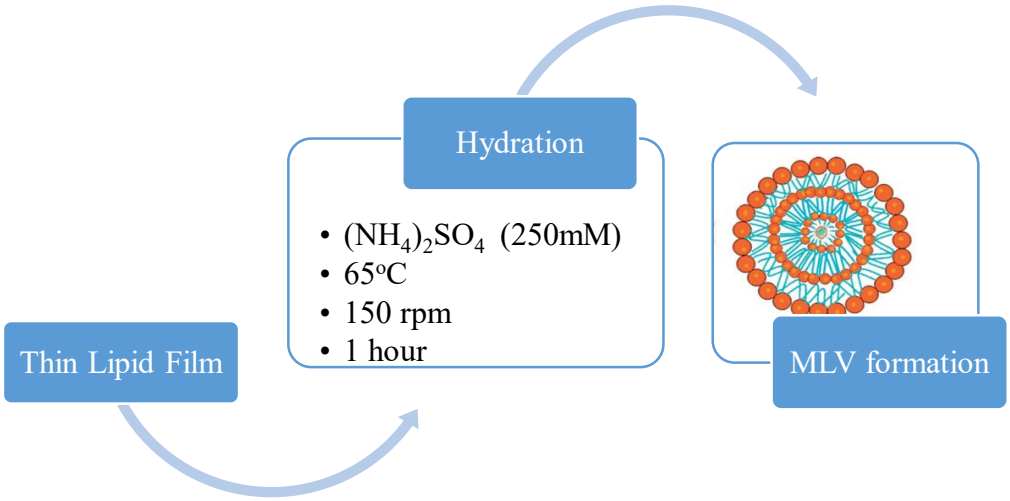


Figure 3.3. Hydration of the thin lipid film for liposome (Source: Tajes et al., 2014)

After the second step of hydration of the thin lipid film and formation of MLV, the extrusion step was applied. The schematic diagram of the extrusion process was depicted in Figure 3.4. Extrusion of hydrated lipid films is a common method for the production of smaller liposomes on a laboratory scale. During the extrusion process, large multilamellar vesicles were pushed through polycarbonate membrane filters at many times. From this, small unilamellar vesicles were formed. The mechanism of the extrusion process is in Figure 3.4. Figure 3.4a illustrates the transformation of multilamellar vesicles to small unilamellar vesicles through the passing 100 nm polycarbonate membrane. Figure 3.4b shows that vesicle extrusion for blowing bubbles through circular pores. The force due to the applied pressure is balanced by a force caused by line tension around the neck of the vesicle. In Figure 3.4c, it was shown that vesicle at the entrance of the pore. The small fragment of the vesicle pulled into the pore has a radius equal to the radius of the pore R_p, whereas the large fragment of the vesicle outside the pore has radius comparable to the original radius of the vesicle R_o. P_2 is the pressure inside the vesicle and the difference between P_1 and P_0 is the applied pressure.

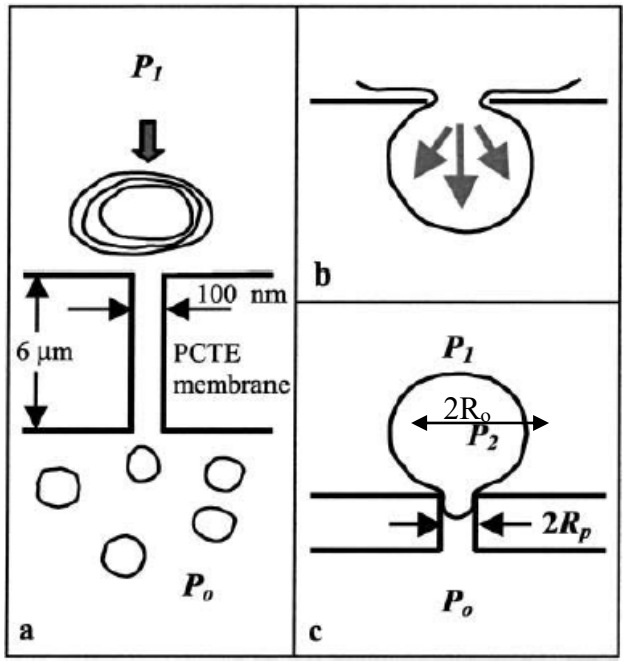


Figure 3.4. Schematic diagram of extrusion process (Source: Patty, & Frisken, 2003).

For the extrusion process, first mini-extruder (Avanti Lipids, Alabama, AL, USA) were assembled as demonstrated in Figure 3.5. The 200 nm of polycarbonate membrane

(Whatman Nucleopore Track-Etch filtration product) was used to reduce the size of liposomes. In addition to these, filter supports were placed in the extruder. Before placing the filter supports, they were wetted with an ammonium sulfate buffer solution.

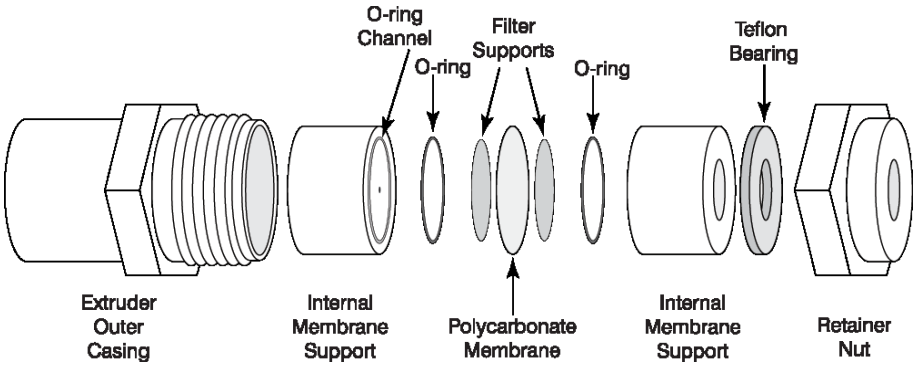


Figure 3.5. Preparation of Extruder (Source: Avanti Lipids, Accessed Feb 25, 2019)

After assembling of the mini-extruder, it was placed on a heater (MTOPS MS300HS) and the heater was set around 73ºC. A thermometer was also situated to the heater. Temperature was tried to hold at 73ºC and avoided rising above 80ºC. At this time, one of the syringes was filled with about 1 mL ammonium sulfate buffer and it was placed on the extruder reciprocally. Ammonium sulfate buffer was passed from one syringe to the other syringe several times. The purpose of this procedure was to decrease void volume inside the extruder. Ammonium sulfate buffer was emptied from the syringe.

The lipid film-buffer mixture was taken from the water bath. One of the two syringes was filled with 1 mL of this hydrated lipid mixture. Syringes were placed to extruder and waited to reach thermal equilibrium. Then, the hydrated lipid mixture was passed through a 200 nm polycarbonate membrane at a constant flow rate of 2.0 mL/min from the syringe to the second syringe with the help of a syringe pump (Longer Syringe Pump LSP02-1B). The hydrated lipid film was passed 10 times through the membrane to obtain a more homogeneous and smaller size liposomes as shown in Figure 3.4a. Extruded liposomes were transferred to an eppendorf tube and stored at 4ºC until use. Characterization of liposomes was done to determine the size distribution and average size of the liposome by using Zeta Sizer (Malvern, NanoZS).

3.2.2. DOX Loading into the Liposomes

Before DOX loading, liposomes were dialyzed against 0.9% NaCl solution using a dialysis bag (Spectra/Por® 6, Spectrum Laboratories, Inc., MWCO: 10000 KD) to remove the ammonium sulfate between the liposomes. The reason for using the NaCl solution for dialysis was to create osmotic pressure and prevent the membrane from swelling or disruption. Dialysate (0.9% NaCl solution) was continuously mixed on a stir plate and replaced with the fresh one after the first 90 minutes. The second dialysate change was performed after 2 hours, and the last change just before loading overnight. Dialyzed liposomes were stored at 4°C until immediately used the DOX loading process. Illustration for dialysis of the liposome against 0.9% NaCl solution as shown in Figure 3.6. Red points show ammonium sulfate loaded liposomes and green points show unloaded ammonium sulfate into liposomes.

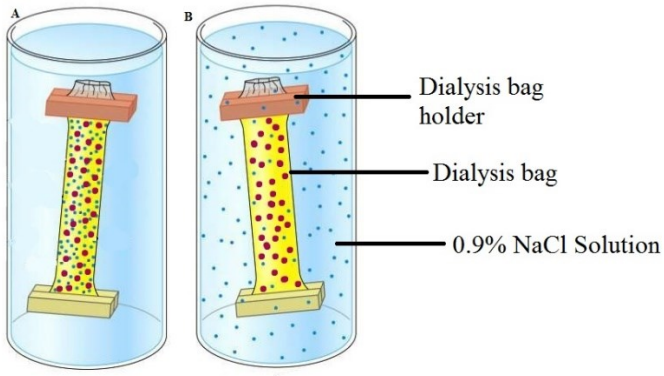


Figure 3.6. Dialysis of ammonium sulfate loaded liposomes A) Before dialysis, B) After dialysis (Source: Foundations of Clinical Sciences, Accessed March 19, 2019)

For DOX loading, DOX solutions of concentrations of 3.0, .0, 1.5, 1.0 and 0.5 mg/mL was prepared. An appropriate amount of DOX was weighed and dissolved in fresh ultra-pure water. DOX solution and dialyzed liposomes were mixed at an equal volume and incubated at 65°C in a water bath shaken at 150 rpm for 90 minutes.

DOX-HCl and ammonium sulfate in liposomes are in balance. DOX and SO_4 combine and they create a crystal structure inside of liposomes. This crystal structure

cannot diffuse to the exterior side of liposomes, they stuck inside. Thus, DOX is loaded into liposome with this mechanism as shown in Figure 3.7.

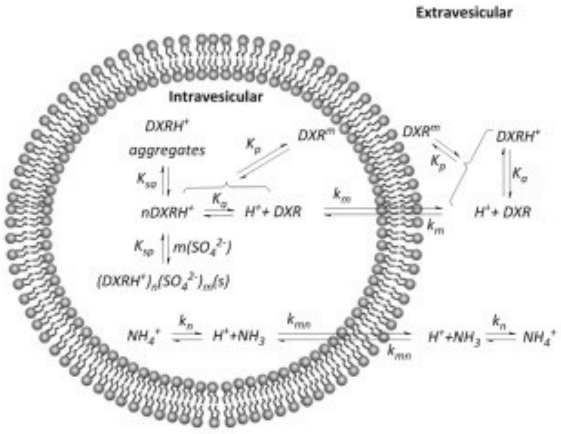


Figure 3.7. Mechanism of DOX loading into liposomes (Source: Fugit et al., 2015)

Unloaded DOX was separated from the DOX-loaded liposome solution (LipoDOX) by dialysis. However, in this step, fresh distilled water was used in the dialysate instead of a 0.9% NaCl solution. LipoDOX not contained unencapsulated DOX, was stored at 4ºC.

3.2.3. Microbubble Preparation

To prepare microubble, DSPC and PEG_{40} Stearate ($PEG_{40}St$) were used. DSPC and $PEG_{40}St$ were mixed with a mole ratio of 5:5. For coupling of LipoDOX and microbubble, DSPE-PEG_{2000}-Biotin was also added to DSPC and $PEG_{40}St$ mixture in a mole ratio of 0.05%.

To prepare thin lipid film, firstly predetermined amount of DSPC, $PEG_{40}St$ and DSPE-PEG_{2000}-Biotin were weighed via precision balance in a glass vial as to be 40 µmol in total. Then, an appropriate amount of chloroform was added to this powder mixture to dissolve and obtain a homogeneous mixture. The chloroform was evaporated by a flowing stream of N_2 on a shaker (Yellowline OS 10) about 1 hour at 250 rpm to obtain a lipid /emulsifier film. After removal of almost all chloroform was evaporated, lipid film was placed in vacuum oven overnight to remove remaining chloroform. Lipid films taken

from a vacuum oven were stored at -24ºC. Thus, thin lipid film preparation step was completed as shown in Figure 3.8.

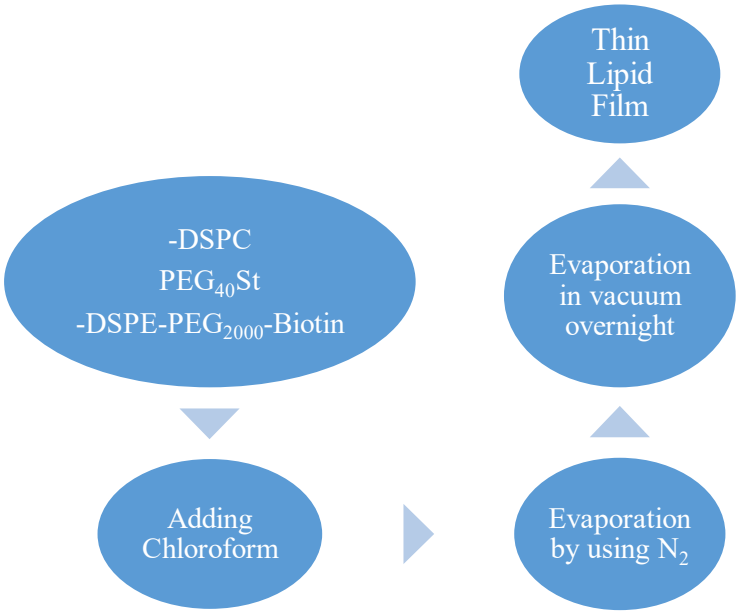


Figure 3.8. The formation steps of thin lipid film for microubble

The second step for microbubble preparation was hydration of the thin lipid film as shown in Figure 3.9. The formed lipid film was hydrated with 4 mL of phosphate buffered saline (PBS, 50 mM, pH: 7.2) and propylene glycol (PG) mixture (volume ratio of 4:1) at a temperature of 65ºC using orbital shaking water bath (WiseClean) at 150 rpm for about 1 hour.

The sonication method was used to produce microbubble by using sonicator (Misonix S4000). The sonicator is a transducer transforming the electric energy to the mechanical vibration with a piezoelectric crystal. Before starting to sonication process, the probe of the sonicator was cleaned with ethanol and then with distilled water. The amplitude of the sonicator was set to 50 kV. A plastic bag was filled with perfluorocarbon gas and the glass vial containing the hydrated thin lipid film mixture was put into this plastic bag. The sonicator probe was entirely immersed into the lipid film mixture and then the formation of the bubbles was provided by mixing the probe up and down motion about 15 seconds. After sonication, about 5 mL of cold PBS buffer kept in the freezer at 4ºC was added to the microbubble suspension in a closed glass vial. The microbubble suspension and PBS buffer were mixed, and the milky solution was withdrawn via a

syringe. The collected microbubble suspension was transferred to a plastic sealed bag previously filled with a few of perfluorocarbon gas. 2 mL of cold PBS buffer was added to the plastic bag again to dilute the microbubble, and then they were kept at 4°C for about 20 min. After 20 min, the bottom phase of the plastic bag was again collected via needle syringe and discarded. This process was first washing. 10 mL of cold PBS buffer was added to a plastic sealed bag and again kept at 4°C for about 20 min. The bottom phase of the bag was collected via needle syringe and discarded. Lastly, 5 mL of PBS-PG mixture was added to a plastic sealed bag and the solution was homogenized before allocation of the microbubbles into crimp-top vials.

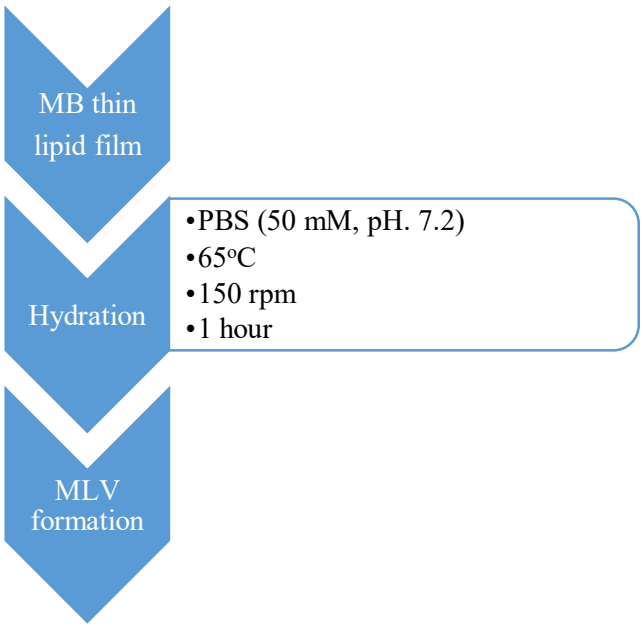


Figure 3.9. Hydration of the thin lipid film for microbubble

3.2.4. Coupling of LipoDOX with Microbubbles

During liposome preparation, besides DSPC, Cholesterol, DSPE-PEG_{2000}, Biotinylated DSPE-PEG_{2000} were added to produce biotinylated liposomes. DOX loaded liposomes were prepared as described in Section 3.2.2. For coupling, Strept Avidin was used. LipoDOX and MBs were combined by using streptavidin-biotin conjugation. Streptavidin is a protein and biotin is a water-soluble vitamin. They make a very strong bond with each other (Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010).

In the literature, Streptavidin is added to MB and then combined with LipoDOX. However, this situation may cause the crosslinking of MBs causing less LipoDOX to be attached to MBs (Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010). In this study, streptavidin was added to LipoDOX to avoid crosslinking in our experiments. Predetermined amount of Streptavidin was added to biotinylated LipoDOX and mixed for a few second and then predetermined amount of MB solution added to Avidinylated LipoDOX. The mixture was placed to the shaker and incubated at 4^{o}C for about 30 minutes. At the end of the 30 minutes, the LipoDOX-MB mixture was washed with PBS at 4^{o}C and centrifuged to remove unbound LipoDOX from LipoDOX-MB complex. This step was repeated twice.

3.2.5. Characterization of the Samples

3.2.5.1. ZetaSizer

In all the laboratory experiments, Malvern Zetasizer Nano Series (ZS, Malvern Instruments) was used to the characterization of liposome, and based on Dynamic Light Scattering (DLS) principle.

DLS known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS) is one of the most popular, non-invasive, well established light scattering techniques to measure size and size distribution of molecules and particles including proteins, polymers, micelles, carbohydrates and nanoparticles in the submicron region because it allows particle size down to 1 nm diameter. DLS measurements depend on the size of the particle core, the size of the surface structures, particle concentration, and the type of ions in the medium.

The sample is enlightened by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle θ by a fast photon detector. DLS measures the speed of particles undergoing Brownian motion. Dynamic Light Scattering principle and Brownian motion were shown in Figure 3.10. The one on the right is DLS principle and the one on the left is Brownian motion.

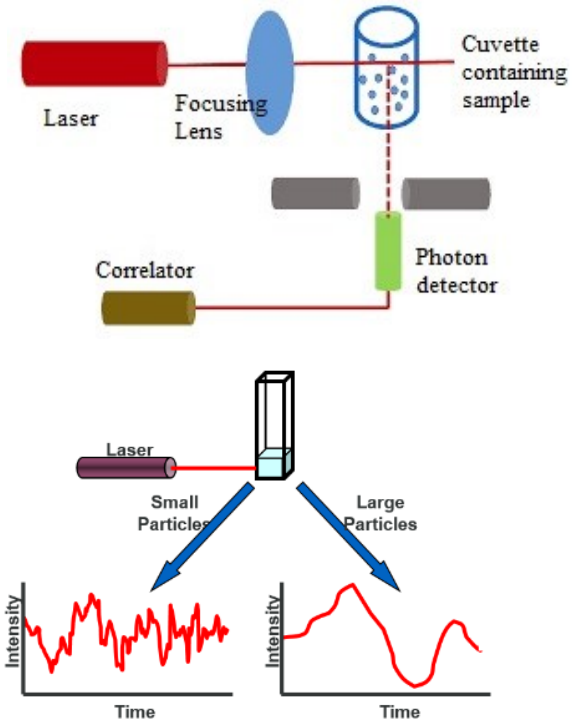


Figure 3.10. Dynamic Light Scattering principle and Brownian motion (Source: Malvern, Accessed Feb 13, 2019)

The smaller particles have faster Brownian motion, and the larger particles have slower Brownian motion. The velocity of the Brownian motion is defined by the translational diffusion coefficient (D) and the translational diffusion coefficient can be converted into a particle size using the Stokes-Einstein equation as shown in Eq. (3.1).

$$d_H = \frac{k*T}{3\pi*\eta*D} \tag{3.1}$$

where;

d_H= Hydrodynamic diameter (m)

k= Boltzmann constant (J/K=kg.m^2/s^2.K)

T= Absolute temperature (K)

η= Solvent viscosity (kg/m.s)

D= Diffusion coefficient (velocity of Brownian motion, m^2/s)

3.2.5.2. Fluorescence Spectrophotometer (Microplate Reader)

Fluorescence spectrometry is a fast, simple and inexpensive method to determine the concentration of an analyte in solution based on its fluorescent properties. It can be used for relatively simple analyses, where the type of compound to be analyzed 'analyte' is known, to do a quantitative analysis to determine the concentration of the analytes. Fluorescence is used mainly for measuring compounds in solution.

In fluorescence spectroscopy, a beam with a wavelength varying between 180 and ~800 nm passes through a solution in a cuvette. We then measure - from an angle - the light that is emitted by the sample. In fluorescence spectrometry both an excitation spectrum (the light that is absorbed by the sample) and/or an emission spectrum (the light emitted by the sample) can be measured. The concentration of the analyte is directly proportional with the intensity of the emission. There are several parameters influencing the intensity and shape of the spectra. When recording an emission spectrum the intensity is dependent on the excitation wavelength, concentration of the analyte solvent, path length of the cuvette and self- absorption of the sample.

During the experiments, the concentration of DOX was estimated with using fluorescence spectrophotometer (Synergy HTX Multi-Mode Micro-plate Reader, BioTek Instruments). The absorbance wavelength of DOX was 480 nm and the fluorescence wavelengths were 480 nm of exciation and 590 nm of emission. Encapsulation efficiency and release rate of DOX were calculated with using fluorescence value of DOX. Encapsulation efficiency of DOX was shown in Eq. (3.2).

$$Encapsulation\ Efficiency\ (\%) = \frac{F_T}{F_{T_0}} * 100 \tag{3.2}$$

where F_T is the fluorescence intensity of DOX after dialysis and F_{T_0} is the fluorescence intensity of DOX before dialysis. The release rate of DOX was shown in Eq. (3.3).

$$Release\ Rate\ (\%) = \frac{F_t - F_0}{F_{T_{x-100}} - F_0} * 100 \tag{3.3}$$

where F_t is the fluorescence intensity of DOX at time t, F_0 is the initial fluorescence intensity of DOX, $F_{T_{x-100}}$ is the fluorescence intensity of DOX after addition of Triton X-100.

Microplate Reader is a compact system for 6- to 384-well microplates automating absorbance, fluorescence and luminescence measurements. Synergy HTX is a single-channel absorbance, fluorescence and irradiation microplate reader and is designed with dual optics to measure microplate format samples. Micro-plate uses a Xenon flash lamp. It operates at wavelengths from 200 to 999 nm in 1 nm increments. Gen5 Data Analysis Software is used for data collection, analysis, exporting and reporting. The principle of fluorescence spectrophotometer was shown in Figure 3.11.

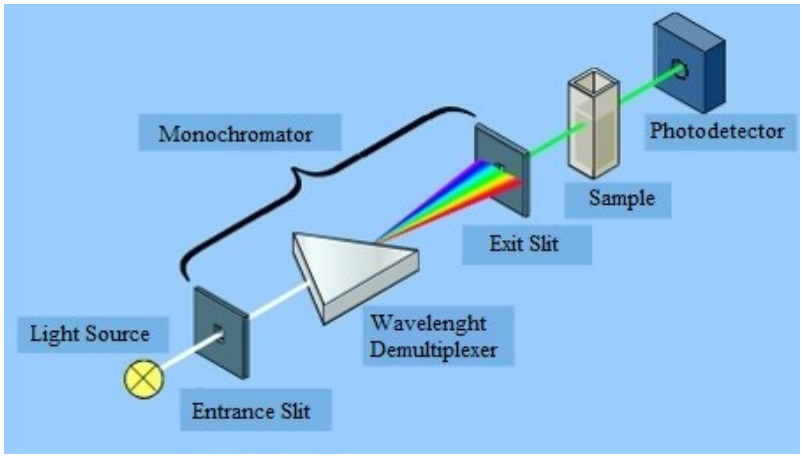


Figure 3.11. The principle of fluorescence spectrophotometer (Source: Chromedia Analytical Sciences, March 27, 2019)

3.2.5.3. Multisizer/Coulter Counter

In the whole experiment, to determine the size distribution and count of the microbubbles, coulter counter instrument (Beckman Coulter Multisizer 4, Coulter Counter, Life Sciences) was used.

A coulter counter is an instrument that can determine the size distribution of electrically non-conducting particles, and their count suspended in a conducting medium. Two electrodes passing constant current are placed on either side of a small hole or aperture through which the suspension is sucked. Because of the smallness of the aperture, the major resistance in the circuit is at the aperture and when a non-conducting particle

passes through, the resistance is changed giving rise to an electrical pulse. The number of pulses matches the number of cells counted and the strength of the signal is directly proportional to the cell volume. Each particle is measured individually. Results are not affected by particle colour, shape, composition or refractive index. The working principle of Beckman Coulter Multisizer was shown in Figure 3.12.

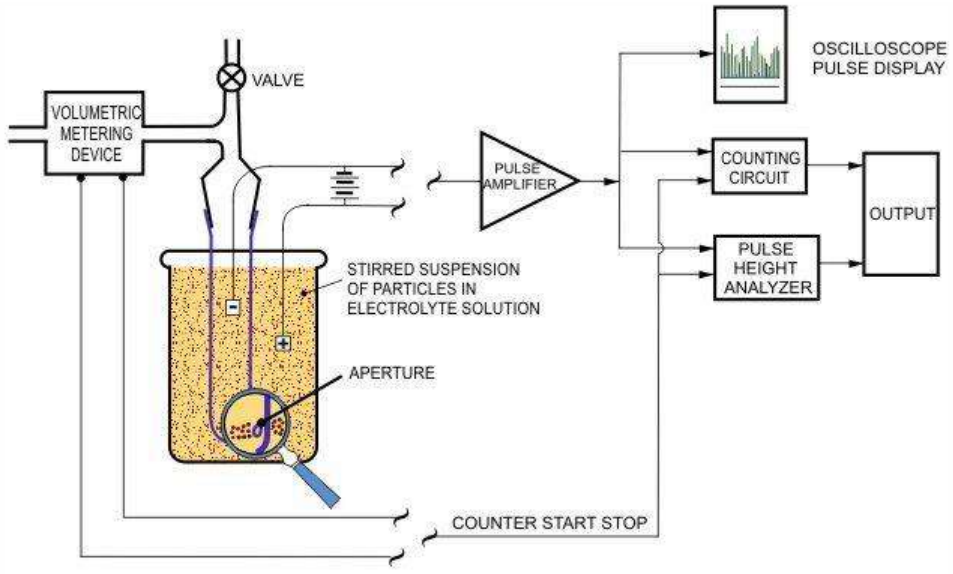


Figure 3.12.The working principle of Multisizer (Source: Thermopedia, Accessed March 13, 2019)

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Ideal Liposome Production

Liposomes have an important role as a drug delivery vehicle. Then, ideal liposome preparation is very important. To produce ideal liposome, some parameters were investigated in this study. These parameters are the size and composition of liposomes. Liposomes were produced by using the extrusion method. To optimize the size of liposomes, pass number, velocity, and polycarbonate membrane pore size were studied. Beyond these, it was investigated how hydration concentration and lipid composition affect the size of the liposomes.

4.1.1. Parameters for Filter Extrusion affecting Liposome Size & Size Distribution

The filter extrusion process is a very important stage for liposomes because of the role of the liposomes in drug delivery. Before applying the filter extrusion process to lipid solution, they are called as Multilamellar Vesicles (MLVs). Their size is more than 0.5 μm. The pore cutoff size in healthy tissues and tumor cells is between 4-25 nm and 380-780 nm, respectively (Nam et al., 2018). MLVs are too large to penetrate healthy and tumor tissues/cells. With the filter extrusion of MLVs, liposomes can be obtained in desired sizes. Liposomes cannot penetrate the healthy cells because of their size but can easily penetrate into the tumor cells. Thus, the extrusion process is very important to achieve penetration of liposomes into targeted sites. Before filter extrusion, the size distribution of MLVs has a wide range. However, after filter extrusion, the size distribution of liposomes is in a narrow range. So, the filter extrusion provides a more monodispersed liposomes.

4.1.1.1. Pass Number

Liposome sizes at the end of 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} passes and as well as prior to any pass are given in Table 4.1 and plotted in Figure 4.1. After every pass, size measurement were performed three times in DLS. Based on the data it can be said that after the 6^{th} pass, liposome size did not change significantly, resulting in liposomes with average size of about 190 nm but the smallest standard deviation was gained as 1.47 after 10^{th} pass. Therefore, 10 pass was chosen as the pass number in our experiments. When pass number was less than 10, liposomes were not reached to desired particle size. On the other hand, when pass number was more than 10 times through polycarbonate membranes it was not effective. Also, in the literature, 11 passes through polycarbonate membranes was reported to be optimum to decrease particle size of liposomes (Isailović et al., 2013). Therefore, our results are in good agreement with the literature pass number values.

4.1.1.2. Velocity

The velocity experiments were performed to understand if there is any effect of extrusion velocity on the particle size of liposomes. For this purpose, liposomes were extruded 10 times at velocities of 0.25, 0.50, 0.75, 1.5, 2 mL/min as well as manually. Liposome solutions were extruded at these predetermined velocities by pushing the plunger of the syringe by means of a syringe pump. The picture of the extruder & syringe pump set-up was given in Figure 4.2.

Table 4.1. Influence of extruding pass number on liposome size

Pass number	Meas. 1	Meas. 2	Meas. 3	Average	STD
4	211.6	215.4	214.9	213.97	2.06
6	179.7	188.4	194.7	187.60	7.53
8	183.7	189.2	196.4	189.77	6.70
10	188.0	190.6	190.5	189.70	1.47
12	185.2	185.4	189.2	186.60	2.25

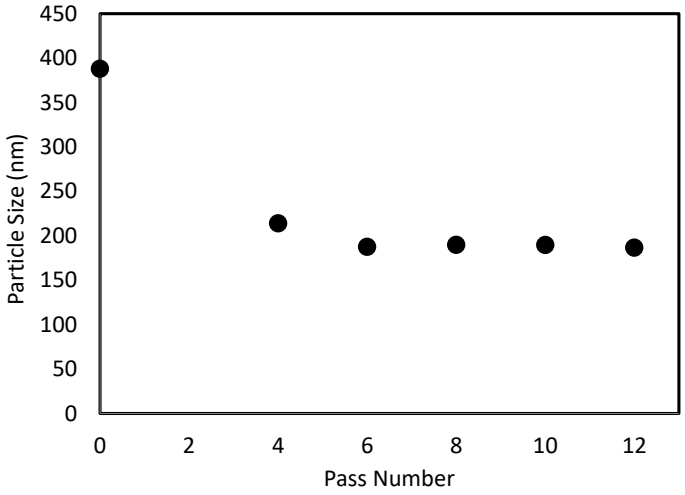


Figure 4.1. Effect of extrusion pass number on particle size (by using 200 nm membrane)

Figure 4.2. Extrusion set-up with syringe pump (in left hand-side) and mini-extruder (in right hand-side, on the top of heater)

The results are given in Table 4.2 and Figure 4.3. In these experiments, the polycarbonate membrane with pore size of 200 nm was used. When we look to average size of liposome given in Table 4.2, it is clearly seen that when the velocity of the syringe pump increases in the extrusion process, the particle size of liposomes get closer to the desired size (200 nm) and the liposome particle size distribution is narrower. Therefore, the velocity was chosen as 2.0 mL/min in our experiments. Moreover, when the liposome solutions were extruded manually (without controlling extrusion speed), it was seen that reproduction of liposomes with the same size and size distribution was not possible.

Table 4.2. Influence of extrusion velocity on liposomes

Velocity (mL/min)	Meas. 1 (nm)	Meas. 2 (nm)	Meas. 3 (nm)	Average (nm)	STD
0.25	243.8	242.5	252.2	246.17	5.27
0.50	367.2	362.3	338.8	356.10	15.18
0.75	244.0	237.8	236.5	239.43	4.01
1.00	231.9	224.4	222.9	226.40	4.82
1.50	234.3	225.3	224.7	228.10	5.38
2.00	241.1	221.4	201.2	221.23	19.95
Manual	308.9	292.3	294.4	298.53	9.04

According to Bernoulli equation, velocity of liquids is related to pressure. Regarding to study of Patty and Frisken showing the pressure dependence of liposome size, we considered that velocity is an effective parameter. They showed that increasing pressure reduced liposome size up to a certain value, but beyond that, liposome size remained unchanged. As shown in Figure 4.3, velocity and liposome size inversely proportional with each other. This result is in good agreement with Patty and Frisken's result, but as mentioned above we could not reach maximum velocity because of the limitations of the syringe pump (Patty, & Frisken, 2003).

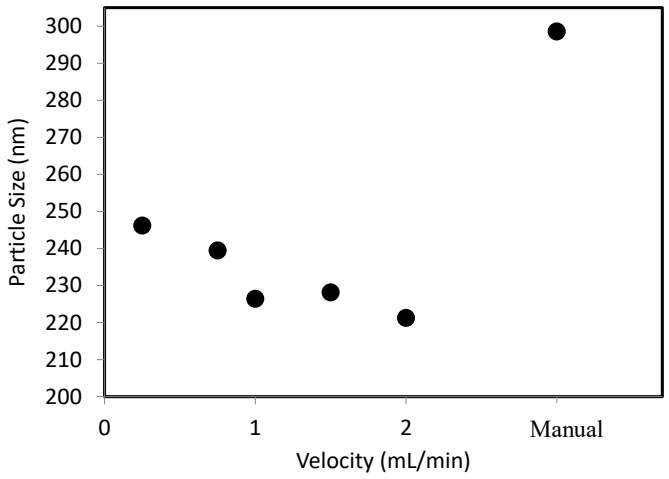


Figure 4.3. Velocity effect on particle size of liposomes

4.1.1.3. Pore Size

We also investigated the effect of polycarbonate membrane pore sizes on the resulting liposome size and distribution. Lipid solutions were passed through 100 nm, 200 nm, and 400 nm and sequentially through 400-200-100 and 200-100 nm membrane. In the experiment, hydration concentration was 20 µM. The experiment results are in Figure 4.4.

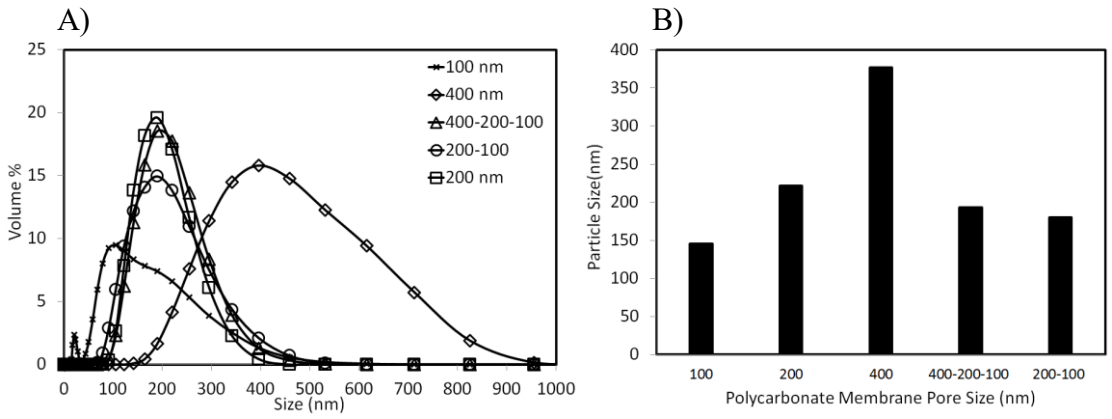


Figure 4.4. Effect of polycarbonate membrane pore size on particle size of liposomes (A) Size distribution (B) Particle size vs. polycarbonate membrane pore size

By the experiment results, 200 nm polycarbonate membrane was selected because of giving the desired size. In Figure 4.4A, the size distribution of liposomes is given. The results of 200 nm and 200-100 nm given the narrowest distribution. Figure 4.4B was also examined, the liposomes prepared by using 400-200-100 nm, 200-100 nm and 200 nm polycarbonate membranes given the best results in terms of approaching the desired particle size. However, the liposomes prepared by using 100 nm polycarbonate membrane are not suitable for DOX encapsulation because of its small size. If the liposome size becomes smaller, the amount of DOX loaded also decreases. Therefore, 200 nm polycarbonate membrane was selected for preparing liposomes.

The values of average particle size and standard deviation obtained by passing through different pore diameters such as 100 nm, 200 nm, etc. by using extruder were shown in Table 4.3.

Table 4.3. Influence of polycarbonate membrane pore size on liposomes size

Membrane Pore Size (nm)	Average Particle Size (nm)				
	Meas.1	Meas.2	Meas.3	Average	Standard Dev.
100	155.5	140.4	141.5	145.8	8.42
200	241.1	221.4	201.2	221.2	19.95
400	360.4	397.3	372.6	376.8	18.80
400-200-100	193.5	196.1	189.5	193.0	3.33
200-100	183.4	178.8	178.2	180.1	2.85

4.1.2. Effect of Hydration Concentration on Liposome Size

To investigate the effect of hydration concentration on liposome size, lipid solutions at concentration of 10 µM, 20 µM and 40 µM were prepared. Liposomes were prepared by using DSPC:Chol:DSPE-PEG_{2000} lipid combination with 57:38:05 molar ratio. Extrusion process was applied with 10-pass, at velocity of 2 mL/min and through 200 nm polycarbonate membrane. In the Figure 4.5, effect of the hydration concentration on particle size of the liposomes are demonstrated.

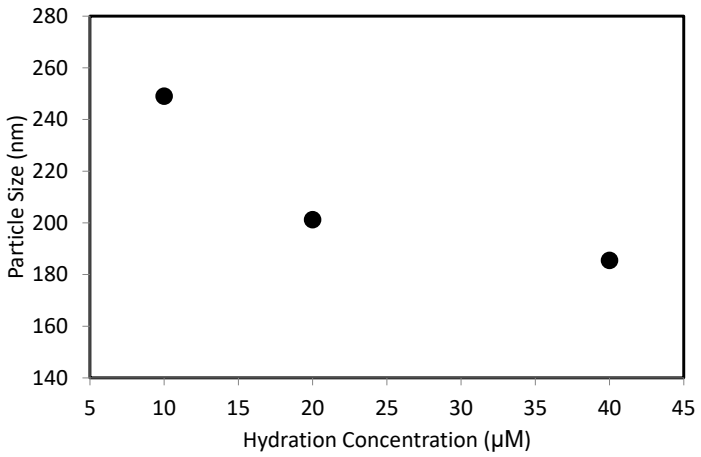


Figure 4.5. Influence of lipid concentration on particle size of the liposomes

As seen in Figure 4.5, there was an inverse proportion between hydration concentration and particle size. When the hydration concentration was 10 μM, the particle size of the liposomes was observed to be about 250 nm, despite 200 nm polycarbonate membrane was used in the extrusion step. When the hydration concentration was 20 μM, the particle size of the liposomes was about 200 nm. When the hydration concentration was 40 μM, the particle size of the liposomes was about 180 nm. In our experiments, we did not prefer the liposomes with the particle size of more than 200 nm for entering of liposomes to the tumor site easily. Also, we did not prefer the liposomes with a particle size of less than 200 nm for encapsulating of the more DOX. Therefore, we chose 20 μM hydration concentration in our experiments.

4.1.3. Effect of PEG Chain Length on Particle Size

PEGylation of liposome surface is very important to obtain stealth function, because PEGylated lipid increases the circulation time of liposome in blood. When the PEGylated lipid was used in liposome structure, liposome is not perceived as a foreign molecule by the body's defense system and it is not destructed. So, drug loaded liposome may remain in the body for longer times. Therefore, we also added a PEGylated lipid into our lipid solutions for making liposome.

Also, polyethylene glycol (PEG) molecular mass is a very important parameter. PEG molecular mass affects liposome size, drug loading capacity of the liposome, cellular uptake efficiency in cancer cells, the flexibility of liposome, interaction with target cells, etc. (Garbuzenko, Barenholz, & Priev, 2005). PEGylation increases the circulation time in blood. However, PEGylation partially inhibits cellular uptake and endosomal release of the drug (Ma, & Yu, 2006). In this study, liposomes were prepared by using DSPC:Chol:DSPE-PEG_{2000} lipid combination with 57:38:05 molar ratio. PEG_{350}, PEG_{1000} and PEG_{2000} were used to understand how liposome size changes with increasing molecular weight of polyethylene glycol in the lipopolymer. The size of three liposomes prepared by using lipopolymers with different molecular weights of PEG, was measured in Dynamic Light Scattering and the results were given in Figure 4.6. As shown in Figure 4.6, liposome with broader size distribution were produced if PEG with lower molecular weight was attached to the lipid. In the figure, the liposome with PEG_{2000} has narrower size distribution and it is more monodispersed than the others. Therefore, PEG_{2000} was

used for preparation of PEGylated liposome in our experiments. Indeed, PEG $_{2000}$ has high cellular uptake in cancer cells and approved as “gold standard” for liposomal formulation in the literature (Pozzi et al., 2013; Saw et al., 2015).

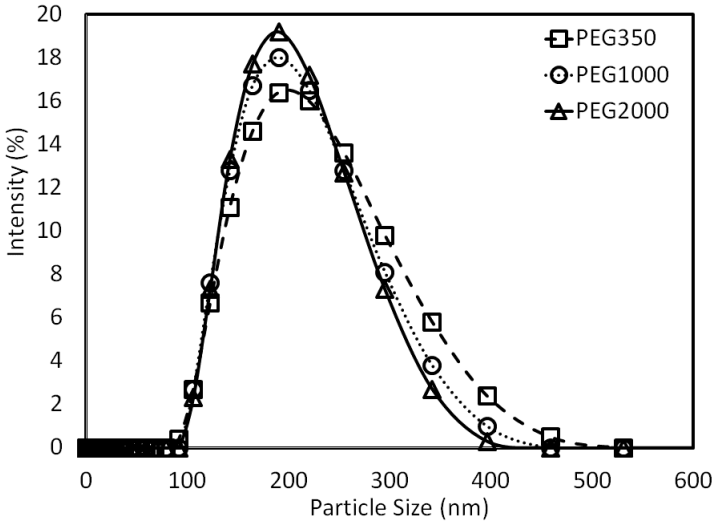


Figure 4.6. Size distribution of liposomes with different PEG chain length

4.1.4. Lipid Composition

To elucidate if the composition of lipid film is a parameter affecting the particle size of liposomes, lipid films compared of A, B, C and D in Figure 4.7 were prepared. In this experiment, lipid mixtures composed of DSPC:Chol:DSPE-PEG$_{2000}$, DPPC:Chol:DSPE-PEG$_{2000}$, DSPC:Chol:DPPE-PEG$_{2000}$ and DPPC:Chol:DPPE-PEG$_{2000}$ were prepared at molar ratios of 80:15:05, 75:20:05, 67:28:05 and 57:38:05. In these experiments, lipopolymer (DSPE-PEG$_{2000}$ and DPPE-PEG$_{2000}$) molar ratios were held constant at 5% to keep away from bilayer-to-monolayer transformation. Thus, effects of Cholesterol, DPPC and DSPC were observed on particle size of liposome.

DSPC:Chol:DSPE-PEG$_{2000}$ lipid combination with different molar ratios was shown in Figure 4.7A. The smallest particle size was obtained at DSPC:Chol:DSPE-PEG$_{2000}$ lipid combination with 57:38:05 molar ratio. When lipid combinations of DPPC: Chol: DSPE-PEG$_{2000}$ with their composition of 80:15.05 was used, the pore size was determined as 210.5 nm and the results were shown in Figure 4.7B. 217.7 nm particle size was obtained as the smallest one by using type of DSPC:Chol: DPPE-PEG$_{2000}$ at composition of 80:15:05, and the results was in Figure 4.7C. When combination of DPPC:

Chol: DPPE-PEG$_{2000}$ with their composition of 57:38:05 was carried out, the smallest liposome size was evaluated as 189.5 nm and the results were shown in Figure 4.7D.

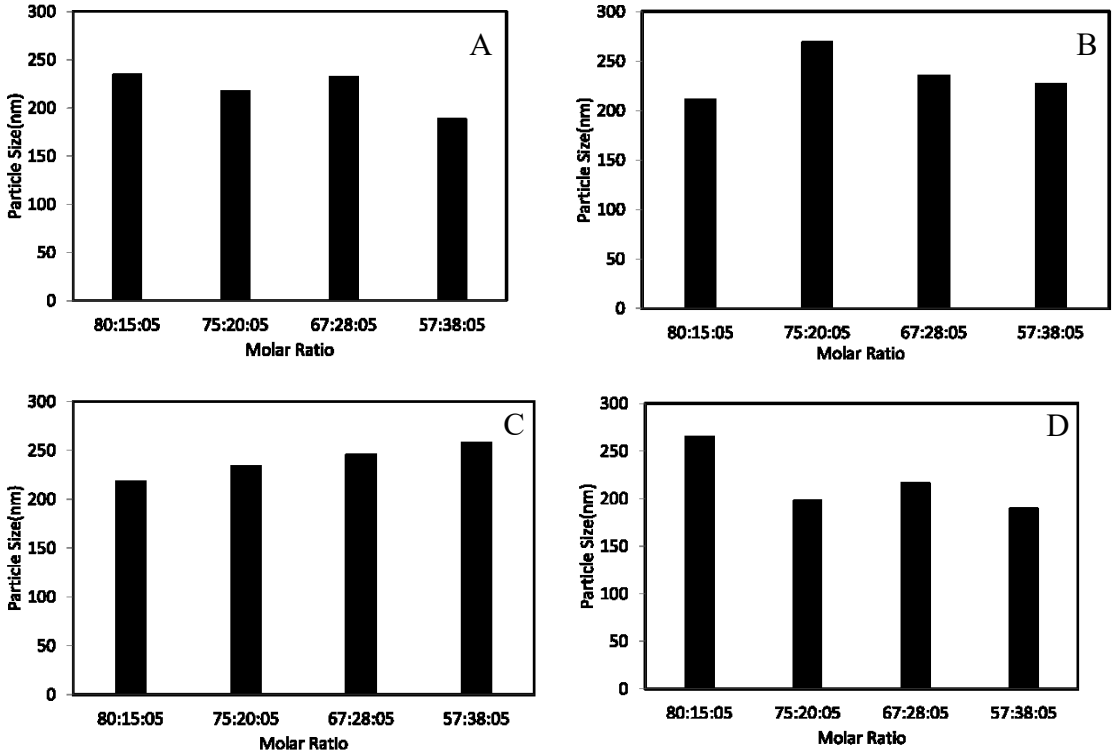


Figure 4.7. Comparison of different molar ratio of lipids A) DSPC:Chol:DSPE-PEG$_{2000}$, B) DPPC:Chol:DSPE-PEG$_{2000}$, C) DSPC:Chol:DPPE-PEG$_{2000}$, D) DPPC:Chol:DPPE-PEG$_{2000}$

In addition, DSPC and DPPC were changed and also molar ratio of lipids was altered as well. Compared to the molar ratio of 80:15:05, the best result was found in lipid types of DPPC:Chol:DSPE-PEG$_{2000}$ since particle size of its was less than the other. It is known that lipid composition influence the structure of liposome, impact on membrane fluidity, permeability and stability.

Based on the results, DSPC:Chol:DSPE-PEG$_{2000}$ and DPPC:Chol:DPPE-PEG$_{2000}$ lipid combinations with 57:38:05 molar ratio are suitable in our experiments by their particle size. In the literature, the most of studies is progressed by using DSPE-PEG$_{2000}$ and 57:38:05 molar ratio. Thus, DSPC:Chol:DSPE-PEG$_{2000}$ lipid mixture at 57:38:05 molar ratio were chosen as the optimum composition to be used in the subsequent experiments.

4.2. Liposome Stability

Liposome stability is important parameter for delivery of therapeutic agents. Moreover, stability of liposome in biological fluids ought to be controlled for their implementations in vivo when serum or plasma components have devastating effect on the bilayer structure.

Liposome stability experiments were carried out with liposome types of DSPC:Chol:DPPE-PEG_{2000} (A), DSPC:Chol:DSPE-PEG_{2000} (B), DPPC:Chol:DSPE-PEG_{2000} (C), and their molar ratios were varied as 80:15:05, 75:20:05, 67:28:05, 57:38:05 in each liposome combinations. As noted above, lipopolymer content (DSPE-PEG_{2000} and DPPE-PEG_{2000}) in the formulation was held constant at 5% to avoid bilayer-to-monolayer transformation.

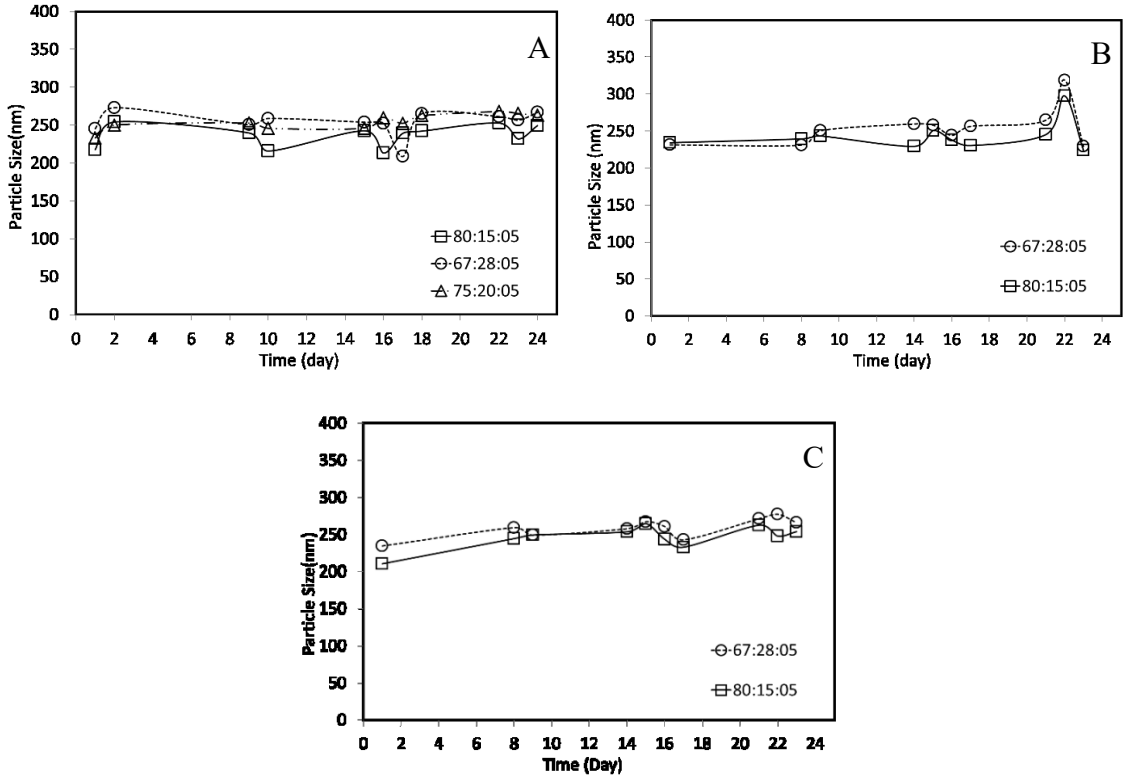


Figure 4.8. Change of particle size of A) DSPC:Chol:DPPE-PEG_{2000} liposome B) DSPC:Chol:DSPE-PEG_{2000} and C) DPPC:Chol:DSPE-PEG_{2000}

Particle size of the liposomes stored at 4ºC was monitored over time using Dynamic Light Scattering (DLS). As seen in Figure 4.8A, the particle size of liposomes

remained between 200 nm and 300 nm for each composition. The most stable liposome types were found as the one at molar ratio of 67:28:05.

Particle size of DSPC:Chol:DSPE-PEG$_{2000}$ liposome was examined at molar ratios of 67:28:05 and 80:15:05. The size measurements were observed to be changing between 200 nm and 300 nm except one trial as indicated in Figure 4.8B. These liposome types were shown similar trend among these two liposomes because particle size measurements by DLS were found close to each other well enough.

Liposomes of DPPC:Chol:DSPE-PEG $_{2000}$ was prepared at molar ratio of 67:27:05 and 80:15:05 were performed. Particle size of these liposomes was found between 200 nm and 250 nm. As Figure 4.8C was examined, particle size of this liposome type was remained almost constant at the end of 23rd day. In this study, it was observed that liposomes are relatively stable in terms of their size regardless of their composition.

To understand that how hydration concentration affect liposome stability, three different liposomes were prepared at 10, 20 and 40 mM of lipid concentration. In this study, liposomes were prepared by using DSPC:Chol:DSPE-PEG$_{2000}$ lipid combination at 57:38:05 molar ratio. The particle size of these prepared liposomes was monitored periodically using DLS. In Figure 4.9, particle size results were demonstrated.

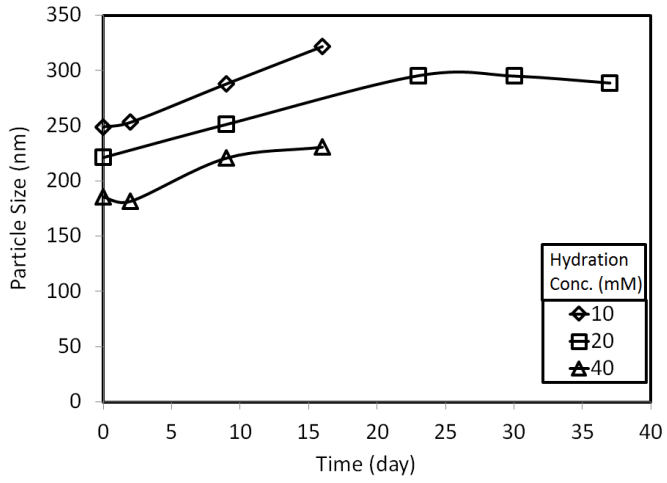


Figure 4.9. Effect of hydration concentration on liposome stability

As seen in Figure 4.9, particle size of the liposomes increased over time. While hydration concentration of liposomes was increased, the increment of particle size decreased. As hydration concentration was 40 µM, particle size of liposomes was 185.5 nm. After 16 days, the particle size raised to 230.6 nm.

For hydration concentration of 20 µM, while particle size was 221 nm at first day, particle size of liposomes was measured as 288 nm after 37 days. The maximum increment was observed in 10 mM of hydration concentration. The particle size of liposomes increased from 250 nm to 320 nm in 15 days.

3 different polycarbonate membrane having 100, 200 and 400 nm pore size were used to understand effect of polycarbonate membrane pore size on liposome stability. After the preparation of lipid thin film, it was hydrated by using ammonium sulfate buffer at 20 µM hydration concentration, and multi lamellar vesicles (MLVs) were produced to obtain liposome from MLVs. First, the solution was extruded by using polycarbonate membrane with pore size of 100 nm, 200 nm and 400 nm, separately and 400-200-100 nm & 200-100 nm systems. The particle size of these liposomes was measured periodically using DLS.

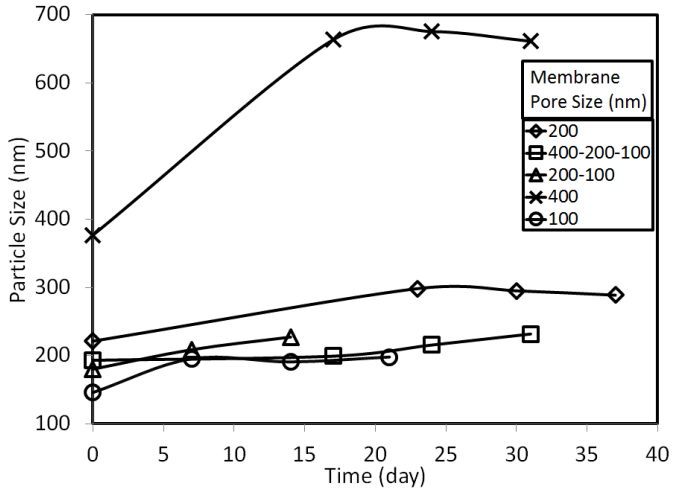


Figure 4.10. Effect of pore size of polycarbonate membrane on liposome stability

In Figure 4.10, effect of pore size of polycarbonate membrane on liposomes stability was demonstrated. Except for the liposomes prepared by using a 400 nm polycarbonate membrane, the increase of particle size in other liposomes was between 40 nm and 60 nm. However, there is a tremendous increase in the particle size of liposomes prepared by using 400 nm polycarbonate membrane over time. As a result, the liposomes prepared using 400 nm could not remain stable over time, but other liposomes remained stable.

4.3. Anti-cancer Drug Loading into Liposome

Considering all the studies, the characteristics of the ideal liposome are listed below:

- Lipid solution hydration concentration : 20 μM
- Liposome formulation : DSPC:Chol:DSPE PEG-$_{2000}$ at molar ratio of 57:38:05
- Pass number in the extrusion process : 10
- The extruder syringe velocity : 2 mL/min.
- Polycarbonate membrane pore size : 200 nm.

After obtaining the ideal liposome and carrying out the stability tests, the drug loading experiments were started. Our aim was to produce the ideal liposome and to load the drug at the maximum level to these liposomes. For this purpose, factors affecting drug loading to liposomes were investigated. Doxorubicin Hydrochloride (DOX) was used in experiments as anti-cancer drug. Factors affecting the loading of DOX to liposome were investigated.

4.3.1. Effect of Temperature on DOX Loading into Liposome

The incubation temperature is a very important parameter in the process of loading the DOX into liposome. Because lipids go to liquid phase passing the phase transition temperatures and so permeability of the lipid membrane increases. Thus, the entry of therapeutic agents through the monolayer or bilayer gets easier.

In this experiment, DSPC was used for preparation of liposome. Liposomes were prepared by using DSPC:Chol:DSPE-PEG$_{2000}$ lipid combination with 57:38:05 molar ratio. The change of DOX loading into liposome was investigated at 22, 35, 45, 55 and 65ºC. Liposome and DOX solutions were mixed equally in volume and then this mixture was put into water bath at 22ºC for during 90 minutes. After 90 min., DOX concentration was measured by fluorescence spectrophotometer. These results are reported as before dialysis and indicated in Figure 4.11A. After before dialysis measurement, LipoDOX solution was dialyzed overnight to remove unloaded/free DOX from liposome and then DOX concentration was measured. These results are reported as after dialysis and demonstrated in Figure 4.11A. All of these processes were repeated for 35, 45, 55 and 65ºC. Also, encapsulation efficiency was calculated and the results were given in Figure 4.11B.

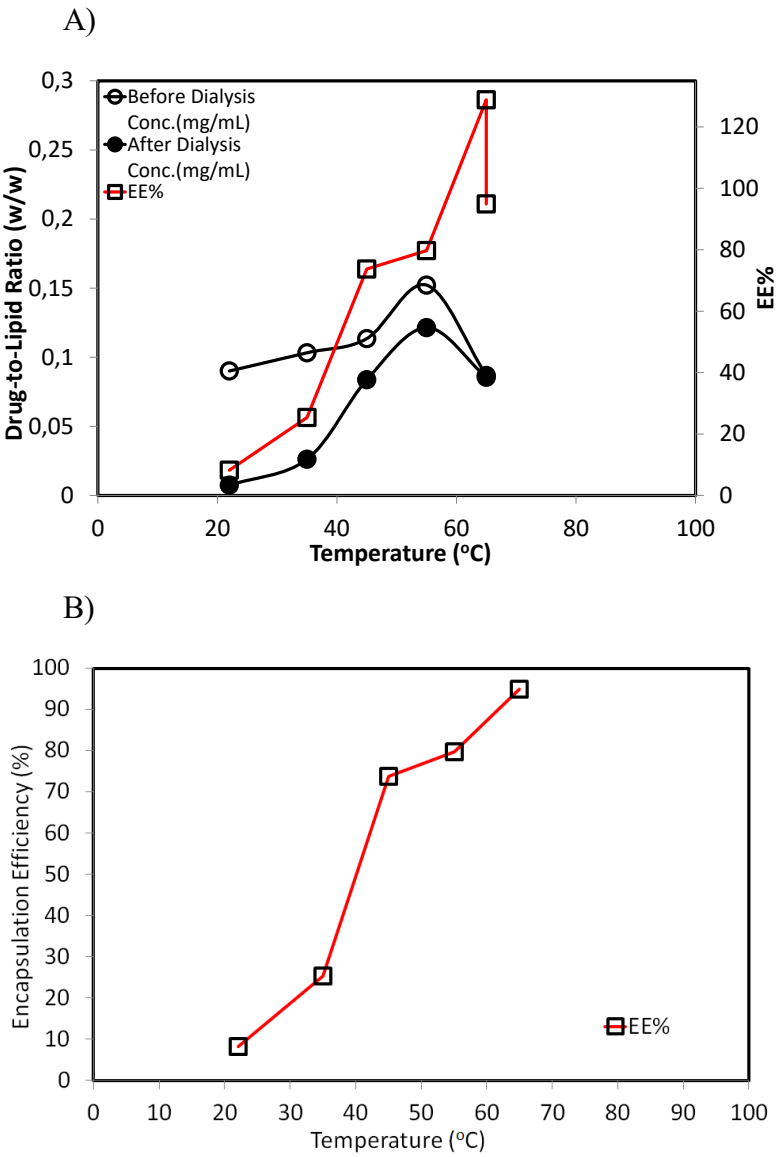


Figure 4.11. Effect of temperature on DOX (DOX concentration : 0.5 mg/mL) loading into liposome, A) Before and after dialysis measurement, B) Encapsulation efficiency

As seen in Figure 4.11A, when incubation temperature was increased from 22°C to 55°C, the amount of loaded DOX also increased as expected, because phase transition temperature of DSPC is 55°C. When the temperature was increased, encapsulation efficiency of DOX also increased proportional with temperature (Figure 4.11B). Unexpectedly, the amount of loaded DOX into liposome decreased at 65°C. In fact, the loading of DOX into liposome was expected to increase even more at 65°C. In the

literature, the incubation temperature is usually set to 60 and 65ºC in the process of loading the DOX into the DSPC liposomes.

Luo and co-workers used DSPC:Chol:Pyro-lipid:DSPE-PEG_{2000} with molar ratio of 53:40:2:5 to prepare liposome in their studies and incubation temperature of DOX and liposome was set as 60ºC. They found the encapsulation efficiency to be close to 100% (Luo et al., 2016). Shaikh and co-workers also used DSPC:Chol with molar ratio of 55:45 to prepare liposome. In their experiments, incubation temperature was set as 60ºC (Shaikh et al., 2013). Lastly, Ong and co-workers used DSPC:Chol:DSPE-PEG_{2000} with molar ratio of 55:40:5 and incubation temperature was determined as 65ºC in their studies (Ong, Sun, & Chan, 2011). To determine the DOX concentration, the calibration curve was used. DOX calibration curve was shown in Figure 4.12.

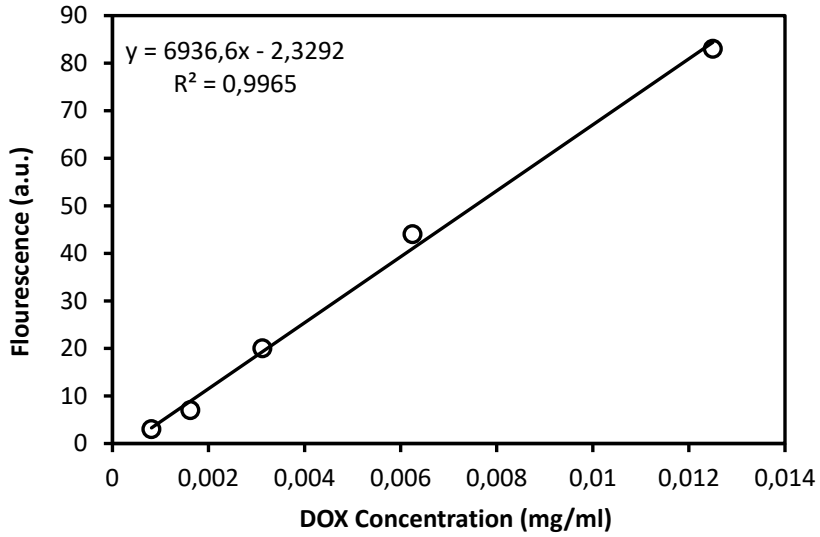


Figure 4.12. The calibration curve of DOX

The fluorescence values between 10 and 80 is valid because of linearfor determining DOX concentration. Because after 80, the graph is not linear. Although the DOX concentration increases, the graph begins to decrease. Quenching is observed and DOX concentration does not measure truely. Therefore, while the determining DOX concentration, DOX is diluted in order to be in between 10 and 80.

Thus, to understand effect of temperature along with DOX concentration on DOX loading into liposome, another experiment was done. In this experiment, DSPC was used to prepare liposome. Incubation temperature of DOX and liposome mixture was set as 55, 60 and 65ºC. Also, DOX concentration during loading was varied as 1.5, 1.0, 0.75,

0.5 and 0.25 mg/mL. Through this experiment, both temperature and DOX concentration effect on loading was determined. The results were shown in Figure 4.13.

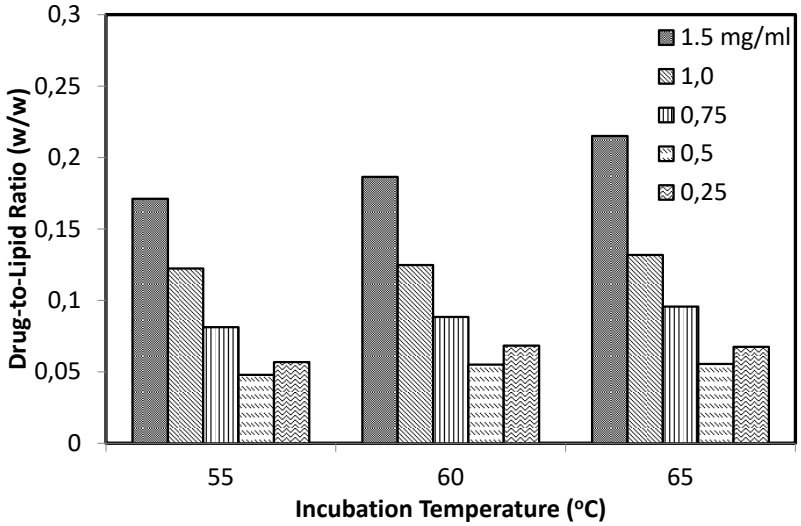


Figure 4.13. Effect of temperature and DOX concentration on DOX loading into liposome

As seen in Figure 4.13, it was observed that the DOX loading increased as the temperature was increased. Especially, this increase was more pronounced at high DOX concentrations. It was seen that the maximum DOX loading into liposome was at 65°C. However, there was no difference in DOX loading between 60 and 65°C at low concentrations, especially at 0.5 and 0.25 mg/mL. In our studies, we usually used DOX concentration of 0.5 mg/mL. According to these results, temperatures of 60 and 65°C were ideal for incubation of DOX and liposome.

4.3.2. Effect of Temperature on Pure Doxorubicin Hydrochloride

To understand if temperature has any effect on pure DOX, different concentrations of DOX were prepared and fluorescence measurements were taken at different temperatures. Firstly, 0.5 mg/mL stock DOX solution was prepared. Then, serial dilutions were made from this stock solution and different concentrations of DOX solutions were obtained. Fluorescence measurements of these DOX solutions were measured at 25, 28, 30, 32, 34, 36, 38 and 40°C by using fluorescence spectrophotometer. The results are shown in Figure 4.14.

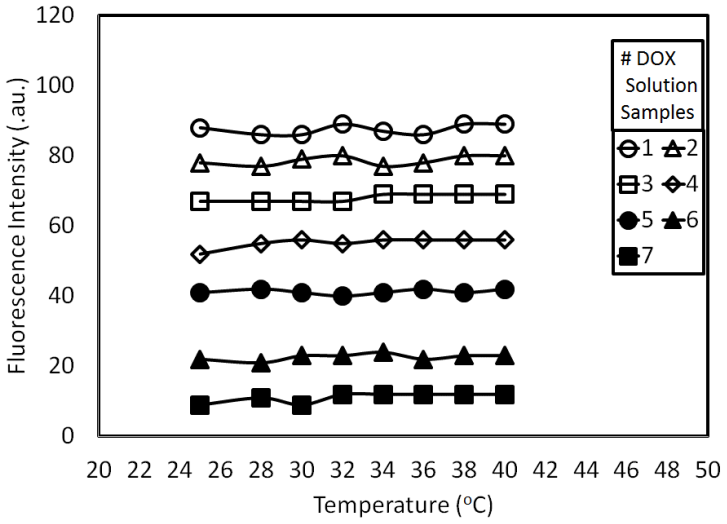


Figure 4.14. Effect of temperature on pure DOX

Figure 4.14 shows the fluorescence values of different concentrations of DOX solutions at different temperatures. In the figure, DOX solution with number 1 was obtained by diluting the stock solution 93.75 fold. The other DOX solutions with numbered 2, 3, 4, 5, 6 and 7 were obtained by diluting 112.5, 140.625, 187.5, 281.25, 562.5 and 1406.25 fold, respectively. When the graph was examined for each different DOX solution, the change in temperature did not affect the fluorescence value of DOX. The temperature did not affect the fluorescence value of DOX.

4.3.2. Effect of High Temperature on Pure DOX with time

In this experiment, our purpose was to understand the effect of high temperature on pure DOX. For this purpose, the DOX solution was prepared at 0.5 mg/mL. Then this prepared solution was incubated in the water bath at 65ºC and 150 rpm and fluorescence measurement was taken at 30 min intervals. Figure 4.15 indicates the concentration change of DOX measured every 30 minutes at 65ºC during 4 hours. As seen in the figure, no changes were observed in DOX concentration during 4 hours. However, according to Janssen and co-workers results indicated that, when the temperature was increased, decomposition of DOX was also increased. They reached this conclusion by taking measurements at 37, 61 and 72ºC for 30 hours. In our study, DOX exposed to temperature

of 65ºC for only 4 hours (Janssen et al., 1985). As a result, since the high temperature did not cause any damage to the liposome and DOX, we decided to use temperature of 65ºC in the incubation studies

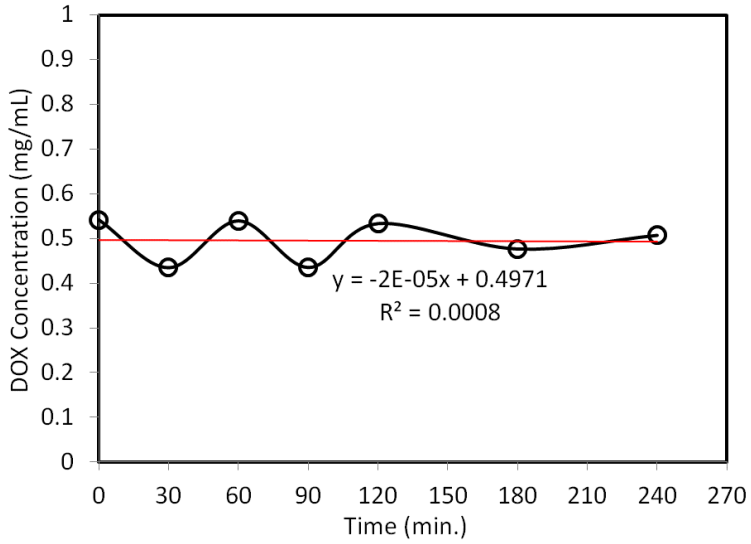


Figure 4.15. Effect of high temperature on pure DOX with time

4.3.3. Effect of Pretreatment on DOX Loading into Liposome

In this experiment, it was investigated that whether pretreatment has an effect on loading DOX to liposomes. Normally, DOX solution and liposome are mixed in equivalent volume at room temperature without any heat treatment and then they are incubated in the water bath at 65ºC and 150 rpm to load DOX into liposomes. However, in this study, the liposome and DOX solution were preheated separately in a water bath at 20, 30, 40, 50, 60 and 65ºC. After the temperature of liposome and DOX reached to desired temperature, they were mixed in equivalent volume and then placed in a water bath at 65ºC and 150 rpm for loading process. This process was done for 30 minutes and 90 minutes. In the Figure 4.16 effect of pretreatment on DOX loading into liposome was demonstrated.

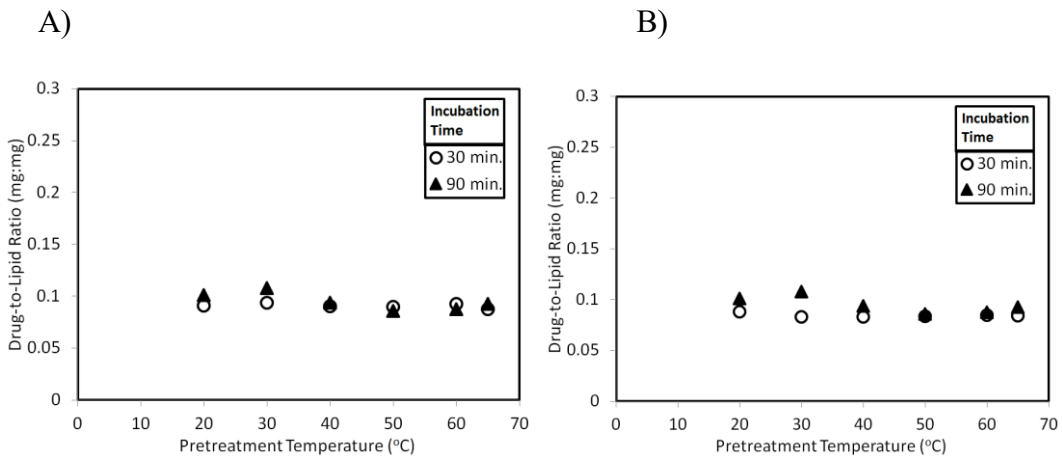


Figure 4.16. Effect of pretreatment on DOX loading into liposome. A) Before dialysis measurement, B) After dialysis measurement

The purpose of this experiment was to determine whether pretreatment had an impact on DOX loading. The phase transition temperature of DSPC lipid is 55ºC (Chen, Dusa, Witos, Ruokonen, & Wiedmer, 2018). As the liposome is exposed to the temperature, as especially it approaches 55ºC, it goes to the liquid phase increasing membrane permeability.

In the Figure 4.16A, DOX-to-Lipid ratio prior to dialysis is indicated. During incubation of liposome and DOX, DOX is loaded into both core of the liposomes and same is adsorbed around the liposomes. To remove the unloaded DOX, dialysis process was applied.

As seen in the Figure 4.16A and B, pretreatment did not affect DOX loading into liposome. For each temperature, drug-to-lipid ratio did not change. Also, incubation time which is 30 and 90 min. did not affect DOX loading. As a result, we understand that the DOX loading into liposome does not depend on the pretreatment.

4.3.4. Effect of Incubation Time on DOX Loading into Liposome

In this experiment, the optimum incubation time for DOX loading into liposome was investigated. In the literature, when the DOX loading studies were examined, we saw that incubation time of DOX loading into liposome varies between 10 minutes and 6 hours. In this study, DSPC:Chol:DSPE-PEG_{2000} with molar ratio of 57:38:5 was used to

prepare liposomes. DOX solution was prepared at 1.0 mg/mL and incubated with liposomes at varying durations changing from 15 minutes to 210 minutes. After each incubation, DOX concentration was measured by using fluorescence spectrophotometer. Encapsulation efficiency of DOX was also determined and all results were demonstrated in Figure 4.17.

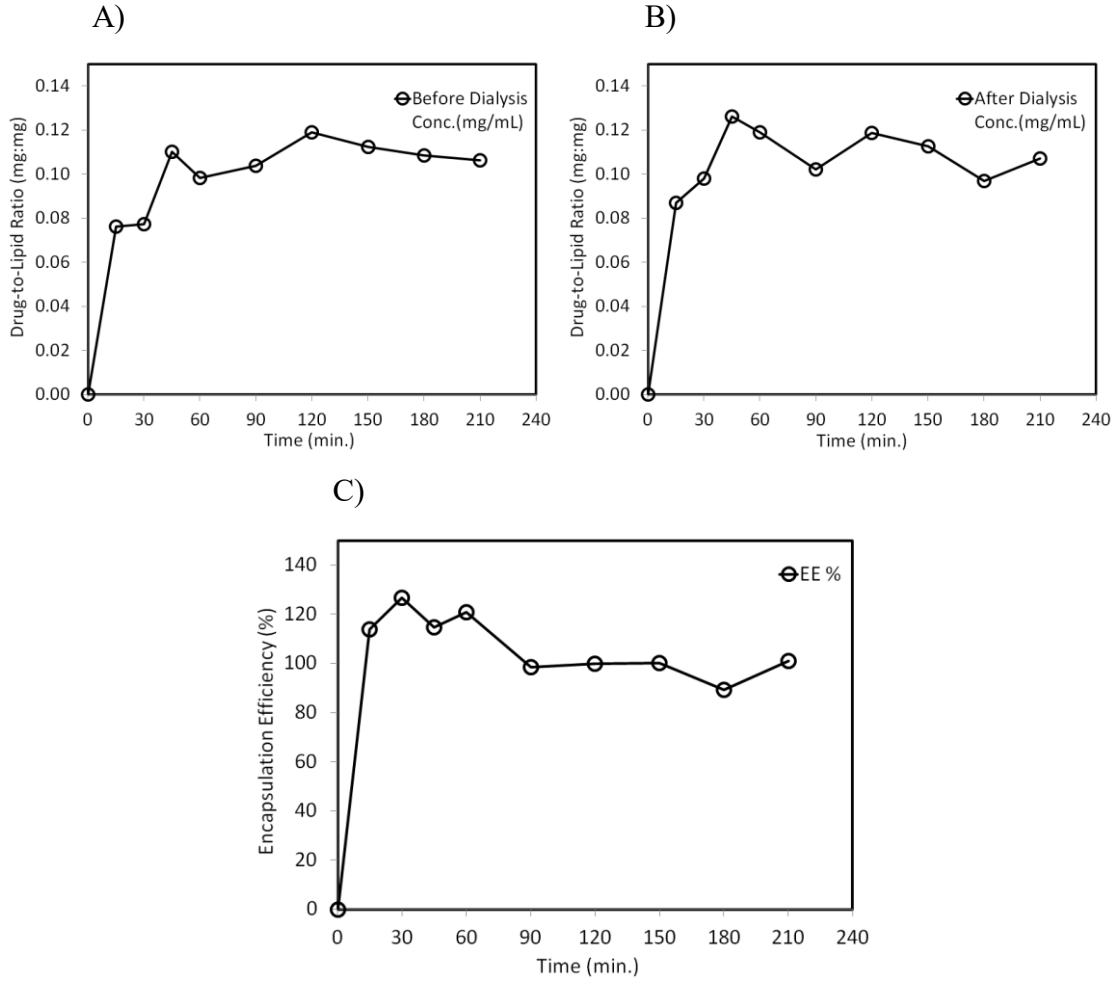


Figure 4.17. Effect of incubation time of DOX and Liposome on DOX loading into liposome containing 38% Cholesterol A) Before dialysis measurement of LipoDOX, B) After dialysis measurement of LipoDOX, C) Encapsulation efficiency of DOX

As seen in Figure 4.17A and B, the loading of DOX into the liposome seems toincrease within the first 45 minutes then a plateau was reached for incubation time of DOX-liposome mixture after 90 minutes and there was no change in the drug-to-lipid

ratio. However, EE% above 100% may mean that between 15-90 minutes DOX is not reached to liposome core, but some remained as adsorbed on liposome surface, resulting in lower fluorescence values due to quenching. This period should be a minimum of 90 minutes and this is clearly evident from the graphs. However, there are many studies in the literature. Boman and co-workers carried out incubation process of DOX and liposome at 60ºC and 10 minutes and they obtained 100% encapsulation efficiency (Boman et al., 1993).

Yu et al. also carried out DOX loading process at 65ºC and 30 minutes and obtain the high encapsulation efficiency (Yu et al., 2015). However, according to our results, the loading process was not completed before 90 minutes. Sheela et al. (2005) made liposomes using DMPC lipid and investigated the amount of loaded DOX into the liposome at different temperatures and times. They incubated liposome with DOX at 20, 40 and 60ºC. The measurements of DOX concentration were taken at 5, 10, 20, 40, and 80 minutes and the drug-to-lipid ratio was determined. According to their results, maximum amount of DOX was loaded into liposome at 60ºC and 40 minutes (Sheela et al., 2005). The phase transition temperature of DMPC is 24ºC (Avanti Lipids). Thus, DOX loading could be completed at 40 minutes.

When we look at Figure 4.17C, it is seen that encapsulation efficiency is more than 100% in the first 90 minutes. However, after 90 minutes, the encapsulation efficiency of DOX was fixed at a value close to 100%. Deng et al. completed the loading process of DOX into liposome at 65ºC and 4 hours (Deng et al., 2014). Also, Ong and co-workers (2011) reported that loading process was completed at 65ºC in 2 hours (Ong et al., 2011). Shaikh et al. (2013) prepared DSPC: Chol lipoosmes at molar ratio of 55:45. The DOX and liposomes were heated separately at 60ºC for 10 minutes before mixing together. Then DOX loading process was performed at 60°C. At the end of the experiment, it was seen that the drug loading was completed in 90 min. They reported the encapsulation efficiency of more than 90% (Shaikh et al., 2013). As seen, various incubation times are reported in the literature for DOX loading.

Figure 4.18 shows that how the LipoDOX size changes with incubation time of liposome-DOX mixture. The size of LipoDOX does not change with incubation time.

The same study was performed by using liposomes prepared with different cholesterol ratios. It was aimed to investigate whether the amount of cholesterol in liposome affects incubation time. In addition, incubation time of liposome-DOX mixture

was investigated in the absence of cholesterol. For this purpose, liposomes containing 0% and 26% cholesterol were prepared.

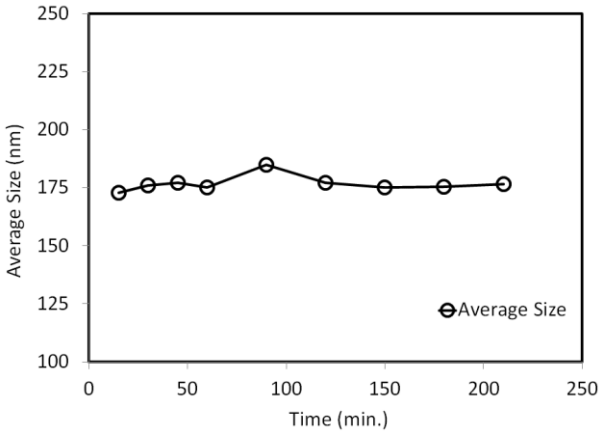


Figure 4.18. Effect of incubation time of DOX & Liposome on average size of LipoDOX

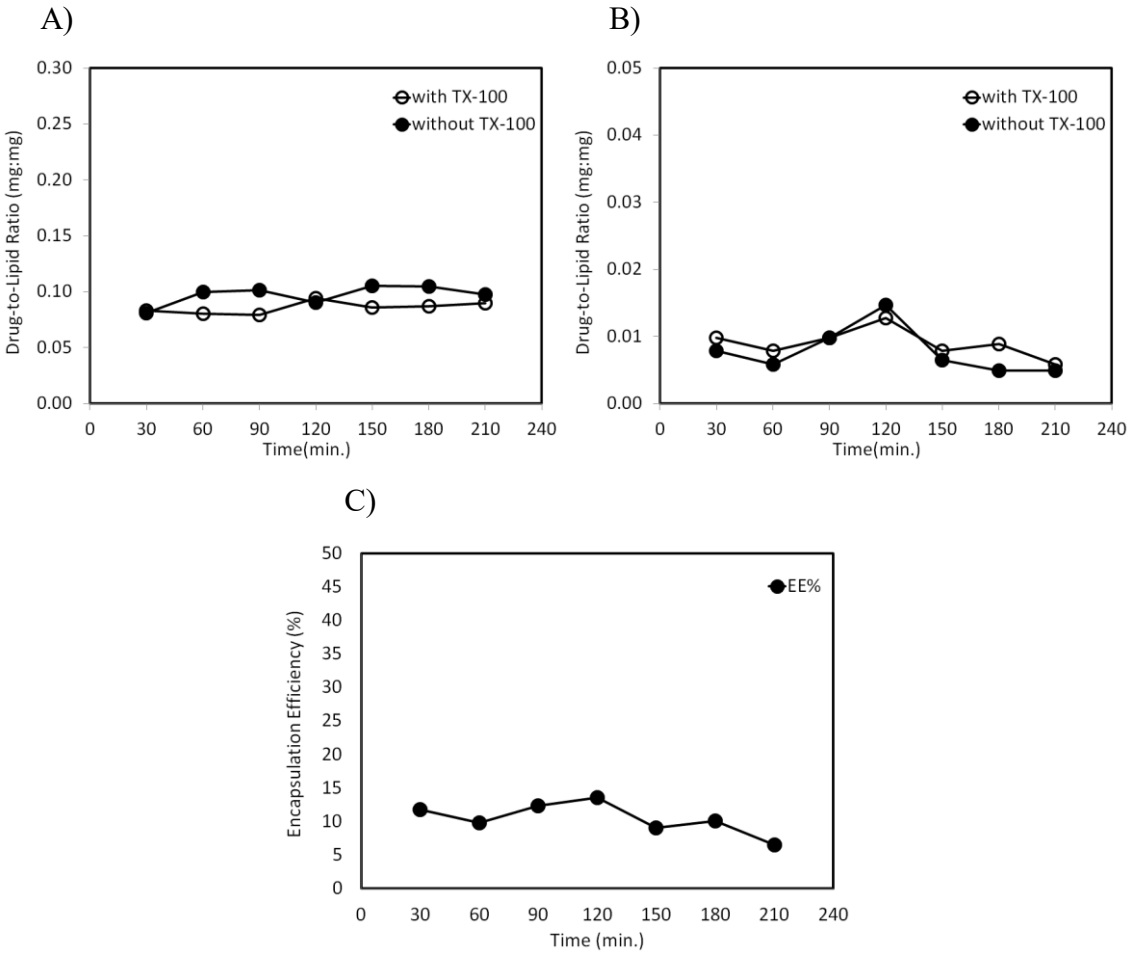


Figure 4.19. The change of DOX concentration in 0% Cholesterol LipoDOX with and without TX-100 A) Before Dialysis, B) After Dialysis
C) Encapsulation efficiency of DOX

In Figure 4.19, the amount of DOX in LipoDOX was measured both using with and without TritonX-100 (TX-100). When we look at Figure 4.19A, it is seen that the results of the measurement with and without TX-100 are the same. This indicates that could not enter into the liposomes and but remained adsorbed on liposomes. The loading of DOX into the liposomes failed in this case. We can also understand this from the encapsulation efficiency graph, Figure 4.19C. However, when we look at the graph showing the encapsulation efficiency of DOX, we see that encapsulation efficiency is very low about 10% for liposome prepared without cholesterol. It is understood that, cholesterol should be used while liposomes are prepared to obtain high efficiency in DOX loading.

Figure 4.19B shows the measurement results of the DOX concentration after dialysis process. In this graph, the measurements with and without TX-100 were given. The results of measurements are the same as in Figure 4.19A. In this graph, it was observed that the results of measurements with and without TX-100 were the same.

Because of this, DOX which could not be loaded into the liposomes, was removed by the dialysis process and only the DOX loaded into the liposomes was measured. However, when we compared Figure 4.19A and Figure 4.19B, it was seen that the amount of measured DOX was lowered after dialysis. While the drug-to-lipid ratio was about 0.1, this rate decreased to 0.01 and below after dialysis. Thus, too much DOX could not be loaded into the liposomes and removed by dialysis. It was also understood from this graph that the DOX loading process was not efficient in the absence of cholesterol.

The same experiment was also done for the liposomes with 26% cholesterol. In Figure 4.20, the results obtained by loading DOX in different incubation times to liposome prepared with using 26% cholesterol were demonstrated.

In Figure 4.20, the amount of DOX in lipoDOX was measured both using with and without TritonX-100 (TX-100). When we look at Figure 4.20A, DOX was measured without dialysis after DOX loading process. In addition, Figure 4.20B shows the measurement results of the DOX concentration after dialysis process.

In Figure 4.20A, the measurements with and without TX-100 were taken to find the amount of DOX loaded into the liposome before dialysis of lipoDOX. It was clearly seen that the drug-to-lipid ratios obtained from the measurements with and without TX-100 were different from each other. The measurement with TX-100 gives the amount of DOX which was both loaded and unloaded into the liposomes and the drug-to-lipid ratio was calculated as 0.12. The measurement without TX-100 gives the amount of DOX

unloaded into the liposomes and the drug-to-lipid ratio was 0.09. Being different from each other of the values obtained from the results of the measurements with and without TX-100 means that only a certain amount of DOX is loaded into liposomes. Figure 4.20B shows the results of the measurements with and without TX-100 after dialysis of lipoDOX. As seen in the figure, the drug-to-lipid ratio is 0.06 in the measurement with TX-100 and 0.006 in the measurement done without TX-100. Being low value in the measurement done without TX-100 indicates that the dialysis procedure was successful. However, the drug-to-lipid ratio was 0.12 in the before dialysis measurement, whereas after dialysis this value decreased to 0.06. Figure 4.21 shows the encapsulation efficiency of DOX.

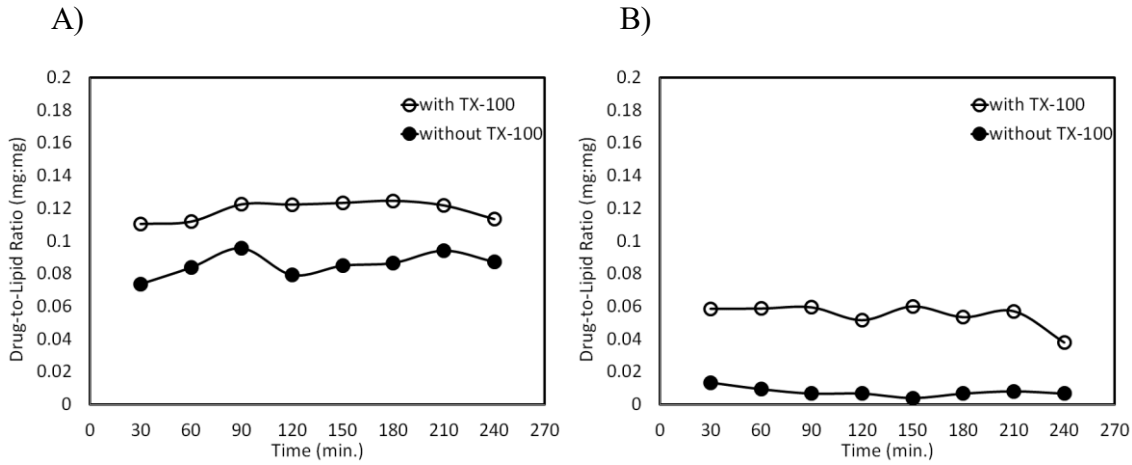


Figure 4.20. The change of DOX concentration in 26% Cholesterol LipoDOX with and without TX-100 A) Before Dialysis, B) After Dialysis

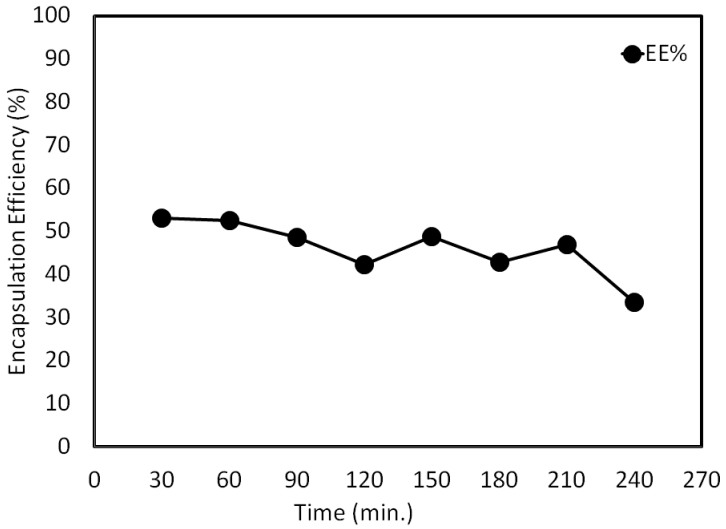


Figure 4.21. Encapsulation efficiency of DOX

When we also look at Figure 4.21 showing the encapsulation efficiency of DOX, we see that encapsulation efficiency is about between 40-50% for liposome prepared with 26% cholesterol.

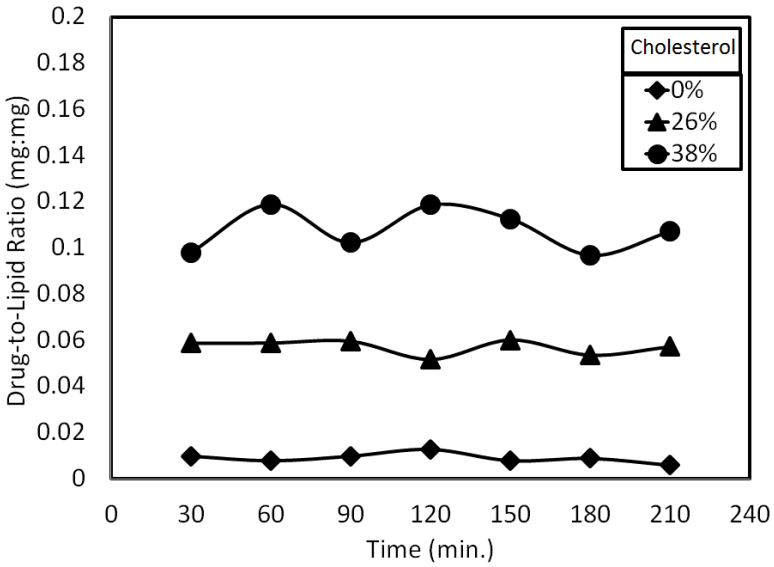


Figure 4.22. The change of DOX concentration in LipoDOX with changing cholesterol ratio in liposome with TX-100

When all studies of DOX loading into liposomes prepared using 38%, 26% and 0% cholesterol are considered. In Figure 4.22, it seems that the amount of cholesterol in liposome is increased DOX loading and, the encapsulation efficiency of DOX also increased.

4.3.6. Effect of PEGylation on DOX Loading into Liposome

PEGylation of liposomes increases the blood circulation times, providing stealth properties. PEG allows liposome to extend their circulation half-life and, consequently, increasing the accumulation of liposomes within tumors. Also, we know that the bilayer-to-monolayer transformation is observed when the liposome has more than 8% PEG in its structure. So PEG can be used between 0% and 5% in liposome. In order to decide how much we should use it, we need to know how PEGylation has affected the DOX loading into liposomes. Therefore, different liposomes were prepared using different percentages of DSPE-PEG_{2000}. The ratio of cholesterol in these liposomes was kept constant. Liposomes were incubated under the same conditions and with the same amount

of DOX. The DSPC:Chol:DSPE-PEG$_{2000}$ ratios in the liposomes were set as 62: 38: 0, 61: 38: 1, 60: 38: 2, 59: 38: 3, 58: 38: 4 and 57: 38: 5. The results were given in Figure 4.23.

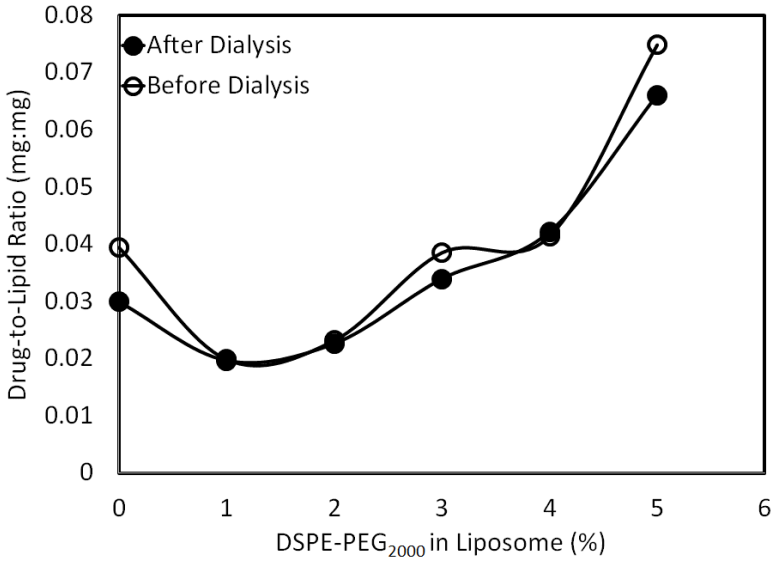


Figure 4.23. The effect of the ratio of DSPE-PEG$_{2000}$ in liposome on DOX loading

As seen in Figure 4.23, when the PEG ratio in the liposome increased, the amount of DOX loaded into liposome increased. The highest loading was observed by using the ratio of 57:38:5 of DSPC:Chol:DSPE-PEG$_{2000}$. For liposome contained 5% DSPE-PEG$_{2000}$, encapsulation efficiency of DOX was obtained as more 95%. But, encapsulation efficiency of DOX was between 80-90% for other liposomes with less than 5% DSPE-PEG$_{2000}$ molar ratio.

In studies in the literature, PEG molar ratio in liposome was usually set as 5%, while liposome was prepared. This is because the amount of DOX loaded into liposomes and the encapsulation efficiency of DOX reduce, when the molar ratio of PEG in liposome is kept less than 5%. Likewise, when more than 5% PEG is also used to liposome preparation, the amount of DOX loaded into liposomes decreases. In addition, when liposome is prepared using more than 5% PEG, micelle formation is observed, especially molar ratio of 8% and more. This is undesirable situation. Because DOX-HCl is a hydrophilic molecule. DOX cannot be loaded into micelles due to micelles are hydrophobic nanoparticles. Luo et al. (2016) prepared different liposomes with using 0%, 1%, 3%, 5% and 8% molar ratio of DSPE-PEG$_{2000}$ and investigated the effect of PEG on

DOX loading into liposomes. Also, release of DOX from liposomes was investigated. According to their results, the maximum drug loading was obtained for 5% molar ratio of DSPE-PEG_{2000}. In addition, encapsulation efficiency of DOX was in the maximum value for 5% PEG. When the PEG molar ratio in liposome increased, the amount of drug loaded into liposomes increased. However, the reduction was observed in the amount of loaded DOX for 8% molar ratio of DSPE-PEG_{2000}. In addition to these, DOX release from liposomes was in the maximum for 8% DSPE-PEG_{2000} by comparison other liposomes with different PEG molar ratio. It is not desirable that DOX which presents in LipoDOX released in blood as soon as it enters the body. Because DOX also damages healthy cells. This is also why liposomes are used. The goal is to achieve controlled release of DOX when the LipoDOX arrives at the targeted tumor site. In the other study performed by Wehbe et al. (2016), 0.5%, 1% and 5% molar ratio of DSPE-PEG_{2000} were used to prepare liposomes. They were observed that when the PEG molar ratio in liposome structure increased, the drug loading efficiency increased in direct proportion. It was obtained maximum loading efficiency and maximum amount of the drug loaded into liposome, when the 5% molar ratio of DSPE-PEG_{2000} in liposome structure (Wehbe et al., 2016).

In our study, it was investigated the effect of different DSPE-PEG_{2000} ratios on liposome size. In Figure 4.24, liposomes size results were demonstrated.

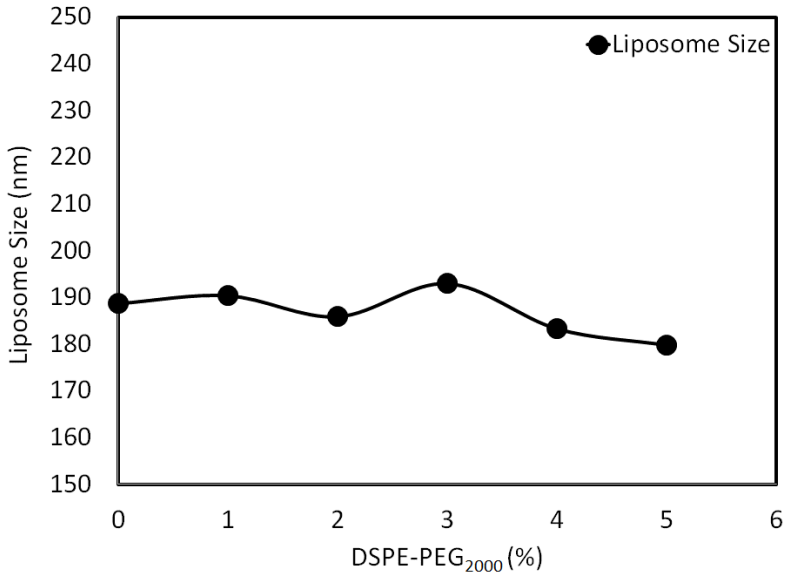


Figure 4.24. The effect of different DSPE-PEG_{2000} ratios on liposome size

As seen in Figure 4.24, size of the liposomes did not change with changing molar ratio of DSPE-PEG_{2000} in liposome. For each PEG ratios, liposome size was about between 180-190 nm.

As the molar ratio of PEG in liposome has an effect on DOX loading into liposomes, the PEG chain length also has a significant effect on drug loading. For this purpose, the study was performed to understand that how the PEG chain length effects on DOX loading. DSPE-PEGs with 3 different chain lengths were used. These are DSPE-PEG_{2000}, DSPE-PEG_{1000} and DSPE-PEG_{350}. Liposomes were prepared by using PEGs with these 3 different chain lengths and PEG molar ratio in liposome was kept constant as 5%. DSPC:Chol:DSPE-PEG molar ratio in liposomes was set as 57:38:05. And each liposome was prepared in 3 different sizes; 400, 200 and 100 nm. Thus, both the effect of PEG chain length on DOX loading and the effect of liposome size on DOX loading were investigated. The results were shown in Figure 4.25.

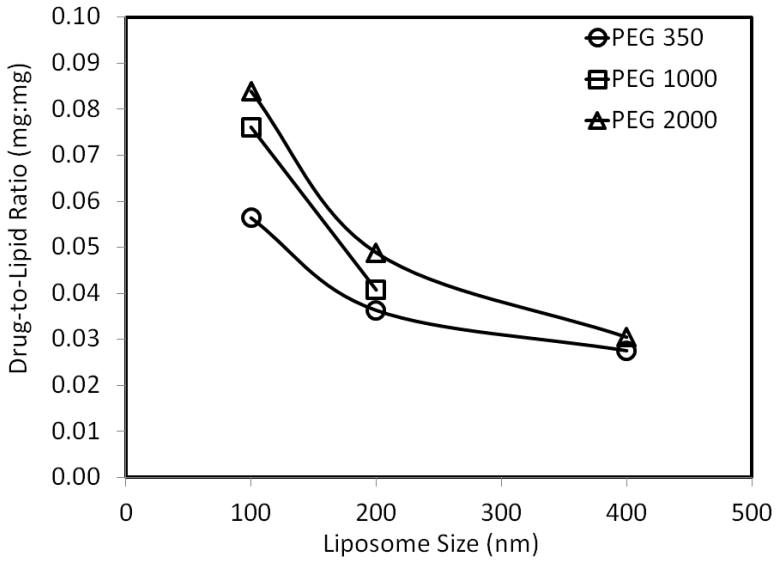


Figure 4.25. The effect of PEG chain length on DOX loading

For drug delivery systems, “PEG dilemma” is one of the most important problem. There are many drug loading studies performed with liposome prepared without using PEG, as well as there are many drug loading studies using PEGylated liposome. Generally, 5% molar ratio of PEG_{2000} is used to prepare liposomes in most studies. In the literature, PEG_{2000} is accepted as “gold standard”. In our experiment, we investigated that why PEG_{2000} is gold standard.

According to the graph, when the PEG chain length increased, drug-to-lipid ratio also increased. For each liposomes in 3 different sizes, the amount of DOX loaded into liposomes prepared by using DSPE-PEG_{2000} was more than the other PEGylated liposomes (DSPE-PEG_{1000} and DSPE-PEG_{350}). Also, when the liposome size decreased, the difference between the drug-to-lipid ratios of each different PEGylated liposomes was more noticeable. When the liposome size increased, this difference was almost nonexistent. In addition to this, DOX loading increased with decreasing the size of the liposomes. Drug loading is higher at smaller liposomes. This can be explained by the number of liposomes. When the number of liposomes increases, the surface area will also increase. And DOX cannot be loaded into liposomes, may be adsorbed on the surface of the liposomes.

Pozzi et al. (2013) used DSPE-PEG_{1000}, DSPE-PEG_{2000} and DSPE-PEG_{5000} in their experiment and they investigated that the effect of PEG chain length on tumor cell viability. It was observed that when the PEG chain length in liposome structure increased, liposome size decreased. In their study, PEGylated liposomes with DSPE-PEG_{2000} showed the high cellular uptake in tumor cells (Pozzi et al., 2013). Abe et al. (2015) used PEG molecular weights of 750, 2000, and 5000 to prepare lipsoomes in their experiments. On the contrary of Pozzi et al., they proved that when the molecular weight of PEG (PEG chain length) increased, liposome size was also increased (Abe, 2015 & Pozzi, 2013). The liposome size results which changes depending on PEG chain length were demonstrated in Figure 4.26.

In the graphs, size distribution of liposomes with different size (400 nm, 200 nm and 100 nm) was given. According to Figure 4.26A, B and C, the size of the liposomes prepared by using different PEG did not change with changing PEG chain length. When we examined the graphs separately, the liposomes in the same size and prepared with different PEG demonstrated the same size distribution. Their size distribution curve overlapped. Consequently, PEG chain length did not affect the liposome size.

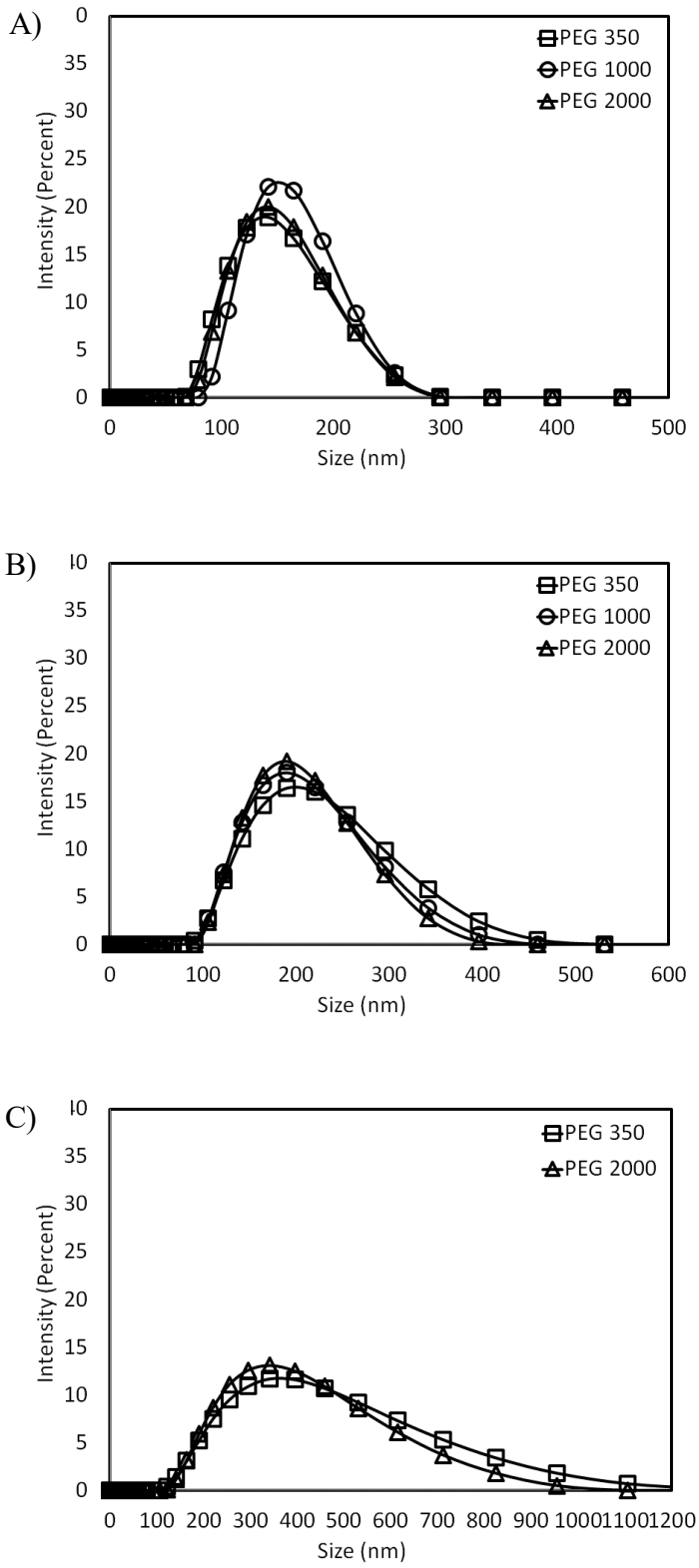


Figure 4.26. The effect of PEG chain length on liposome size with A)100 nm, B) 200 nm, C) 400 nm liposomes

4.3.7. Effect of Liposome Size on DOX Loading into Liposome

To understand the effect of liposome size on DOX loading, liposomes with different size were prepared and DOX was loaded into these liposomes in the same condition and amount. The polycarbonate membranes of 400, 200 and 100 nm were used in this purpose and obtain liposomes in 3 different sizes. In addition to these liposomes, different liposomes were prepared through 3 different procedures. First, the liposome was passed through a 400 nm polycarbonate membrane and then it was passed through a 200 nm polycarbonate membrane. Another liposome was first passed through a 400 nm polycarbonate membrane and then passed through a 100 nm polycarbonate membrane. The final liposome was first passed through a polycarbonate membrane of 200 nm and then passed through a polycarbonate membrane of 100 nm. In other words, we have 3 liposomes with a size of 100 nm, 2 liposomes with a size of 200 nm and 1 liposome with a size of 400 nm. These 6 liposomes were incubated with DOX at the same concentration and amount. The DOX loading results for these 6 different liposomes are given in Figure 4.27.

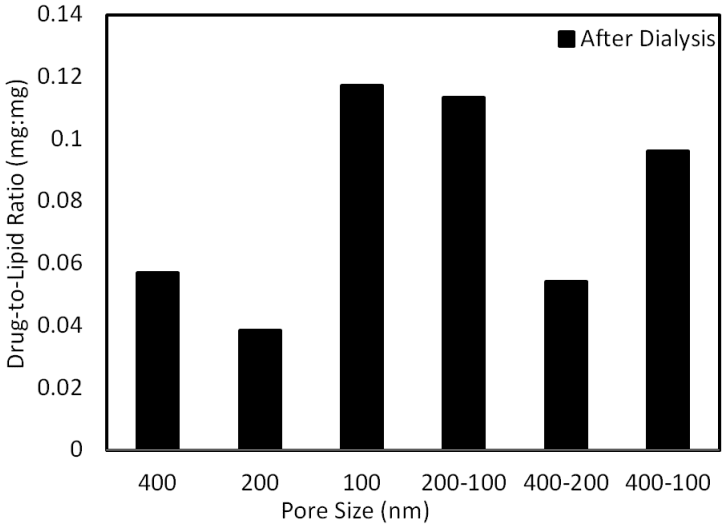


Figure 4.27. Effect of liposome size on DOX loading into liposome

According to Figure 4.27, DOX loading is increasing while liposome size is decreasing. The amount of loaded DOX is in the maximum value for the liposomes of 100 nm. This result shows us, drug loading is higher at smaller liposomes. This can be explained by the number of liposomes. When using same amount of film solution, there

will be more liposomes when we extrude them with a polycarbonate membrane of 100 nm. When the number of liposomes increases, the surface area will also increase.
We understand from this that some amount of the DOX tried to load into liposomes is actually adsorbed on the surface of the liposomes.

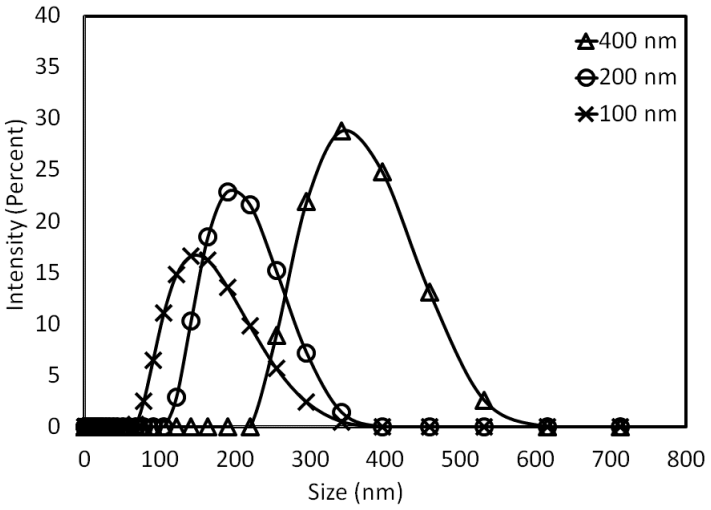


Figure 4.28. Effect of liposome size on DOX loading into liposome

4.3.8. Effect of Lipid Composition on DOX Loading into Liposomes

Despite there are many parameters that have an effect on drug loading, lipid composition in the liposome structure is the most significant parameter for drug loading. The lipid-cholesterol ratio in liposome structure changes the drug loading capacity too much. Cholesterol plays a critical role in liposomes composition. There are limited studies in the literature for this purpose. Thus, we done experiments to understand that how the lipid-cholesterol ratio changes the amount of DOX loaded into the liposomes. In our study, we kept the amount of DSPE-PEG_{2000} constant to be molar ratio of 5% of the liposomes composition. We also kept the hydration concentration constant to be 20 µmole. Cholesterol content in the liposomes was varied from 0% to 50%. The molar ratio of DSPC in the liposome structure has changed with changing in the amount of cholesterol. DSPC:Chol:DSPE-PEG_{2000} molar ratios were as follows: 95:00:05, 75:20:05, 67:28:05, 57:38:05 and45:50:05. After liposomes preparation, liposomes prepared in 5 different compositions were mixed equally with DOX. DOX was loaded into liposomes at various concentrations using remote active loading strategy (DOX concentration in LipoDOX was 1.5, 1.0, 0.75, 0.5 and 0.25).

Then, liposomes and DOX were incubated at 65ºC, 150 rpm water bath for 90 minutes. The experimental results were given in Figure 4.29.

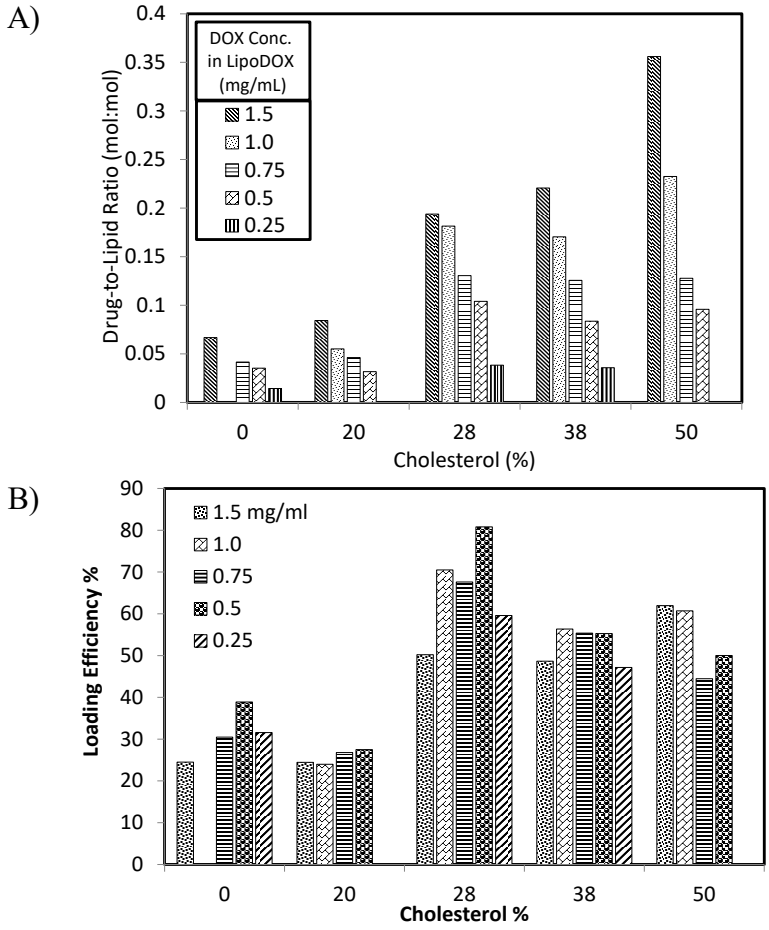


Figure 4.29. The effect of Cholesterol content on drug loading at different drug concentrations A) Drug-to-Lipid Ratio, B) Loading Efficiency

In Figure 4.29, the drug-to-lipid ratio and the loading efficiency at different drug concentrations were demonstrated. As seen in Figure 4.29A, when the cholesterol content in the liposomes increased, the amount of DOX loaded into the liposomes increased in direct proportion. The maximum amount of DOX loaded into the liposomes was obtained by using the liposomes contained 50% cholesterol. Also, the drug concentration increased, drug-to-lipid ratio increased. However, in Figure 4.29B, drug loading efficiency was maximum in 28% cholesterol content. The reason is that, Figure 4.29A shows the "after dialysis results" of LipoDOX. Figure 4.29B also shows the ratio of "after dialysis results" to "before dialysis" results and this is shown in the following equation.

$$EE\% = \left(\frac{Amount\ of\ DOX\ after\ dialysis}{Amount\ of\ DOX\ before\ dialysis}\right) * 100 \quad \text{(E.5)}$$

When we compared these two graphs, whence we can understand that before dialysis DOX concentration increased for the liposomes contained 38% and 50% cholesterol in their structure whereas the before dialysis results should remain constant. Normally, the before dialysis DOX concentration should be the same for all liposomes which contain different cholesterol content. Because an equal amount of DOX was added to all liposomes for incubation of liposomes and DOX. There may be an interaction between DOX and cholesterol. The liposomes with high cholesterol may interfere with DOX, so we may be finding more than the loaded DOX. Or, the other scenario may be that in DOX loading to low-cholesterol-contained liposomes, the results of before dialysis may be less than the required value. In low-cholesterol-contained liposomes, DOX may be quench. When the amount of cholesterol is less, DOX may be stacking into the gaps in the liposome's bilayer and quenched and so the loaded DOX may not be measured.

As a result in this experiment, 28% cholesterol content was more efficient than the others for DOX loading. The maximum loading efficiency was obtained in this content.

In the other study performed to understand the effect of cholesterol content on DOX loading into liposomes, only cholesterol molar ratio in liposomes structure was changed. The molar ratio of DSPE-PEG_{2000} and DSPC in the liposomes structure was kept constant. Thus, hydration concentration changed, not constant at 20 μmole. In this experiment, the purpose was to keep constant the number of liposomes and understand only cholesterol effect on DOX loading. Cholesterol content in the liposomes was varied from 0% to 56%. As in previous study, DOX was loaded into liposomes at various concentrations. Then, liposomes and DOX were incubated at 65ºC, 150 rpm water bath for 90 minutes. The experimental results were given in Figure 4.30. According to Figure 4.30A, when the cholesterol ratio in liposomes structure was increased to 32%, the amount of loaded drug increased. However, after the 32% cholesterol content, when the cholesterol ratio was increased to 56%, the amount of loaded drug decreased.The maximum amount of DOX loaded into liposomes was obtained by using 32% cholesterol in liposomes structure. When Figure 4.30B was examined, loading efficiency was between 85-95% by using 32% cholesterol and more. When the low-cholesterol-contained liposomes was used for DOX loading, the amount and loaded DOX and loading

efficiency decreased like at the previous study. We understand that molar ratio of cholesterol in the liposomes structure should be high to obtain the maximum amount of loaded DOX. According to this experiment results, the results of before dialysis measurement of DOX concentration was not the same each other instead of being the same. As in the previous experiment result, there may be an interaction between DOX and cholesterol. Or, DOX may be quench.

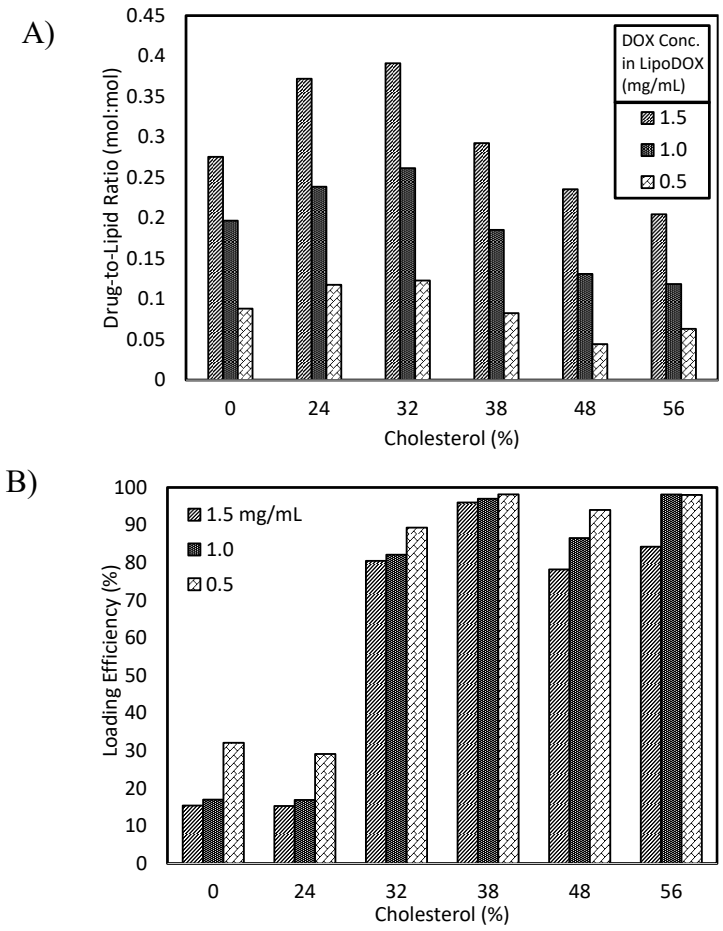


Figure 4.30. The effect of cholesterol content on drug loading at different drug concentrations and constant DSPC content A) Drug-to-Lipid Ratio, B)Loading Efficiency

Considering the results of two experiments, the molar ratio of cholesterol in liposomes structure should be in between 28% and 32%. In this values, the maximum amount of DOX loaded into liposomes was obtained and loading efficiency was high.

In the literature, there are limited studies about this subject. In one of these, to prepare liposomes, DSPC as phospholipid and 5 mol. % DSPE-PEG_{2000} were used. The

molar ratio of cholesterol was changed from 30% to 45% by increasing 5. The same incubation and extrusion processes were done to liposomes. The same results was obtained like in our studies. Encapsulation efficiency was found by them close to 100% for all cholesterol molar ratios in liposomes structure (Fouad, & Aanei, 2017). In our study, as seen in Figure 4.30B, the amount of cholesterol in liposomes increased from 38% to 56% but encapsulation efficiency did not change. In the other study, Farzaneh et al. (2018) used DMPC, DPPC, DSPC and EPC for liposomes preparation. They prepared liposomes both with cholesterol and cholesterol-free for all liposomes types contained different phospholipid. According to their results, for all liposomes, the amount of loaded DOX into cholesterol-containing liposomes was more than the amount of loaded DOX into cholesterol-free liposomes (Farzaneh et al., 2018). According to our results, the amount of loaded DOX into cholesterol-free liposomes was minimum in comparison to the amount of loaded DOX into cholesterol-containing liposomes.

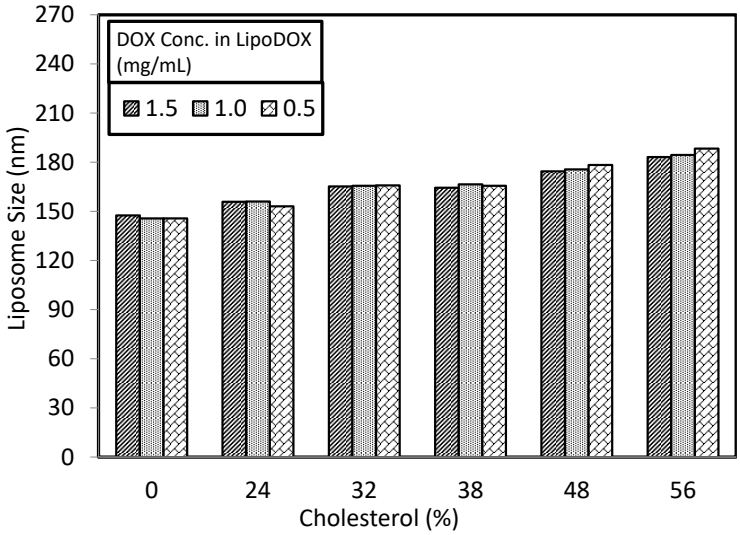


Figure 4.31. The effect of cholesterol content on liposomes size

Figure 4.31 shows that how the amount of cholesterol in liposomes formulation affects the size of the liposomes. There is no change in the liposomes size with changing the concentration of DOX in LipoDOX. However, there is a change in size with changing in the amount of cholesterol in liposomes structure. When the molar ratio of cholesterol in liposomes increased, the size of the liposomes increased in direct proportion. An increasing from 150 nm to 190 nm was observed.

4.4. Release Studies

To understand that how cholesterol ratio in liposomes content affect DOX retention in the liposomes, the release studies were performed by loading DOX at different concentration into Liposomes prepared by using different cholesterol molar ratios. The molar ratios of 0%, 28%, 38% and 50% cholesterol were used to prepare the liposomes. DOX concentration was varied as 0.5 mg/mL, 1.0 mg/mL and 1.5 mg/mL DOX in LipoDOX. DSPE-PEG_{2000} molar ratio in liposomes content was kept constant for all liposomes types. The amount of DSPC changed with changing the amount of cholesterol. Release studies were carried out at 2 different mediums and 37ºC body temperature. These are PBS and PBS + BSA. PBS + BSA medium was used to mimic the blood. The concentration of DOX was measured at 10 min and 30 min. The experimental results were shown in Figure 4.32. Figure 4.32A, B and C demonstrate that the release results of LipoDOX prepared with 0.5 mg/mL, 1.0 mg/mL and 1.5 mg/mL DOX, respectively. As seen in the graphs, the amount of cholesterol in liposomes did not affect the drug retention. There was no release in different liposomes types. The concentration of DOX and experimental medium did not also affect drug retention. There was no increase in the amount of drug release. The ratio of DOX retention is close to 100%. Similar results were observed in other studies in the literature. Rouf et al. (2009) used the molar ratio of 6% DSPE-PEG_{2000} in liposomes structure and, the amount of cholesterol was varied as 5%, 15% and 30%. The release study was performed during 24 h at 37ºC. Rapamycin was used as drug instead of DOX. Rapamycin was released from liposomes very slowly and after24 hours, the percentage of drug release was in the about of 10% for all liposomes types (Rouf, Vural, Renoir, & Hincal, 2009). Huang et al. (2017) used DSPE-PEG_{2000} in different molar ratios; 3%, 4% and 5%. They prepared liposomes with and without cholesterol. Carboxyfluorescein (CF) was used in drug loading stage. The release study was performed during 30 min. at different temperature; 37ºC, 38ºC, 39ºC, 40ºC and 42ºC in PBS medium. Then the amount of CF released from liposomes was determined by measuring the concentration of free CF in the medium. The ratio of CF release was about 3% at 10^{th} min, after 30 min this ratio was about 10% for all liposomes. Also, when the temperature increased, drug release increased (Huang, Bruni, Messa, & Cellesi, 2017).

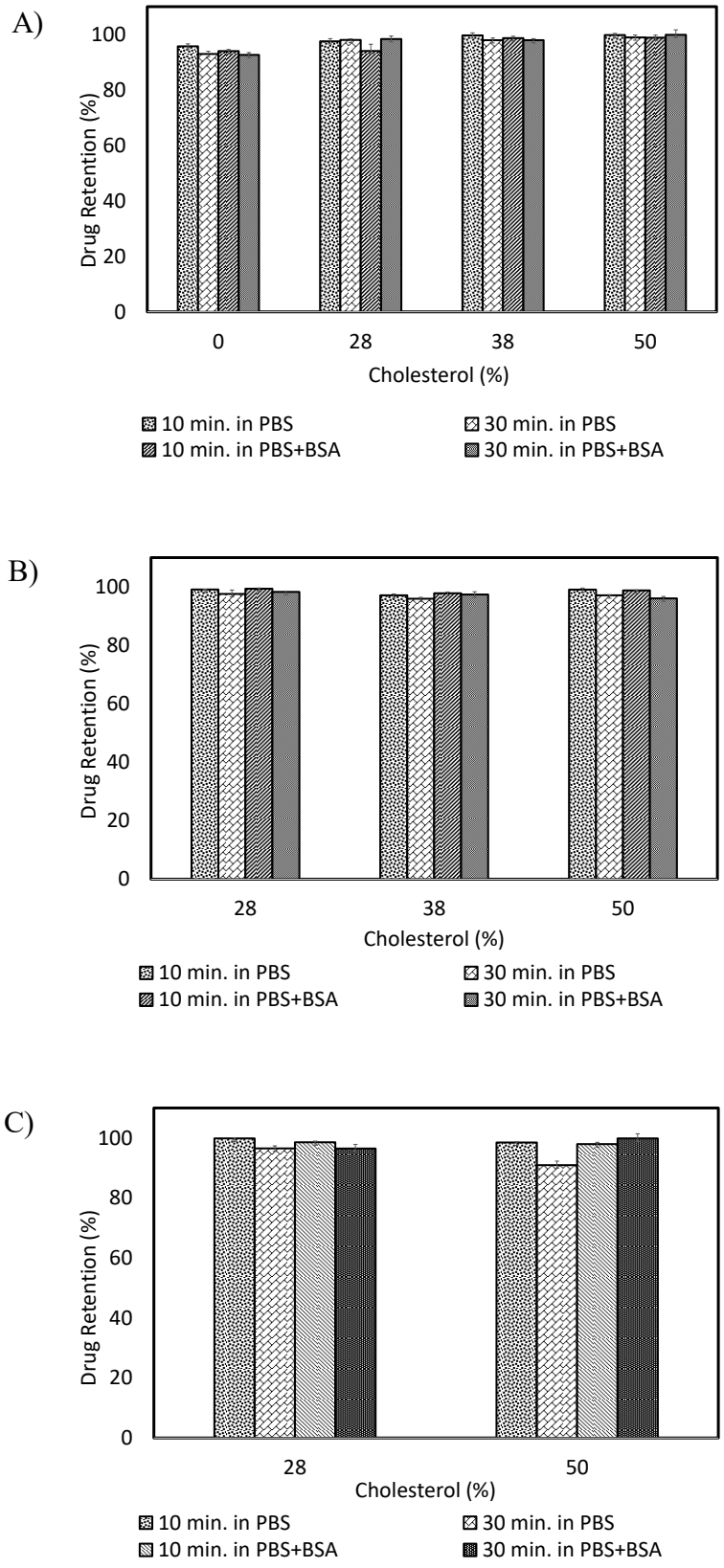


Figure 4.32. Percentage of drug retention vs. time from various liposomal DOX formulation A) 0.5 mg/mL, B) 1.0 mg/mL, C) 1.5 mg/mL DOX in LipoDOX

To understand the change in the size of LipoDOX in different medium, PBS and PBS+BSA mediums were used. Two different liposomes contained 50% and 28% cholesterol were prepared. DOX concentration was changed as 0.5 mg/mL, 1.0 mg/mL and 1.5 mg/mL. The change results of size of the LipoDOX prepared by using 50% cholesterol were shown in Figure 4.33.

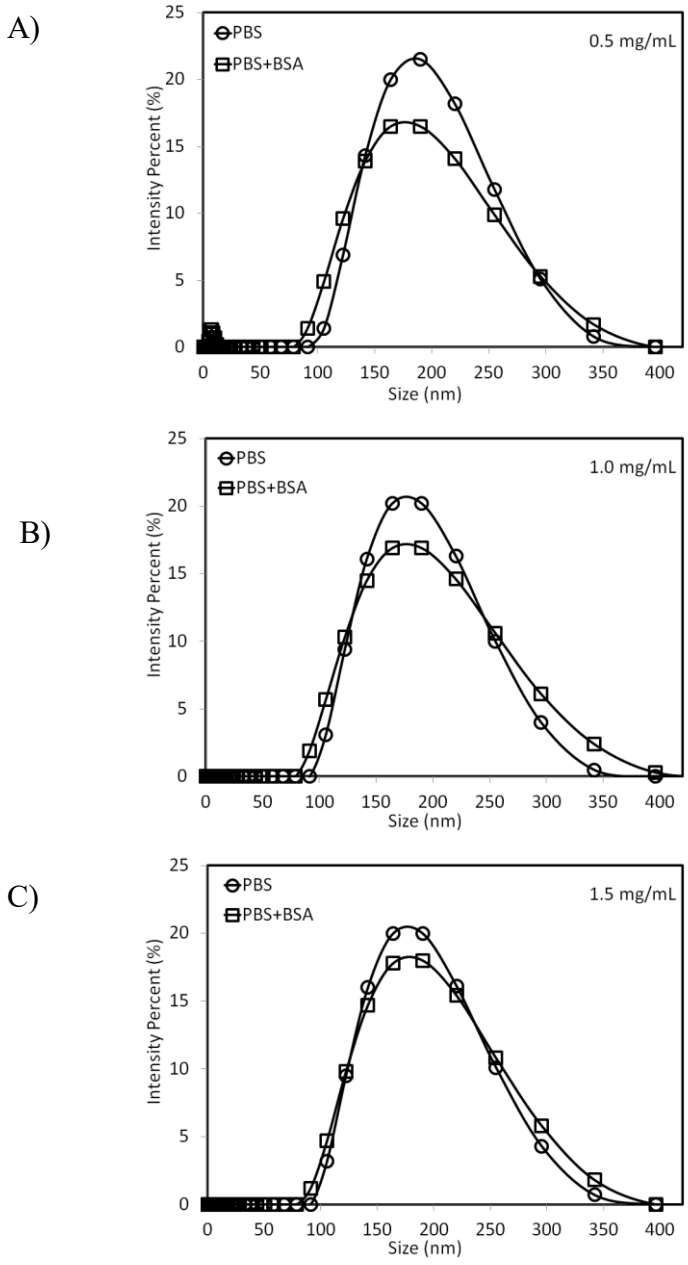


Figure 4.33. The size distribution of LipoDOX contained 50% cholesterol in PBS and PBS+BSA environment A) 0.5 mg/mL, B) 1.0 mg/mL, C) 1.5 mg/mL DOX in LipoDOX

According to Figure 4.33, the size of LipoDOX located in PBS and PBS+BSA mediums was measured different from each other. The size of the LipoDOX with 180 nm in PBS medium decreased slightly in PBS + BSA medium. Whereas, in the PBS medium, the size of the LipoDOX did not change with changing DOX concentration in LipoDOX, the size of the LipoDOX in the BSA-containing medium changed with the variation of the DOX concentration. As DOX concentration increased, the LipoDOX size also increased.

The same study was done for 28% cholesterol-contained LipoDOX. DOX concentration was only changed as 0.5 mg/mL and 1.0 mg/mL. The change results of size of the LipoDOX prepared by using 28% cholesterol were shown in Figure 4.34.

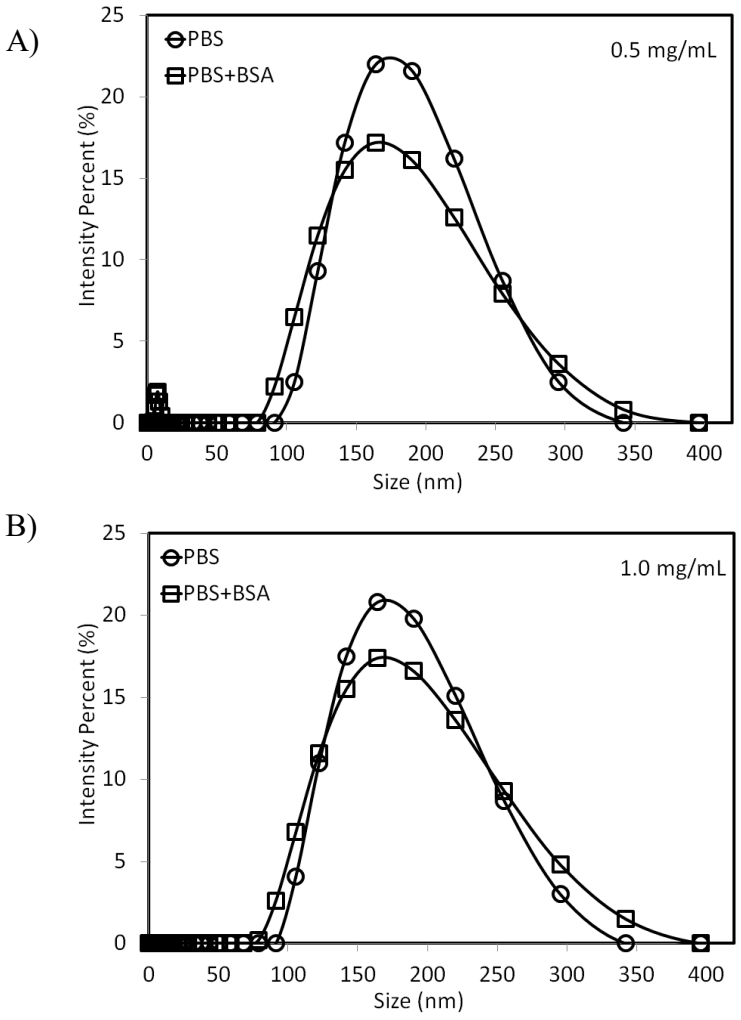


Figure 4.34. The size distribution of LipoDOX contained 28% cholesterol in PBS and PBS+BSA environment A) 0.5 mg/mL, B) 1.0 mg/mL DOX in LipoDOX

In Figure 4.34, the size of LipoDOX in PBS and PBS+BSA mediums was measured differently from each other like as in LipoDOX contained 50% cholesterol. The LipoDOX size was about 170 nm. However, it decreased in PBS+BSA medium. DOX concentration did not effect on the size of the LipoDOX in PBS, but LipoDOX size changed with changing DOX concentration in PBS+BSA medium. While DOX concentration increased, the size of the LipoDOX also increased. In addition to all, when Figure 4.33 and Figure 4.34 are considered together, we prove that as the cholesterol content in liposomes increases, the size of the liposomes also increases like as in Figure 4.31.

4.5. LipoDOX-Microbubble Coupling Studies

For coupling studies, biotinylated LipoDOX and biotinylated Microbubbles were used. In the coupling process of LipoDOX and Microbubbles, Strept Avidin (StAv) was used to bind LipoDOX and microbubbles each other. Amount of StAv is very important. StAv-to-Biotin ratio affects the binding ratio of LipoDOX and microbubbles each other. Therefore, experiments was performed to determine StAv-to-Biotin ratio and experimental results were shown in Figure 4.35.

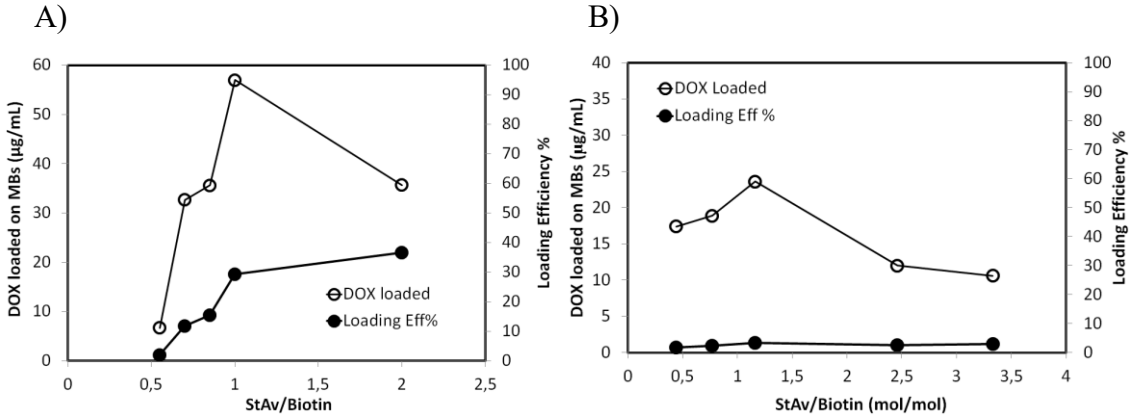


Figure 4.35. Effect of StAv/Biotin ratio to DOX loaded on MBs

Figure 4.35A and Figure 4.35B demonstrated the effect of the same experiments (by using the same biotinylated lipoDOX and MBs) on binding by loading different amounts of DOX and using different StAv/Biotin ratio. In these experiments, 0.05% biotinylated lipoDOX and 8% biotinylated microbubbles were used. StAv-to-Biotin ratio

was changed as 0.5, 0.7, 0.85, 1.0 and 2.0 in the experiment given its results in Figure 4.35A. StAv-to-Biotin ratio was changed as 0.4, 0.8, 1.1, 2.5 and 3.3 in Figure 4.35B. According to these two figures, when the StAv-to-Biotin ratio was 1.0 (in other words, the amount of StAv in lipoDOX equal to the amount of Biotin in lipoDOX), the maximum amount of DOX loaded on MBs was observed.

The similar experiment was done by changing StAv-to-Biotin ratio as 1.0, 1.5 and 2.0. In addition to the previous study, 0.1% and 0.05% biotinylated lipoDOX was used in this study. The amount of biotin in biotinylated microbubbles were kept constant as 8%. The effect of both StAv/Biotin ratio and biotin molar ratio in lipoDOX on DOX loaded on MBs were investigated. And the results were demonstrated in Figure 4.36.

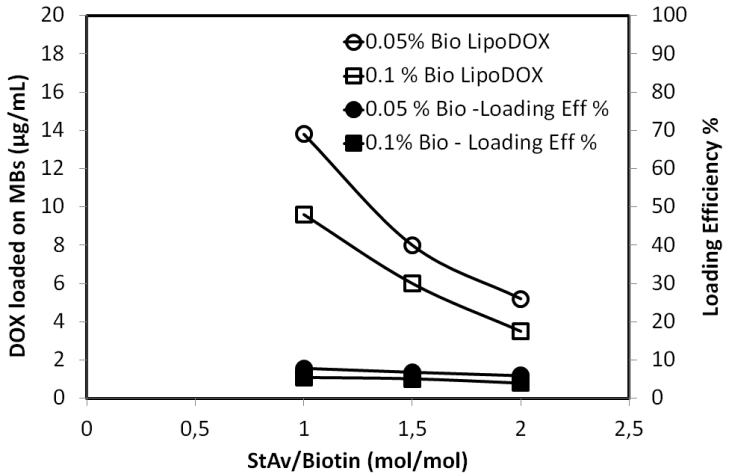


Figure 4.36. Effect of StAv/Biotin ratio and Biotin molar ratio in LipoDOX on DOX loaded complex

From the above figure, two different results were obtained. When the molar ratio of biotin in lipoDOX was increased from 0.05% to 0.1% the binding of lipoDOX and microbubbles each other decreased and so the amount of DOX loaded on MBs also decreased. Moreover, while StAv-to-Biotin ratio in lipoDOX was increased, the amount of DOX loaded on MBs decreased, too.

When Figure 4.35 and Figure 4.36 were examined together, the amount of optimum StAv should have been equal to the amount of biotin in lipoDOX, in other words StAv-to-Biotin ratio should have been 1.0. In this way, maximum binding was obtained and so maximum DOX loaded on MBs was provided. Hence, StAv-to-Biotin ratio was

kept constant as 1.0 in all of the next experiments. Considering all the results in Figure 4.35 and Figure 4.36, when 0.05% biotinylated lipoDOX was used instead of using 0.1% biotinylated lipoDOX for lipoDOX-MB coupling study, more binding of lipoDOX and MBs was demonstrated.

To understand effect of StAv to biotin ratio on the size of liposomes, an experiment included different liposomes with different StAv-to-biotin ratio was designed. In this experiment, 0.05% biotinylated lipid solution were prepared and 200 nm polycarbonate membrane was used to form liposomes. Different amount of avidin was added to these liposomes and so liposomes had different StAv/Biotin ratio were obtained. Size measurement was carried out for these different liposomes. The experimental results were shown in Figure 4.37.

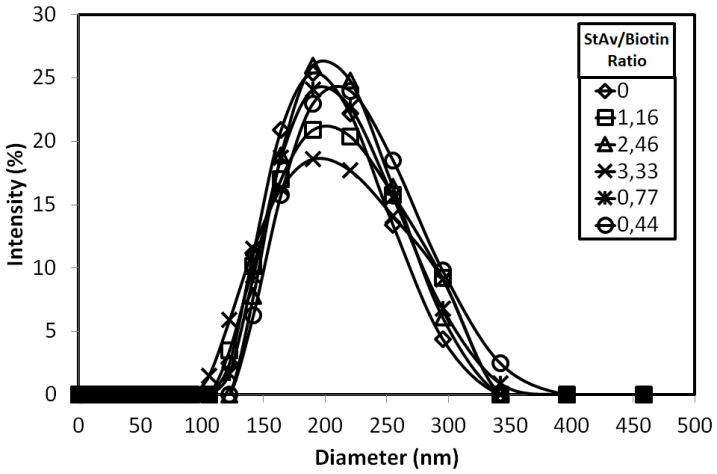


Figure 4.37. Effect of StAv-to-Biotin ratio on the size of liposomes

Figure 4.37 shows that how the effect of StAv to biotin ratio on liposomes' diameter. As seen in the Figure, the change of StAv-to-Biotin ratio did not affect the diameter of liposomes. The diameter of liposomes was 200 nm for all liposomes types included different StAv/Biotin ratio. Also, this graph shows that the liposomes did not crosslink with each other.

To understand the effect of amount of biotin in MBs on lipoDOX-MB binding and so the amount of loaded DOX to the complex, the amount of biotin in LipoDOX structure was kept constant as 0.05% but the amount of biotin in MBs structure was changed as 2%, 5% and 8%. In addition, the volume of lipoDOX added for obtaining lipoDOX-MB complex was changed in during all experiments. By this means, the effect

of lipoDOX volume, and biotin ratio in MBs were investigated. The experimental results were demonstrated in Figure 4.38.

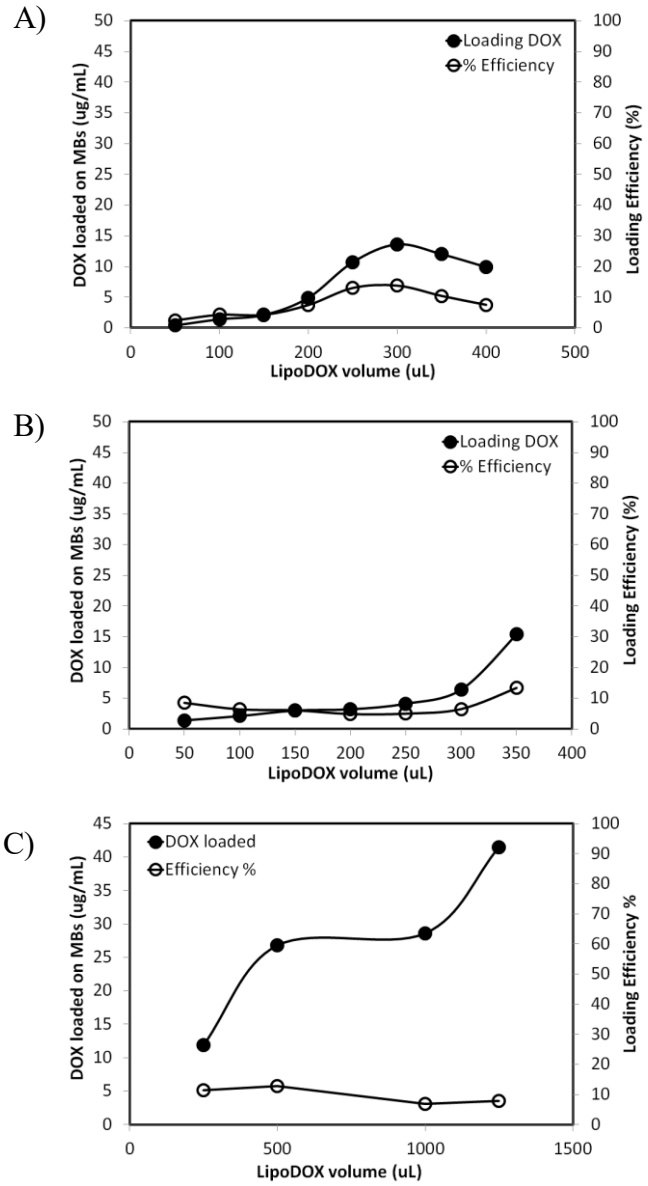


Figure 4.38. Effect of Liposome volume and Biotin molar ratio in MBs on DOX loaded complex, A) 2% Biotinylated MBs, B) 5% Biotinylated MBs and C)8% Biotinylated MBs

In Figure 4.38A, B and C, 2%, 5% and 8% biotinylated MBs and 0.05% biotinylated lipoDOX were used, respectively. Also, liposome volume was increased for each lipoDOX-MBs couple and the effect of liposome volume on DOX loading was

investigated. StAv-to-biotin ratio was kept constant as 1.0 for all experiments. When the biotin molar ratio was 2% and 5% in MBs, the amount of DOX loaded on MBs was about between 5 and 10 µg/mL. Also, while the lipoDOX volume was increased, the amount of DOX loaded increased in direct proportion. When Figure 4.38C was examined, the considerable increase in the amount of DOX loaded on 8% biotinylated MBs was observed. The amount of DOX loaded on microbubbles was obtained about 40 µg/mL. Figure 4.39 shows fluorescence microscope views of these MB-LipoDOX Couples.

In the literature, Streptavidin is added to MB and then combine with LipoDOX. However, this situation causes the crosslink of MBs. Due to the crosslink of the MBs, less LipoDOX can be attached to MB and so the effectiveness of the MB-LipoDOX complex on tumor cells reduces (Lentacker, Geers, Demeester, De Smedt, & Sanders, 2010). Therefore, streptavidin was added to LipoDOX to avoid crosslink in our experiments. Figure 4.39A, Figure 4.39B and Figure 4.39C shows the fluorescence microscope views of MB-LipoDOX Couple for 2% Biotinylated MBs, 5% Biotinylated MBs and 8% Biotinylated MBs, respectively. It is clearly that there is not any crosslink in MBs and Liposomes.

To sum up by considering all experiments with lipoDOX-MBs, when StAv-to-biotin ratio was 1.0, 0.05% biotinylated lipoDOX, 8% biotinylated MBs and high amount of lipoDOX were used for coupling, maximum amount of DOX loaded on MBs was obtained. The reason is that, the more biotin on the surface of the microbubbles allows more lipoDOX to bind. Also, the number of lipoDOX to be bound to the MB surface will be more by increasing lipoDOX volume in complex.

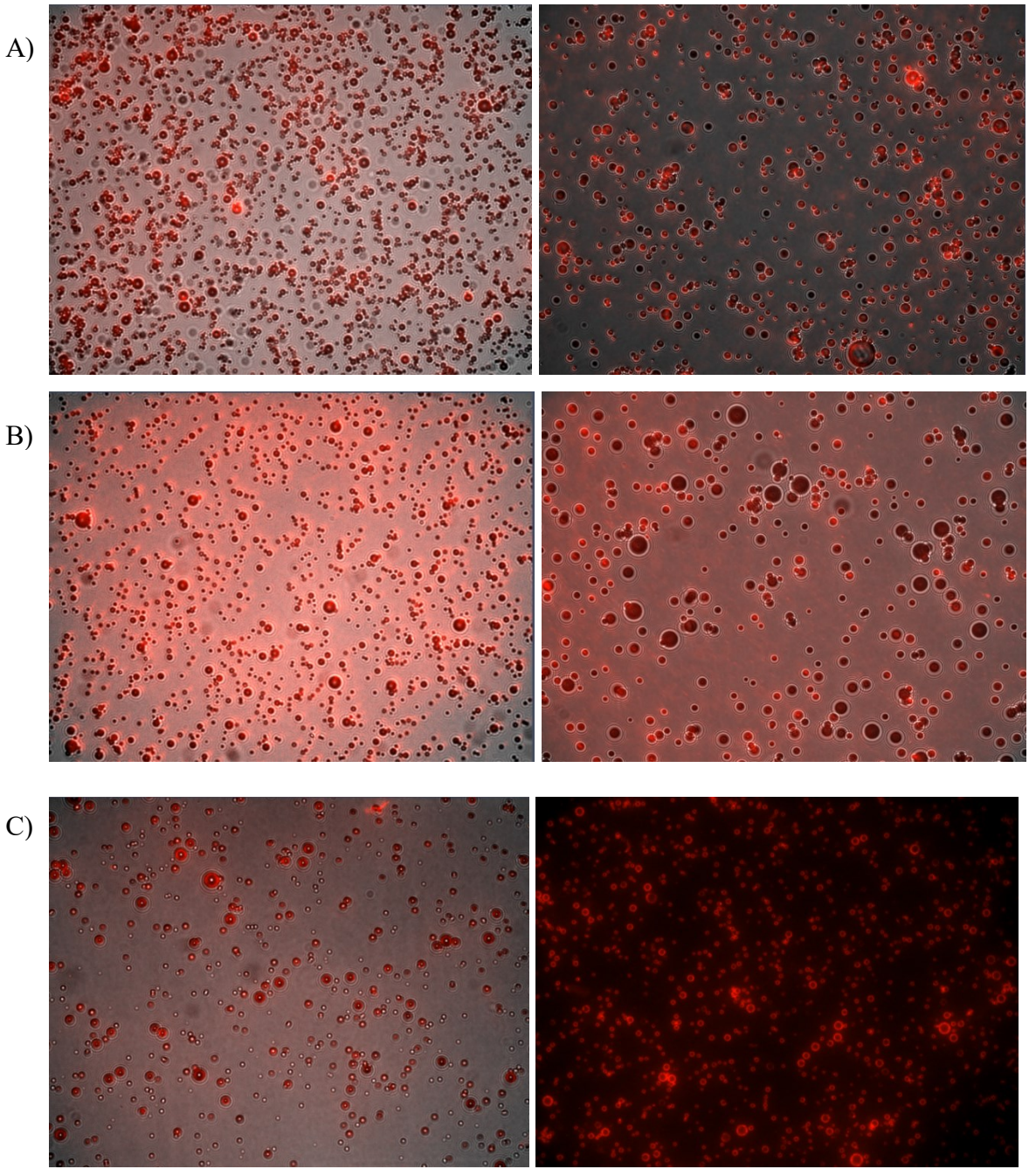


Figure 4.39. Fluorescence Microscope views of MB-LipoDOX Couple A) 2% Biotinylated MBs, B) 5% Biotinylated MBs and C) 8% Biotinylated MBs

CHAPTER 5

CONCLUSIONS

In this study, liposomes and DOX loaded liposome-microbubble complex as drug delivery systems for cancer treatment, were designed and characterized by Dynamic Light Scattering (DLS) method, Fluorescence Spectrometry method and Coulter Counter. First of all, an ideal liposomes was tried to be produced. For this purpose, conditions providing ideal liposome size, maximum DOX loading and liposome stability, were determined by using different sized polycarbonate membrane in extrusion step. Optimum extrusion pass number and velocity were investigated for the same purpose. As a result of these experiments, ideal liposome size was determined as at around 200 nm. Extrusion pass number and velocity was also determined as 10 passes and 2 mL/min, respectively. Liposome size was found to be related to the pore size of polycarbonate membrane used in the extrusion. Another important parameter for ideal liposomes is polyethylene glycol (PEG) chain length in PEGylated lipid. PEG $_{350}$, PEG $_{1000}$ and PEG_{2000} were used to understand that how liposome size changes with changing molecular weight of polyethylene glycol. It was found that when the PEG_{2000} was used in liposome formulation, particle size distribution is more monodispersed than the others.

After ideal liposome optimization, some experiments were carried out for optimization of amount of anticancer agent (DOX) to be encapsulated into liposomes. Effect of incubation temperature and incubation time on DOX loading into liposomes was studied. The maximum drug loading was obtained at 65ºC and in 90 min. duration. It was found that the liposome size did not change with changing temperature and time.

Lipid composition in the liposome is the most significant parameter for drug loading. It was seen that the lipid-cholesterol ratio in liposome structure changes the drug loading capacity significantly. Cholesterol plays a critical role in drug loading. Cholesterol molar ratio was changed between 0%, - 56%, and DSPE-PEG_{2000} was kept constant at 5% in all formulations. According to all experimental results, when the molar ratio of cholesterol in liposome structure was between 28% and 32%, the maximum amount of DOX was loaded into liposomes and loading efficiency was found to be the highest.

After DOX loading studies, lipoDOX-microbubble coupling studies were done. When the StAv-to-Biotin ratio was 1.0, maximum binding was obtained, resulting in maximum DOX loaded liposomes-MBs complex. In addition, the optimum amount of biotin in liposome and microbubble structure was also investigated. When 0.05% biotinylated liposome and 8% biotinylated microbubble were used for coupling studies, the maximum amount of DOX loaded liposome-microbubble complex was obtained. To sum up, DSPC/Cholesterol/DSPE-PEG_{2000}/DESPE-PEG_{2000}-Biotin at molar ratio of 57/38/4.95/0.05 for liposome preparation, 0.5 mg/mL DOX concentration in LipoDOX, DSPC/PEG_{40}St/DESPE-PEG_{2000}-Biotin at molar ratio of 50/42/8 were used.

REFERENCES

Abraham, S. A., Waterhouse, D. N., Mayer, L. D., Cullis, P. R., Madden, T. D., & Bally, M. B. (2005). The Liposomal Formulation of Doxorubicin. *Methods in Enzymology, 391*, 71-97.

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S. W., & Zarghami, N. (2013). Liposome: classification, preparation, and applications. *Nanoscale Research Letters, 8*(102).

Allen, C., Dos Santos, N., Gallagher, R., Chiu, G. N., Shu, Y., Li, W. M., Johnstone, S. A., Janoff, A. S., Mayer, L. D., & Webb, M.S., et al. (2002) Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). *Bioscience Reports, 22* (2), 225–250.

Baskar, R., Dai, J., Wenlong, N., Yeo, R., & Yeoh, K.-W. (2014). Biological response of cancer cells to radiation treatment. *Frontiersin molecular bioscieces, 1*(24), 1-10.

Bergström, K., Osterberg, E., Holmberg, K., Hoffman, A. S., Schuman, T. P., Kozlowski, A. & Harris, J. H. (1994). Effects of branching and molecular weight of surface bound poly (ethylene oxide) on protein rejection. *J Biomater Sci Polym Ed*, *6*, 123–132.

Cavalli, R., Bisazza, A., & Lembo, D. (2013). Micro- and nanobubbles: A versatile non-viral platform for gene delivery. *International Journal of Pharmaceutics, 456*(2), 437–445.

Chen, W., Duša, F., Witos, J., Ruokonen, S.-K., & Wiedmer, S. K. (2018). Determination of the Main Phase Transition Temperature of Phospholipids by Nanoplasmonic Sensing. *Scientific Reports, 8*(1).

Cheson, B.D. (2017). What Is the Role of Chemotherapy in Patients With Chronic Lymphocytic Leukemia? *Clinical Lymphoma, Myeloma&Leukemia, 17*(11), 723-7.

Cheung, C. L. (1998). Loading of Doxorubicin into Liposomes by Forming Mn^{2+}-Drug Complexes.

Conniot, J., Silva, J. M., Fernandes, J. G., Silva, L. C., Gaspar, R., Brocchini, S., Florindo, H. F. & Barata, T. S. (2014). Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking. *Frontiers in Chemistry, 2*(105), 1-27.

Cooper, G. M., & Hausman, R. E. (2007). *The cell: A molecular approach*: Sinauer Associates, Fourth Edition.

Damen, J., Regts, J., & Scherphof, G. (1981). Transfer and exchange of phospholipid between small unilamellar liposomes and rat plasma high density lipoproteins. Dependence on cholesterol content and phospholipid composition. *Biochimica Biophysics Acta, 665*, 538–545.

Deng, Z., Yan, F., Jin, Q., Li, F., Wu, J., Liu, X., & Zheng, H. (2014). Reversal of multidrug resistance phenotype in human breast cancer cells using doxorubicin-liposome–microbubble complexes assisted by ultrasound. *Journal of Controlled Release, 174*, 109–116.

Desai, M.P., Labhasetwar, V., Amidon, G.L., & Levy, R.J. (1996). Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharmaceutical Research, 13*(12), 1838–1845.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. Cell Metabolism.

Di, Y., Gao, Y., Gai, X., Wang, D., Wang, Y., Yang, X., Zhang, D. Pan, W., & Yang, X. (2017). Co-delivery of hydrophilic gemcitabine and hydrophobic paclitaxel into novel polymeric micelles for cancer treatment. *The Royal Society of Chemistry, 7,* 24030–24039.

Di Saia, P. J., & Creasman, W. T. (2012). Clinical Gynecologic Oncology: *Elsevier Health Sciences.*

Farzaneh, H., Ebrahimi Nik, M., Mashreghi, M., Saberi, Z., Reza Jaafari, M., & Teymouri, M. (2018). A study on the role of cholesterol and phosphatidylcholine in various features of liposomal doxorubicin: from liposomal preparation to therapy. *International Journal of Pharmaceutics.*

Flaherty, K. T. (2006). Chemotherapy and Targeted Therapy Combinations in Advanced Melanoma. *Clinical Cancer Research, 12*(7), 2366–2370.

Fonseca, M. J., van Winden, E. C. A., & Crommelin, D. J. A. (1996). Doxorubicin induces aggregation of small negatively charged liposomes. *European Journal of Pharmaceutics and Biopharmaceutics, 43,* 9-17.

Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *Am J Cancer Res, 7*(5), 1016-1036.

Fritze, A., Hens, F., Kimpfler, A., Schubert, R., & Peschka-Süss, R. (2006). Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient. *Biochimica et Biophysica Acta, 1758,* 1633–1640.

Garbuzenko, O., Barenholz, Y., & Priev, A. (2005). Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chemistry and Physics of Lipids, 135*, 117–129.

Gbadamosi, J. K., Hunter, A. C., & Moghimi, S. M. (2002). PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. *FEBS Letters 532*, 338–344.

Gubernator, J. (2011). Active methods of drug loading into liposomes: recent strategies for stable drug entrapment and increased in vivo activity. *Expert Opinion on Drug Delivery, 8*(5), 565–580.

Gültekin, M., & Boztaş, G. (2014). *Türkiye kanser istatistikleri* (Vol. 43): Sağlık Bakanlığı, Türkiye Halk Sağlığı Kurumu.

Hernot, S., & Klibanov, A. L. (2008). Microbubbles in ultrasound-triggered drug and gene delivery. *Advanced Drug Delivery Reviews, 60*(10), 1153–1166.

Huang, X., Li, M., Bruni, R., Messa, P., & Cellesi, F. (2017). The effect of thermosensitive liposomal formulations on loading and release of high molecular weight biomolecules. *International Journal of Pharmaceutics, 524*(1-2), 279–289.

Ilbasmis-Tamer, S., Unsal, H., Tugcu-Demiroz, F., Kalaycioglu, G. D., Degim, İ. T., & Aydogan, N. (2016). Stimuli-responsive lipid nanotubes in gel formulations for the delivery of doxorubicin. *Colloids and Surfaces B: Biointerfaces 143*, 406–414.

Isailović, B. D., Kostić, I. T., Zvonar, A., Đorđević, V. B., Gašperlin, M., Nedović, V. A., & Bugarski, B. M. (2013). Resveratrol loaded liposomes produced by different techniques. *Innovative Food Science & Emerging Technologies, 19,* 181–189.

Ishaque M. Shaikh 1, Kuan-Boone Tan1, Anumita Chaudhury, Yuanjie Liu, Bee-Jen Tan, Bernice M.J. Tan, Gigi N.C. Chiu. (2013). Liposome co-encapsulation of synergistic combination of irinotecan and doxorubicin for the treatment of intraperitoneally grown ovarian tumor xenograft. *Journal of Controlled Release 172,* 852–861.

Janssen, M., Crommelin, D., Storm, G., & Hulshoff, A. (1985). Doxorubicin decomposition on storage. Effect of pH, type of buffer and liposome encapsulation. *International Journal of Pharmaceutics, 23*(1), 1–11.

Joo, W. D., Visintin, I., & Mor, G. (2013). Targeted cancer therapy – Are the days of systemic chemotherapy numbered? *Maturitas, 76*(4), 308–314.

Kasper, Dennis L., Anthony S. Fauci, and Stephen L. (2015). Hauser. Harrison's Principles of Internal Medicine. New York: *Mc Graw Hill education.*

Kirby, C. and Gregoriadis, G. (1980). The effect of the cholesterol content of small unilamellar liposomes on the fate of their lipid components in vitro. *Life Sciences, 27*, 2223–2230.

Kozue Abe, Kenjirou Higashi, Keiko Watabe, Ai Kobayashi, Waree Limwikrant,Keiji Yamamoto, Kunikazu Moribe. (2015). Effects of the PEG molecular weight of a PEG-lipid and cholesterol onPEG chain flexibility on liposome surfaces. *Colloids and Surfaces A: Physicochem. Eng. Aspects 474*, 63–70

Lentacker, I., Geers, B., Demeester, J., De Smedt, S. C., & Sanders, N. N. (2010). Design and Evaluation of Doxorubicin-containing Microbubbles for Ultrasound-triggered Doxorubicin Delivery: Cytotoxicity and Mechanisms Involved. *Molecular Therapy, 18*(1), 101–108.

Lordick, F. and Hacker, U. (2014). Chemotherapy and Targeted Therapy. *Med Radiol Radiat Oncol,* 3-15.

Lu, C.-T., Ying-Zheng Zhao, Shu-Ping Ge, Jin, Y.-G., & Du, L.-N. (2013). Potential and problems in ultrasound-responsive drug delivery systems. *International Journal of Nanomedicine, 1621*.

Luo, D., Carter, K. A., Razi, A., Geng, J., Shao, S., Giraldo, D., Lovell, J. F. (2016). Doxorubicin encapsulated in stealth liposomes conferred with light-triggered drug release. *Biomaterials, 75*, 193–202.

Ma, X., & Yu, H. (2006). Global burden of cancer. *Yale J Biol Med, 79*(3-4), 85-94.

Ma, Y., Liu, D., Wang, D., Wang, Y., Fu, Q., Fallon, J.K., Yang, X., He, Z., & Liu, F. (2014). Combinational Delivery of Hydrophobic and Hydrophilic Anticancer Drugs in Single Nanoemulsions to Treat MDR in Cancer. *Mol. Pharmaceutics, 11*, 2623−2630.

McMurry, J., Castellion, M., Ballantine, D. S., Hoeger, C. A., & Peterson, V. E. (2010). *Fundamentals of general, organic, and biological chemistry*: Pearson Education.

Mediated by Mitotic DNA Synthesis Engages Break-Induced Replication Processes. Molecular and Cellular Biology.

Min, J., Wright, W., and J. Shay. (2017). Alternative Lengthening of Telomeres

Moghimi, S. M., Hunter, A. C., & Murray, J. C. (2001). Long-circulating and target specific nanoparticles: theory to practice. *Pharmacological Reviews, 53*(2), 283–318.

Mohamed Wehbe , Malathi Anantha, Ian Backstrom, Ada Leung, Kent Chen, Armaan Malhotra, Katarina Edwards, Marcel B. Bally. (2016). Nanoscale Reaction Vessels Designed for Synthesis of Copper-Drug Complexes Suitable for Preclinical Development. *PLOS ONE, 11*(4), e0153416.

Nam, L., Coll, C., Erthal, L., de la Torre, C., Serrano, D., Martínez-Máñez, R., Ruiz-Hernández, E. (2018). Drug Delivery Nanosystems for the Localized Treatment of Glioblastoma Multiforme. *Materials, 11*(5), 779.

Nancy L. Boman,' Pieter R Cullis,'Marcel B. Ballu, and Lawrence D. Mayer. (1993). Preclinical And Clinical Activity Of Liposomal Doxorubicin.

Ong, J. C.-L., Sun, F., & Chan, E. (2011). Development of stealth liposome coencapsulating doxorubicin and fluoxetine. *Journal of Liposome Research, 21*(4), 261–271.

Pandey, H., Rani, R., & Agarwal, V. (2016). Liposome and Their Applications in Cancer Therapy. *Braz. Arch. Biol. Technol, 59*.

Patil, Y. P., and Jadhav, S. (2014). Novel methods for liposome preparation. *Chemistry and Physics of Lipids, 177*, 8– 18.

Patty, P. J. and B. J. Frisken (2003). "The pressure-dependence of the size of extruded vesicles." *Biophysical Journal* 85(2): 996-1004.

Pozzi, D., Colapicchioni, V., Caracciolo, G., Piovesana, S., Capriotti, A. L., Palchetti, S., Laganà, A. (2013). Effect of polyethyleneglycol (PEG) chain length on the bio–nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells. *Nanoscale, 6*(5), 2782.

Qian Liu, Q., Zhang, J., Sun, W., Xie, Q.R., Xia, W., & Gu, H. (2012). Delivering hydrophilic and hydrophobic chemotherapeutics simultaneously by magnetic mesoporous silica nanoparticles to inhibit cancer cells. *International Journal of Nanomedicine, 7,* 999–1013.

Rouf, M.A., Vural, I., Renoir, J.M. & Hincal, A.A. (2009). Development and characterization of liposomal formulations for rapamycin delivery and investigation of their antiproliferative effect on MCF7 cells. *Journal of Liposome Research, 19*(4): 322–331.

Rycaj, K., & Tang, D. G. (2015). Cell-of-origin of cancer versus cancer stem cells: Assays and interpretations. *Cancer Research, 75*(19), 4003–4011.

Saw, P. E., Park, J., Lee, E., Ahn, S., Lee, J., Kim, H., Jon, S. (2015). Effect of PEG Pairing on the Efficiency of Cancer-Targeting Liposomes. *Theranostics, 5*(7), 746–754.

Schiller, K. and Combs, S.E. (2018). Use of alternative Treatment Methods for Cancer and Survival Effects. *Strahlentherapie Und Onkologie, 194*(9), 870-871.

Scholtz, J. C. (2010). Preparation, stability and in vitro evaluation of liposomes containing amodiaquine

Schutt, E. G., Klein, D. H., Mattrey, R. M., & Riess, J. G. (2003). Injectable Microbubbles as Contrast Agents for Diagnostic Ultrasound Imaging: The Key Role of Perfluorochemicals. *Angewandte Chemie International Edition, 42*(28), 3218–3235.

Shaikh, I. M., Tan, K.-B., Chaudhury, A., Liu, Y., Tan, B.-J., Tan, B. M. J., & Chiu, G. N. C. (2013). Liposome co-encapsulation of synergistic combination of irinotecan and doxorubicin for the treatment of intraperitoneally grown ovarian tumor xenograft. *Journal of Controlled Release, 172*(3), 852–861.

Sheela A. Abraham, Dawn N. Waterhouse, Lawrence D. Mayer, Pieter R. Cullis, Thomas D. Madden, and Marcel B. Bally

Sirsi, S. R., & Borden, M. A. (2009). Microbubble compositions, properties and biomedical applications. *Bubble Science, Engineering & Technology, 1*(1-2), 3–17.

Tian, B., Al-Jamal, W.T., Al-Jamal, K.T., & Kostarelos, K. (2011). Doxorubicin-loaded lipid-quantum dot hybrids: Surface topography and release properties. *International Journal of Pharmaceutics, 416*, 443– 447.

Van Swaay, D. (2013). Microfluidic methods for forming liposomes. *Lab on a Chip, The Royal Society of Chemistry, 13*(5), 752-767.

Vemuri, S. and Rhodes, C. T. (1995). Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharmaceutics Acta Helvetiae, 70*, 95–111.

Yang, F., Jin, C., Jiang, Y., Li, J., Di, Y., Ni, Q., & Fus, D. (2011). Liposome based delivery systems in pancreatic cancer treatment: From bench to bedside. *Cancer Treatment Review, 37*, 633–642.

Yang, W., Cheng, Y., Xu, T., Wang, X. & Wen, L. (2009). Targeting cancer cells with biotine-dendrimer conjugates. *European Journal of Medicinal Chemistry, 44*, 862-868.

Yu, F. T. H., Chen, X., Wang, J., Qin, B., & Villanueva, F. S. (2015). Low Intensity Ultrasound Mediated Liposomal Doxorubicin Delivery Using Polymer Microbubbles. *Molecular Pharmaceutics, 13*(1), 55–64.

http://www.avantilipids.com, Accessed on February 25th 2019

http://www.chromedia.org, Accessed on March 27th 2019

http://www.comis.med.uvm.edu, Accessed on March 19th 2019

http://www.malvernpanalytical.com, Accessed on February 13th 2019

http://www.medicalnewstoday.com, Accessed on February 13th, 2019

http://www.thermopedia.com, Accessed on March 13th 2019