INCREASING DOXORUBICIN (DOX) RELEASE FROM LIPOSOMES

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ABSTRACT

INCREASING DOXORUBICIN (DOX) RELEASE FROM LIPOSOMES

Cancer is the second most common cause of death in the world and its incidence is increasing day by day. Doxorubicin (DOX) is an anthracycline group drug frequently used in many cancer treatments including breast cancer. However, free DOX has many harmful side effects and need to be encapsulated into nanocarrier such as liposomes. Although liposomal DOX has many advantages over its free form, liposomal DOX has undesirable side effects such as hand and foot syndrome. In this thesis, it was aimed to develop a more effective liposomal DOX delivery and release systems. Liposomes were prepared with alkaline solutions containing tris, sodium carbonate, ammonium chloride, and ammonium sulfate. DOX loading into liposomes and the percentage of release from liposomes were examined. A loading efficiency of about 80% was achieved, while the release was found to be below 13% at room temperature. The release of DOX was found to be enhanced from liposomes in the presence of ammonia (NH_3), whose content was dependent on pH. Temperature was also found an important parameter and enhances DOX release at higher temperatures than the phase transition temperature of the lipid. A two-component liposomal system was proposed where ammonia (NH_3) would be released from one liposome and enhance the DOX release from other liposomes. It was found that temperature, pH, and ammonia (NH_3) concentration affected DOX release from liposomes. As a result, DOX was successfully loaded into liposomes and ready to study their effect on breast cancer cells.

ÖZET

LİPOZOMLARDAN DOKSORUBİSİN (DOX) SALINIMININ ARTIRILMASI

Kanser dünyada ölüm nedenleri arasında ikinci sıradadır ve kanser vakaları gün geçtikçe artmaktadır. Doxorubicin (DOX) meme kanseri dahil birçok kanserin tedavisinde kullanılan antrasiklin grup ilaçlardandır. Ancak serbest DOX'un birçok yan etkisi vardır ve lipozomlar gibi nano taşıyıcılar içierisine hapsedilmesi gerekmektedir. Her ne kadar lipozomal DOX'un serbest haline göre birçok avantajı olsa da el ve ayak sendromu gibi bazı yan etkileri de bulunmaktadır. Bu tezde, daha etkin lipozomal DOX taşıma ve salınım sistemleri geliştirme amaçlanmıştır. Lipozomlar tris, sodyum karbonat, amonyum klorit ve amonyum sülfat içeren alkali solüsyonlar ile hazırlandı. Lipozomlara DOX yüklemesi ve lipozomlardan yüzde salınımları incelendi. Yükleme verimliliği %80 olarak gerçekleştirildi. Salınım hızının oda sıcaklığında %13'den düşük oranlarda olduğu bulundu. Lipozomlardan DOX salınımının ortamda pH'a bağlı amonyak (NH_3) konsantrasyonuna bağlı artırılabileceği bulundu. Sıcaklığın da önemli bir parametre olduğu ve lipidin faz geçiş sıcaklığı üzerindeki sıcaklıklarda DOX salınımını artırdığı bulundu. İki-bileşenli bir lipozomal sistem önerildi, burada amonyak (NH_3) bir lipozomdan salınırken, diğer lipozomlardan DOX salınımını arttıracaktır. Sıcaklığın, pH'ın ve amonyak (NH_3) konsantrasyonunun lipozomlardan DOX salınımını etkilediği bulundu. Sonuçta, DOX lipozomlara başarı ile yüklendi ve meme kanseri hücreleri üzerindeki etki çalışmaları için hazırlandı.

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CHAPTER 1

INTRODUCTION

Chemotherapeutic agents used in the conventional cancer treatment are not specific because they are distributed not only to the target tumor site but also to the normal cells, which limits the effective dose in the tumor. Nanoparticles can increase the intracellular concentration of drugs in cancer cells and reduce or inhibit the toxicity of healthy cells. In addition, these smart drug delivery systems can be designed to provide key-lock compatibility with certain special cell receptors and to increase specificity (Ross et al., 2004; Larsen et al., 2000). Liposomes are drug carriers that are frequently used for this purpose. In particular, the stealth liposomes can increase the accumulation of drugs in the tumor region by providing long circulation times. One of the commonly used anticancer agents of liposomal formulation is doxorubicin hydrochloride (DOX-HCl). This anticancer drug is an anthracyclic antibiotic that is widely used in the treatment of many types of carcinomas, particularly breast cancer. Liposomal doxorubicin provides many advantages over free form. Some of them include reducing the risk of immune system response, allowing the drug to remain in the bloodstream long-term, and providing a targeted drug delivery to only specific tumor cells instead of healthy cells (Allen & Cullis, 2004; Torchilin, 2005).

Although liposomes have many advantages as drug delivery systems, they may remain in the blood circulation for a long time and they accumulate in the capillaries. Liposome accumulation causes undesirable wounds at the fingertips on the hands and feet. This phenomenon called as hand and foot syndrome which is a painful condition for cancer patients. It was proposed that if pH of the tumor microenvironment was increased for a short time, amount of uncharged DOX can be increased and the viability of the tumor cells decreased because uncharged and small molecules can easily pass through the cell membrane while charged and large molecules cannot pass. Accordingly, in this thesis it was aimed to encapsulate DOX-HCl into high pH liposomes and to increase the uptake of intracellular neutral DOX-HCl by cancer cells and so minimizing the side effects of cancer treatment.

CHAPTER 2

LITERATURE SURVEY

2.1. Molecular Mechanism of Cancer

Cancer is a disease caused by abnormal cell growth. This abnormal growth is caused by the degeneration of some important signals required for cell growth and development. The basic mechanism of cancer is based on the uncontrolled division of cells. Generally, even if the DNA has been damaged, the body has the ability to repair this damage. But the damaged DNA in the cancer cells cannot be repaired. The cancer cells continue to grow rather than die as DNA damage occurs. Cancer cells are genotypically and phenotypically complex, causing cancer diversity. Cancer is mostly seen as solid tumors. Early diagnosis of cancer has vital importance. If the diagnosis of cancer is done early, the chance of treatment is high. In addition, the development of many cancer types can be prevented by controlling the environmental factors such as lifestyle, eating habits, non-smoking etc. (Siegel et al., 2015).

Cells in the body multiply by a type of division called mitosis. When proliferating cells are sufficiently crowded, they stop dividing by contact inhibition. If there is a defect or deficiency in any cell, this cell is removed by a controlled cell death called apoptosis. However, in the case of any cell mutation in the cancer process, the cell division cannot be inhibited because it is not possible to stop the DNA cleavage. Thus, cells are divided into infinite boundaries abnormal growth tendency and causes neoplasm structures. These structures differentiate over time and cause tumor formation (Michalopoulou et al., 2016).

Cancer cells need more energy than normal cells with increasing rate of division. This leads to metabolic differences between normal and cancer cells. A clear understanding of these metabolic differences has led to the development of novel treatment methods for cancer treatments (Ward & Thompson, 2012).

Cancer causes the death of millions of people with painful years, and day by day, the rate is increasing. Heart system disorders are the first in the world in terms of cause of death, while cancer is the second.

World Health Organization (WHO) reports that cancer is the first or second cause of death in 91 countries and it is the third or fourth cause of death in 22 countries (Bray et al., 2018). According to the relationship between sex and cancer type, the most diagnosed type of cancer and the first cause of cancer deaths in both sexes is lung cancer. Based on women only, breast cancer is the leading cause of death and diagnosis. On average, a woman is diagnosed with breast cancer every 2 minutes and one woman dies because of breast cancer every 13 minutes (WHO, 2018).

According to the researches, more than 80% of cancer patients have solid tumors, and almost half of these patients die because of cancer, known as malignant tumor (Jain, 1994). Cancer cells are capable of spreading in distant or nearby cells or tissues. This is the most prominent feature that makes it a life threatening. Cancer cells are circulating to the lymphatic or blood vessels which are circulatory system elements, joining the circulation and replicating in another part of the body through this circulation. This feature is known as "metastasis". Some mutations that occur in a cell cause unlimited growth of cells and uncontrolled growing cells can easily spread to neighboring tissues by metastasis. The spread of vascular network is an important factor in the spread of cancer tissue to different regions. This vascular growth involves the formation of new blood and lymph vessels. The process in which new blood vessels are formed is angiogenesis and the process in which new lymphatic vessels occur is lymphangiogenesis. These two systems provide the nutrients and oxygen support needed by the cells and help the removal of waste materials (Folkman, 1971).

On the other hand, the relationship between extracellular matrix (ECM) and cancer is also known. The ECM is the non-cellular core component in the tissues. This structure acts as a support for the adhesion and proliferation of the cells, while on the other hand it provides intercellular communication as shown in Figure 2.1. For this reason, ECM has a very important effect on the growth and development of cells and then the construction of the tissues.

The ECM consists of components such as water, minerals, various proteins and carbohydrates, proteoglycans. And the proportion and type of these components are specific to each tissue (Frantz et al., 2010; Bonnans et al., 2014).

Recent studies have shown that the increased crosslinking and deposition of collagen, one of the key components of ECM, promotes tumor formation by integrin signaling (Levental et al., 2009; Jordan, 2018).

Therefore, the accumulation and deterioration in the ECM structure is an important criterion for cancer diagnosis and treatment.

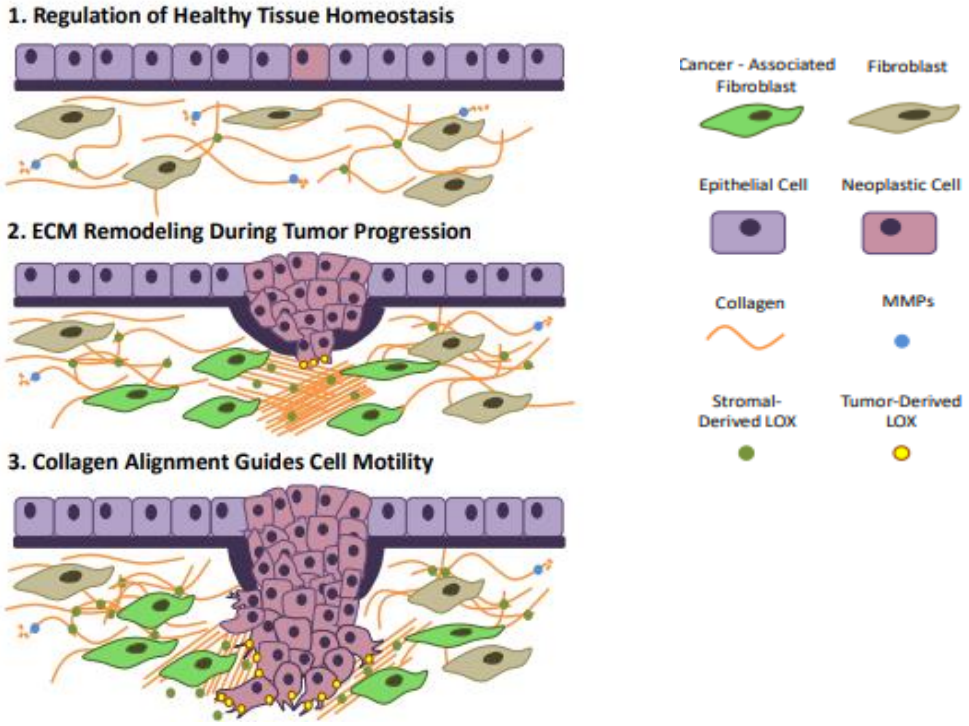


Figure 2.1. Component structure of normal and cancerous tissue
(Source: Kaushik et al., 2019)

It has been reported that the pH of the tumor tissues is lower than that of normal tissue as a result of acidic metabolites, the product of anaeorobic respiration (Solary et al., 1993). As shown in Figure 2.2, the extracellular pH (pH_e) of the tumor cells decreased from about 7.4 to about 6.5. The physiological pH_e of healthy cells is around 7.2. (Hashim et al., 2011). The frequent use of the glycolysis mechanism, which is a short energy producing step in cancer cells, increases intracellular acidity through by-products. The cell carries the internal hydrogen molecules out of the cell with some transport mechanisms to maintain the physiological pH. As a result, extracellular pH varies in cancerous cells while the internal pH in normal cells is lower than the external pH. The internal pH becomes higher than the external pH. The extracellular pH increases invasion of cells to the tumor site, which causes the spread and growth of cancer. On the other hand, increased intracellular pH allows the cancer cell to survive by limiting its access to apoptosis (Moolenaar , 1986; Stubbs et al., 1994; Webb et al., 2011).

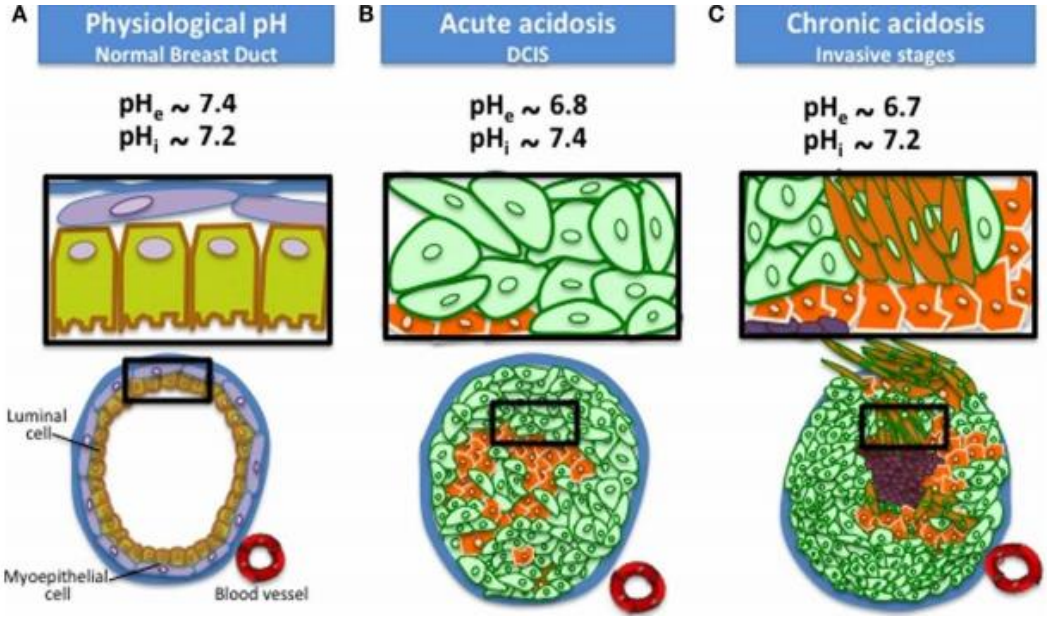


Figure 2.2. Exterior and interior pH values of normal and cancer cells
(Source: Damaghi et al., 2013)

2.2. Cancer Therapy Methods

As cancer is becoming more and more dangerous, there is a need to develop methods to treat it. Some of these methods involve surgery and use of modern technologies such as radiation therapy, chemotherapy, immunotherapy, hormonal therapy, and targeted drug delivery aplications (Sudhakar, 2009). The mostly used methods in the cancer treatment are radiation therapy and surgery. However, these treatment modalities still result in some failures. For example, surgical removal of a solid tumor may result in tumor recurrence. Radiation can damage some healthy organs (Brizel et al., 1994).

Chemotherapy is another common form of therapy used with other treatments. This method is based on the use anticancer agents to kill the cancer cells. Although there are many successes, some disadvantages also exist in the chemotherapy. For example, in chemotherapy the therapeutic dose is limited to the tumor site, because only a small amount, 2% of drug injected, reaches the target area and the rest of the chemotherapeutic drug circulates throughout the body (Chaplin et al., 1996). Application of a chemotherapy that does not harm the healthy tissues and organs is unfortunately still not available (Gabizon et al., 1990).

The side effects of traditional chemotherapy limits the success of this treatment. In addition, the chemotherapeutic agent's access to the tumor has been restricted by some physiological obstacles. Multi drug resistance (MDR) is another of the challenges encountered in chemotherapy. This term involves the development of some resistance mechanisms for cancer cells against chemotherapy drugs (Gillet & Gottesman, 2010; Alfarouk et al., 2015; Nooter & Stoter, 1996).

As a result of such failures, the demand for new and effective drug delivery systems has expanded. These transportation systems, which are one of the most popular subjects of recent development, are nano-sized structures. These systems serves many purposes such as cosmetic, gene therapy, treatment of infectious diseases, treatment of dermatological diseases, especially for drug transportation purposes.

As shown in Figure 2.3, some of these carriers, called smart delivery systems, are liposomes, dendrimers, gold nanoparticles, carbon nanotubes, micelles etc.

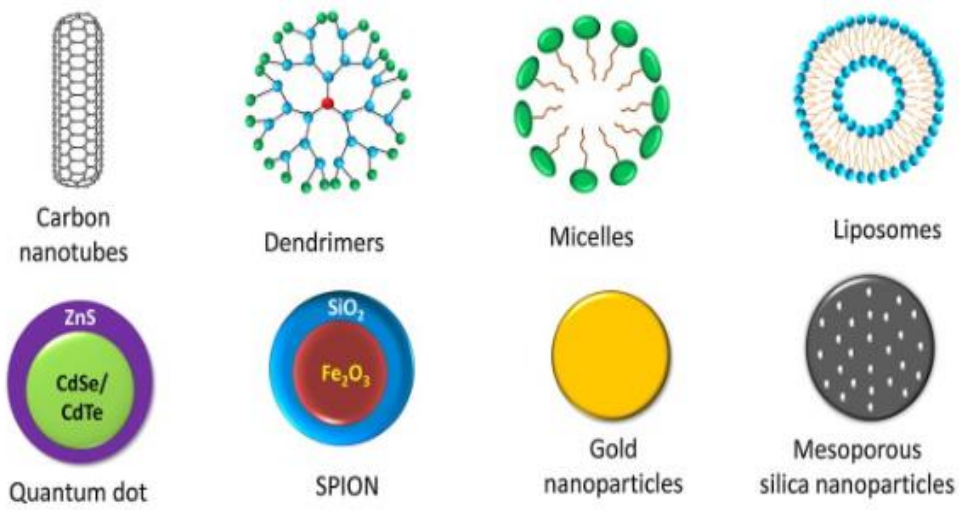


Figure 2.3. Some of the commonly used nanocarriers (Source: Hossen et al., 2019)

For efficient transport, nanoparticles must have some properties. First, smart nanocarriers should not be destroyed by the immune system. They need to be accumulated at the targeted site. Smart nanocarriers should release their load at the targeted site. In addition, they should be suitable for co-delivery of other substances, such as genetic materials, imaging agents, etc. as indicated in Table 2.1 (Gradishar et al., 2005). There are nano carriers for drug delivery and each has its own unique characteristics (Knop et al., 2010; Verhoef & Anchordoquy, 2013; Xu et al., 2014).

Table 2.1. Types of nanocarriers and characteristics for drug delivery
(Source: Gradishar et al., 2005)

System	Structure	Characteristics	Examples of compounds
Polymeric nanoparticles (polymer-drug conjugates)	Drugs are conjugated to the side chain of a linear polymer with a linker (cleavable bond)	(*a*) Water-soluble, nontoxic, biodegradable (*b*) Surface modification (pegylation) (*c*) Selective accumulation and retention in tumor tissue (EPR effect) (*d*) Specific targeting of cancer cells while sparing normal cells—receptor-mediated targeting with a ligand	Albumin-Taxol (Abraxane) PGA-Taxol (Xyotax) PGA-Camptothecin (CT-2106 HPMA-DOX (PK1) HPMA-DOX-galactosamine (PK2)
Polymeric micelles	Amphiphilic block copolymers assemble and form a micelle with a hydrophobic core and hydrophilic shell	(*a*) Suitable carrier for water-insoluble drug (*b*) Biocompatible, self-assembling, biodegradable (*c*) Ease of functional modification (*d*) Targeting potential	PEG-pluronic-DOX PEG-PAA-DOX (NK911) PEG-PLA-Taxol (Genexol-PM)
Dendrimers	Radially emerging hyperbranched synthetic polymer with regular pattern and repeated units	(*a*) Biodistribution and PK can be tuned (*b*) High structural and chemical homogeneity (*c*) Ease of functionalization, high ligand density (*d*) Controlled degradation (e) Multifunctionality	PAMAM-MTX PAMAM-platinate
Liposomes	Self-assembling closed colloidal structures composed of lipid bilayers	(*a*) Amphiphilic, biocompatible (*b*) Ease of modification (*c*) Targeting potential	Pegylated liposomal DOX (Doxil) Non-pegylated liposomal DOX (Myocet) Liposomal daunorubicin (DaunoXome)
Viral nanoparticles	Protein cages, which are multivalent, self-assembled structures	(*a*) Surface modification by mutagenesis or bioconjugation—multivalency (*b*) Specific tumor targeting, multifunctionality (*c*) Defined geometry and remarkable uniformity (*d*) Biological compatibility and inert nature	HSP-DOX CPMV-DOX
Carbon nanotubes	Carbon cylinders composed of benzene ring	(*a*) Water-soluble and biocompatible through chemical modification (organic functionalization) (*b*) Multifunctionality	CNT-MTX CNT-amphotericin B

Abbreviations: PGA, poly-(L-glutamate); HPMA, *N*-(2-hydroxypropyl)-methacrylamide copolymer; PEG, polyethylene glycol; PAA, poly-(L-aspartate); PLA, poly-(L-lactide); PAMAM, poly(amidoamine); DOX, doxorubicin; MTX, methotrexate; PK, pharmacokinetics; EPR, enhanced permeability and retention; CNT, carbon nanotube; HSP, heat shock protein; CPMV, cowpea mosaic virus.

2.3. Doxorubicin

Chemotherapy is a vital method for treating cancer. It is based on using chemicals to prevent growth or kill cancer cells. There are agents used in chemotherapy as shown in Figure 2.4.

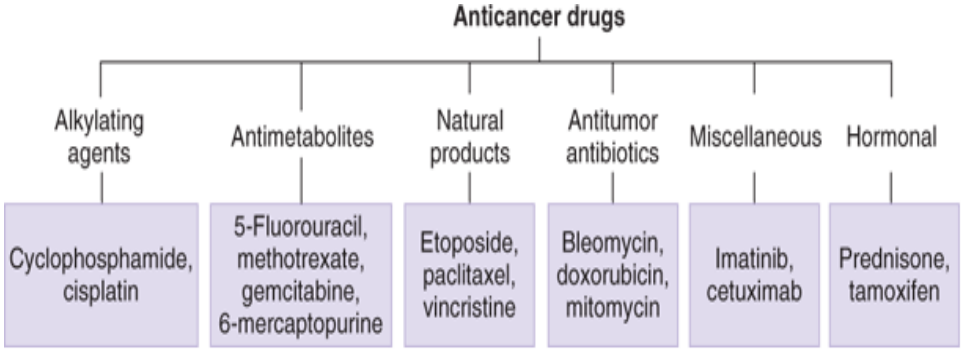


Figure 2.4. Some of the chemotherapy agents (Source:Trevor et al., 1998.)

One of chemotherapeutic drugs is doxorubicin (DXR) which is most widely used and recently combined with the targeted drug delivery system. As shown in Figure 2.5 amphipathic DOX molecule consists of water-soluble group, amino-sugar (Daunosamine: $C_6H_13NO_3$) and a water-insoluble aglycone (Adriamycin: $C_{21}H_{18}O_9$) group.

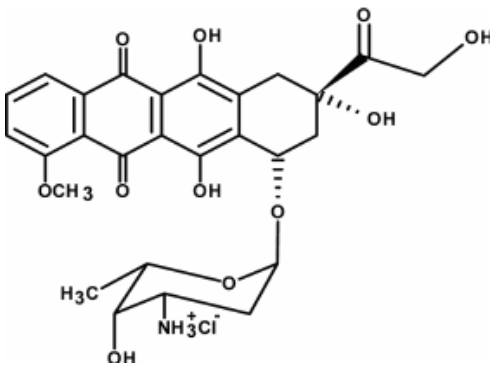


Figure 2.5. Chemical structure of doxorubicin (Source: Bhattacharjee et al., 2008)

Doxorubicin, also known as Adriamycin, is an important cytotoxic antibiotic derivative. Doxorubicin hydrochloride (DOX-HCl) which is a water-soluble form is mosty used in drug delivery systems.

Although this drug was first obtained from bacterium called *Streptomyces peucetius*, it is also possible to synthesize chemically today. DXR is a DNA intercalating agent. It shows its effect by binding to DNA and inhibiting topoisomerase II enzyme (Jain et al., 2017). This enzyme is essential for DNA replication. When the DOX is linked to the DNA molecule, DNA replication is prevented and thus the proliferation of the cells is prohibited (Minotti et al., 2004).

Doxorubicin has three pK_a values, $pK_1 = 8.15$, $pK_2 = 10.16$ and $pK_3 = 13.2$ (NCBI, 2019). The fluorescent property of doxorubicin has made it attractive for its drug delivery systems. This drug can be displayed with an absorbance rate of 470/550 nm. Doxorubicin is an effective drug when used alone or in combination with other drugs. DOX is an antitumor drug for the treatment of sarcomas and lung cancer, esophagus carcinoma, osteosarcoma, kaposisarcoma and soft tissue sarcomas.

However, it has many toxic effects. Cardiotoxicity comes at the beginning of these toxic effects. Other side effects include stomatitis, gastrointestinal toxicity, with vomiting, diarrhea, nausea etc. The cardiotoxicity of doxorubicin is generally manifested in two different tables. First, acute myocarditis-pericarditis syndrome leading to progressive heart failure and arrhythmias in patients; the second is the progressive loss of myocardial function due to the cumulative dose of doxorubicin. The first case was 7-14 days can occur with sudden death. And second one, cumulative dose-dependent myocardial dysfunction is directly proportional to the total dose administered (Blauwet & Cooper, 2010). Patients with a lifelong (cumulative) doxorubicin dose of 450-500 mg/m^2 will unfortunately face this situation **(**Pegram et al., 1999). Another disadvantage of DOX, like other antineplastic drugs, is that the therapeutic index (TI) is low. In other words, when the dose to prevent the growth of the tumor is given to the blood, healthy tissues are also damaged. The fact that free form of the doxorubicin causing side effects has led to the idea of combining this drug with nano-driven drugs. With the development of more efficient drug systems, many of these side effects have begun to be overcome. Liposomal DOX is the most common use of nano carrier mediated drug delivery systems.

2.4. Liposomes as Drug Delivery Systems

The idea that structures consisting of both hydrophobic and hydrophilic units may be useful for cell studies, and it has an old history.

Toward the end of the 19th century, the term "magic bullet" was introduced by bacteriologist Paul Ehrlich. The definition of this term was the chemical carrier biological structures that damage the abnormal cells without affecting normal cells. This definition is the basis of the idea of creating various drug delivery systems based on certain biochemical principles (Alavi et al., 2017). Based on certain biochemical and physical principles, the buildings in 1965 called "bangosomes" were brought to science by A.D. Bangham. This term was updated as "liposomes" (Bangham et al., 1965). Liposomes were firstly used in biological membrane model studies in 1968. Because liposomes consist of phospholipid like cells as shown in Figure 2.6 phospholipids have a fatty acid based hydrophobic tail and a phosphate based hydrophilic head. As shown in the figure liposomes are an aqeuous center wrapped by lipid layers (two or more). Liposomes are amphipathic self assemble closed structures and composed of phospholipid bilayers.

Figure 2.6. Bilayer structure of liposome (Source: Alavi et al., 2017)

Because phospholipids are inherently water-treated, they tend to turn into a spherical structure and form a membrane (Papahadjopoulos et al., 1973). Thus, both hydrophilic and hydrophobic materials can be encapsulated into liposomes. This makes liposomes attractive as a drug delivery system. These biological structures have been used as a drug carrier especially for the last 50 years (Sercombe et al., 2015; Pozzi et al., 1996; Nomura et al., 2001). The effectiveness of liposomes varies depending on the type of lipid, its size, surface charge and physicochemical properties.

Liposomes that have been proven to carry biologically active substances in vitro and in vivo are the most successful nano-guided transport systems known today (Felice et al., 2014). Liposomes can be classified according to their size, preparation technique and number of layers as shown in Figure 2.7.

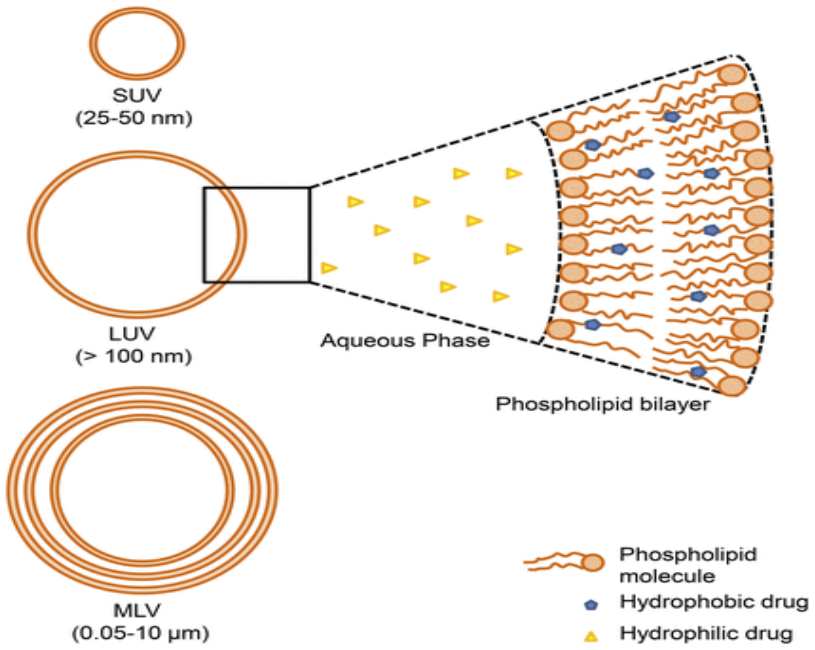


Figure 2.7. Liposome classes in terms of size and hydrophilicity
(Source: A. Sharma & U. S. Sharma, 1997)

Small unilameller vesicles (SUVs) have one lipid bilayer and the size is approximately 25-50 nm. Large unilameller vesicles (LUVs) has also one lipid bilayer and the size is larger than approximately 100 nm and multi lameller vesicles (MLVs) have several bilayers are larger than 500 nm. However, the size of the liposomes used in the medical field ranges from 50 nm to 1000 nm (Etheridge et al., 2013). Liposome size is selected according to purpose of the study. For example, the gap between tumor endothelial cells is wider than normal cells. Liposomes smaller than 200 nm can easily leak through these cavities (Gabizon & Papahadjopoulos, 1988).

ULVs (LUV and SUV) are water-soluble and MLVs are ideal candidates for encapsulating lipid-soluble drugs. In addition to the ability to retain the encapsulated drug according to this solubility difference, the release amounts of the drug in these two types of liposomes may vary. Single-layer liposomes release its content more rapidly than the multi layer liposomes. The main liposome production techniques are vesicle extruded technique and reverse-phase evaporation vesicles (Immordino et al., 2006). High drug

loading capacity, controlled and long-term release, biocompatibility, and high stability in biological environments have been effective in liposomes as the most preferred drug delivery systems. With these advantages, the effectiveness of liposomes in the diagnosis and treatment of many diseases have been reported (Deamer, 2010; Allen & Cullis, 2013; Al-Jamal & Kostarelos, 2011).

Although the first liposomes described in 1965 had many advantages, they had some shortcomings. These first liposomes, which were defined as conventional liposomes, did not have any surface modifications. Therefore, they tended to fuse each other. This caused gradual release of the loaded agent (Marrink & Mark, 2003; Haluska et al., 2006). Serum proteins bounded to these liposomes without surface modification. Thus, the liposomes were detected and eliminated by the body defense system as a foreign matter and circulation of loaded liposomes in blood were decreased. Many new liposome formulations were developed over time to overcome these challenges as shown in Figure 2.8. One of these new formulations is PEGylation. This allows the liposome to escape from the body defense system, the reticuloendothelial system (RES). Thus, liposomes are able to escape from phagocytosis and circulate in the blood for longer. These are known as "stealth liposomes" which can remain in the blood circulation for a long time (Allen & Cullis, 2013).

Liposome size should be chosen according to the study purpose. For example, the gap between tumor endothelial cells is wider than normal cells. Liposomes smaller than 200 nm can easily leak through these cavities. Cholesterol is used to modulate rigidity and to reduce instability caused by binding of serum proteins to the liposome membrane. Antibodies, proteins, vitamins, carbohydrates or drugs can be encapsulated into liposomes. The loaded liposomes can be delivered to the target tumor site by active targeting. The second generation innovative liposomes may release their contents in a controlled manner with the effects of pH changes, ultrasound, enzyme, microwave, light etc. This helps release the drug and other substances in the liposome only at the target site (Lee & Thompson, 2017; Huang & MacDonald, 2004; Jin et al., 2016). The usefulness of the drug loaded into liposomes only occurs if the drug is released. The condition for maximizing the bioavailability of a new drug is to ensure the appropriate release rate and release time. Too slow or too fast release of the drugs affect the treatment negatively. In order to avoid these problems, the lipid bilayer should be modified or the appropriate drugs should be selected.

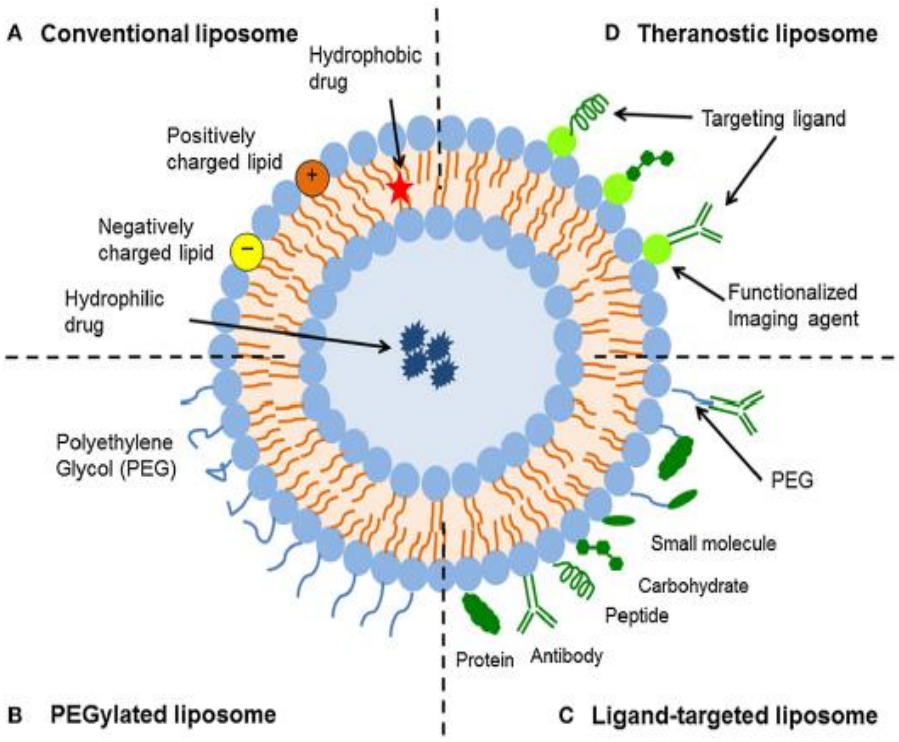


Figure 2.8. An example of liposome varieties (Source: Sercombe et al., 2015)

Cholesterol may be added to the liposome content to convert the phospholipid layer from the liquid phase to the solid phase. Type of lipids in the structure of the liposome should be chosen appropriately. As with other biological membranes, liposomes also show high permeability in hydrophobic drugs and low permeability in hydrophilic drugs (Fahr et al., 2005). The phase transition temperature of the lipid used is another factor affecting liposome rigidity as shown in Figure 2.9.

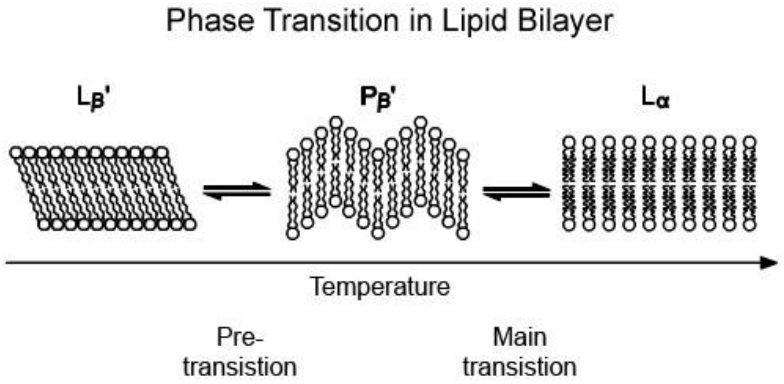


Figure 2.9. Phospholipids according to phase transition temperature (Source: Sigma Aldrich, 2019)

The lipids migrate from the "solid" gel state to a "fluid" liquid above their phase transition temperature (T_m) (Lewis & McElhaney, 2013). Conformational change between C-C single bonds of lipids increases membrane permeability. Because of the permeability of the membrane at the *Tm* temperature, the release of the drug trapped in the liposome increases. Each lipid has a specific T_m value as shown in Table 2.2. Liposomes can be prepared with a single type of lipid or a combination of several lipids (Papahadjopoulos et al., 1973).

Table 2.2. List of phase transition temperatures of phospholipids
(Source: Papahadjopoulos et al., 973)

Phospholipids	Abbreviation	T_c (°C)
Soybean phosphatidylcholine	SPC	−20 to −3
Hydrogenated soybean phosphatidylcholine	HSPC	52
Egg sphingomyelin	ESM	Ca. 40
Egg phosphatidylcholine	EPC	−5 to −15
Dimyristoyl phosphatidylcholine	DMPC	23
Dipalmitoyl phosphatidylcholine	DPPC	41
Dioleoyl phosphatidylcholine	DOPC	−22
Distearoyl phosphatidylcholine	DSPC	55
Dimyristoyl phosphatidylglycerol	DMPG	23
Dipalmitoyl phosphatidylglycerol	DPPG	41
Dioleoyl phosphatidylglycerol	DOPG	−18
Distearoyl phosphatidylglycerol	DSPG	55
Dimyristoyl phosphatidylethanolamine	DMPE	50
Dipalmitoyl phosphatidylethanolamine	DPPE	60
Dioleoyl phosphatidylethanolamine	DOPE	−16
Dimyristoyl phosphatidylserine	DMPS	38
Dipalmitoyl phosphatidylserine	DPPS	51
Dioleoyl phosphatidylserine	DOPS	−10

One of the most remarkable issues of recent times is the development of teranostics, where imaging and therapeutic properties are combined in a single complex. Liposomes have flexible structures that are suitable for modification. The incorporation of both imaging agents and therapeutic agent into the liposome at the same time provides advantages in many respects. Thus, the effects of treatment can be monitored (Kelkar & Reineke, 2011). Liposomes have been used as nano carriers for many medical imaging techniques such as magnetic resonance, nuclear imaging, ultrasound, fluorescence. Fluorescent imaging is the most commonly used method. The technique can be used to determine the activity of an enzyme, expression of a gene, or the position of a biomolecule etc. (Weissleder & Pittet, 2008). Liposomes containing perfluorocarbon gas are called acoustic liposomes. These liposomes serve as the ultrasound imaging probe.

Acoustic liposomes can also be used to determine drug efficacy and antitumor activity (Deckers & Moonen, 2010; Ferrara, et al., 2009).

Although liposomes are biocompatible, efficient drug delivery systems, they can be broken down by some chemicals or enzymes in the cell before they do reach to tumor sites. The loaded drug could be released before it reaches the tumor. In order to overcome these barriers, targeted drug delivery systems are used. In such systems, drug-loaded liposomes can be transported to the tumor site by means of markers such as specific antibodies on the cell surface, ligands (Park et al., 2002).

Immunoliposomes are antibody-targeted liposomes. The ligand-based targeted drug delivery system involves directing liposomes to specific ligands such as certain specific hormones, growth factors, enzymes, peptides on the cell surface. Some liposomes may be conjugated with special polymers for this purpose (Park, 2000; Matteucci & Thrall, 2000).

2.4.1. Transportation of Liposomes to Target Tumor Region

There are two main goals for success in chemotherapeutic cancer treatment. The first one is the chemotherapeutic agent with minimal loss in the blood circulation, passing through the barriers and reaching the tumor area. The other is the controlled releasing of the drug, which reaches the tumor area with minimum loss and maximum activity.

Liposomes are ideal nanoparticles that fulfill desired targets, and these carriers reach the tumor site in two ways. The first is passive targeting and the other is active targeting strategy (Moghimi et al., 2001).

Passive targeting is based on the enhanced permeability and retention effect (EPR) and the tumor microenvironment effect. Liposomes and other nano-carriers and even some macromolecules can accumulate in tumor tissue due to the unique characteristics of the tumor vessels. Some growth factors and indiscriminate development of other regulators on cells at the tumor site with a high tendency to create new vessels lead to some gaps between tumor-forming cells. As shown in Figure 2.10 nanoparticles with molecular weight over 50 kDa and some macroparticles can selectively accumulate in the tumor interstitium by passing through these cavities. This effect, which provides selective permeability due to the nature of the tumor without requiring any surface modification, is called as EPR effect.

Because the space between the tumor cells is about 1 micrometer, liposomes can easily pass through these cavities and reach the tumor and retained in tumor tissues for long periods. Because the space between the normal tissues is narrower, the liposomes pass less throught the normal cells (Torchilin, 2011).

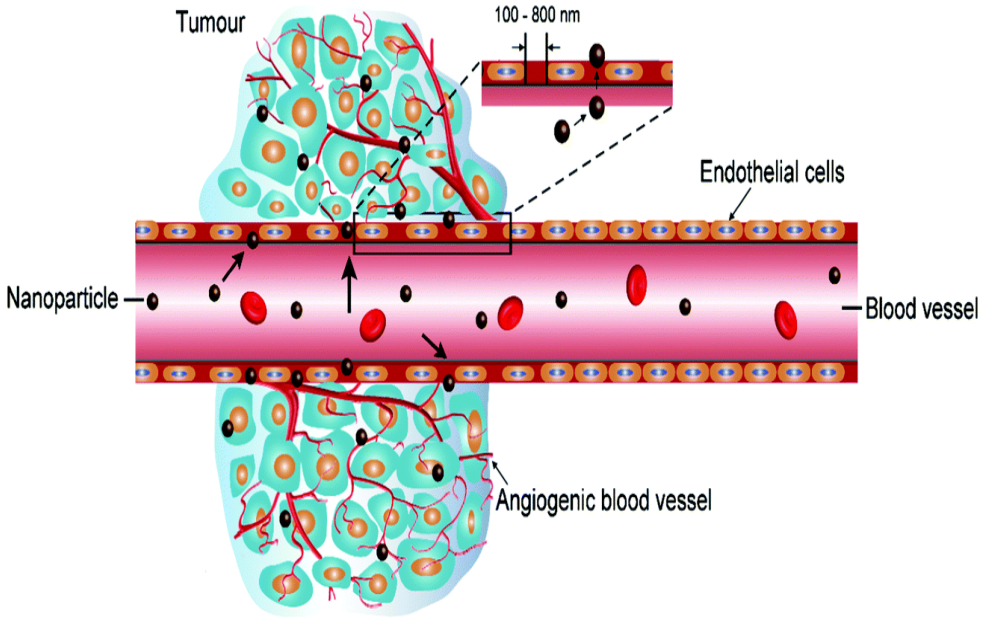


Figure 2.10. EPR effect of blood cells and passive targeting (Source: Dai et al., 2017)

In addition, cancer cells with uncontrolled growth require more oxygen and nutrients than normal cells. In order to obtain these substances, they go the way of producing extra energy by the shortest way. To this end, they cause a high reaction of glycolysis, resulting in an acidic environment. Some pH senitive liposomes can be designed according to this phenomena. This effect is also a result of tumor microenvironment (Pelicano et al., 2006; Yatvin et al., 1980). A drug delivery system based solely on passive guidance causes some nonspecific conditions. Modification of surface ligand or antibody-specific molecules to the liposome surface is quite a trend to avoid such disadvantages. Thus, a more specific drug delivery system is established. This system is called active targeting. Cancer cells have a large amount of various cell receptors or antigen constructs. These structures are expressed very high in tumor cells (Mura et al., 2013). Some conditions are required for successful targeting as shown in Figure 2.11. For example, these ligand or antibody-like keys on the cell surface are specific to cancer cells and should not be present in normal cells. These key molecules

should not be spilled in the blood and they should be homogenously expressed in targeted tumor cells (Leamon & Reddy, 2004).

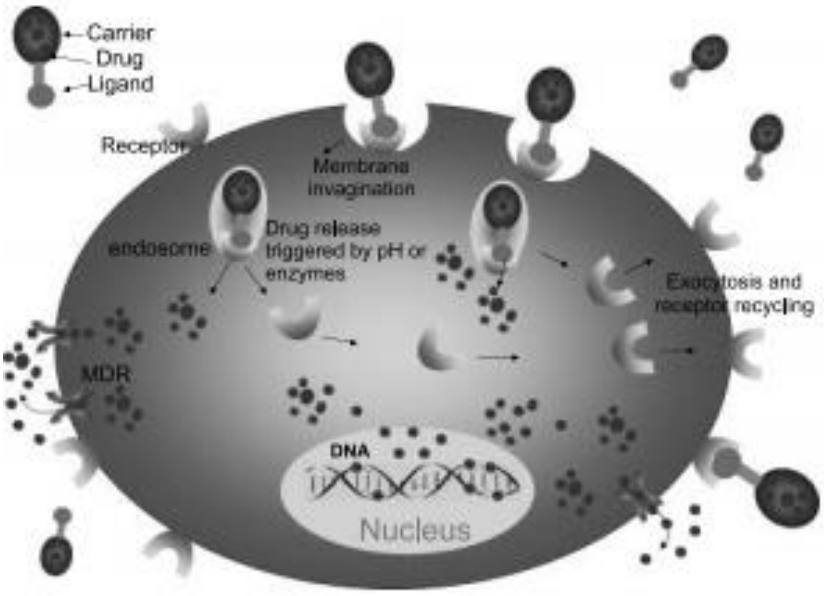


Figure 2. 11. Active targeting method of drug carriers (Source: Leamon & Reddy, 2004)

2.5. Liposomal Doxorubicin

Anthracycline glycosides are antineoplastic drugs with a wide range of effects and use. However, these chemotherapies can give rise to heart toxicity. Therefore, the limiting dose in human is 550 mg/m^2. Liposomal formulations of doxorubicin and other anthracyclines have shown a high therapeutic index (TI) on solid tumors. This formulation also increased the rate of drug accumulation at the tumor site.

Doxorobicin is an ideal candidate for encapsulation into liposomes because it is a drug with high encapsulation efficiency. This loading is carried out by the method called active loading by creating a gradient. The first liposomal doxoroubicin approved in the United States was a formulation called "Doxil". Doxil had 8 times more circulation time and half life than free doxorubicin. In addition, the drug had less toxic side effects than free form (Forssen et al., 1996; Gabizon, 1994).

Liposomal doxorubicin, approved by the American Food and Drug Administration (FDA), has been the main protagonist of many diseases such as uterine cancer, breast cancer, bladder cancer, Karposi's sarcoma. This liposomal doxorubicin is

briefly referred to as "LipoDOX". Free DOX is different from that of the lipoDOX for the targeted cells (Yu et al., 2016). DOX molecules in liposomes appear as long and fiber-like DOX crystals as shown in Figure 2.12. This shape is called as "coffe bean" structure.

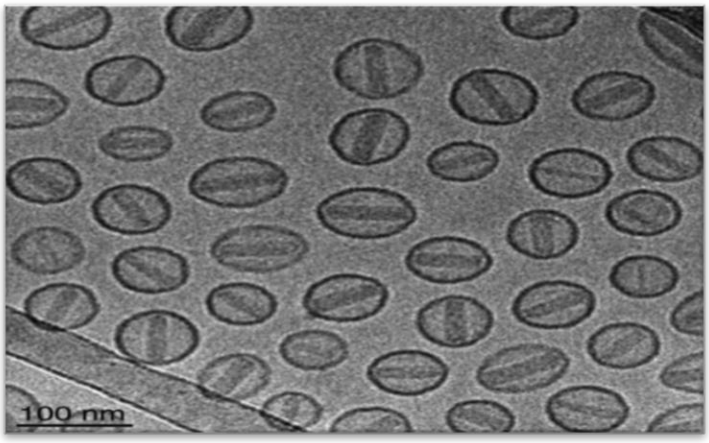


Figure 2.12. Image (cryo-TEM) of commercial Doxil (Source: Barenholz, 2012)

Doxil is also used in the treatment of many types of cancer in more than 80 countries. One of the reasons why it is so widely preferred all over the world is that there is an extensive literature about DOX and lipoDOX (Addeo et al., 2008; Barenholz, 2012).

In Canada and Europe approved counterpart of Doxil is Caelyx. These two LipoDOX also consist of hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol) and PEG-modified phosphatidylethanolamine (55:40:5 molar ratio). Another commercial liposomal anthracycline, Myocet, has a different phospholipid difference (egg phosphatidylcholine). Doxil/Caelyx has a steric barrier through its PEG, which provides a long circulating life and advantage for medicines that are given to the skin. However, it is not valid in the case of the blood.

Although it has many advantages over the free form, when injected into the vein, these drugs accumulate in the veins due to liposomes and cause some disadvantages such as hand and foot syndrome (Waterhouse et al., 2001).

The PEGylated liposomal DOX has the longest circulating time in the blood with a half-life of 55 hours. The lipoDOX accumulated in the tumor region should be released at the appropriate rate.

Very fast or very slow release causes some failure. If doxorubicin is released very rapidly through liposomes, the toxic effects caused by free DOX are repeated and the use of liposomes is of no importance. On the contrary, at least the release can be neither

therapeutic nor toxic for lipoDOX. Many variables, intraliposomal pH such as the type of drug selected, the drug/lipid ratio affects the rate of release. Optimal release can be found by controlling these variables (Wu et al., 2013). For example, the use of cholesterol at a concentration greater than 30% mol reduces the energy required to dissolve lipid acyl chains. This makes the liposome more rigid. Therefore, the amount of release is reduced. The loading of the drug into the liposome is by two ways: passive loading and active loading. The passive loading method comprises the steps of treating the aqueous solution of doxorubicin with the lipid film. Hydrophilic in the aqueous part formed in the center of the liposome and the hydrophobic components are compressed between the bilayer. However, doxorubicin has an amphiphatic molecular structure containing hydrophilic and hydrophobic groups. It can therefore be encapsulated both the liposome center and the between phospholipid tails. The efficiency of passive loading is not very successful, up to maximum 80% encapsulation occurs. Also, passive loading is not suitable for clinical use and production as it requires additional treatments such as removal of the drugs that is not encapsulated and the efficiency of encapsulation depends on the solubility of doxorubicin.

The active loading process comprises the addition of aqueous doxorubicin solution to the previously prepared liposomes. The most important difference from passive loading is that it is based on forming an ion gradient. In this way, DOX molecules are passed through the bilayer and then trapped in the center which is aquous. The active loading provides for encapsulation of about 48,000 doxorubicin molecules per 100 nm diameter liposome (Cullis et al., 1989; Mayer et al., 1994). This means a very high encapsulation efficiency compared to passive probing. The active loading is the encapsulation method used in commercially available liposomal doxorubicin formulations approved by FDA. The basic principle of the active loading method is to form a pH gradient and thus to introduce the external doxorubicin into the liposome. This method eliminates the dependence on drug resolution required by passive loading. The formed pH gradients cause the doxorubicin to collapse with the salts in the liposome aqueous phase or by self-interaction. The liposome is prepared with an acidic buffer to form a gradient. Then the external pH is changed. A basic buffer is added or the outside buffer is removed by methods such as dialysis (Harrigan et al., 1992).

Another method of forming a pH gradient is encapsulating ammonium sulfate ($(NH_4)_2SO_4$) into liposomes as shown in Figure 2.13.

After encapsulating ammonium sulfate into the liposomes, a buffer such as NaCl solution is used to remove the external buffer. Due to its high permeability, NH_3 leaves a proton for every NH_3 molecule passing through the liposome membrane and disappears. The generated $[NH_4^+]_{in}=[NH_4^+]_{out}$ gradient paves the way for loading the doxorubicin (Haran et al., 1993; Bolotin et al., 1994).

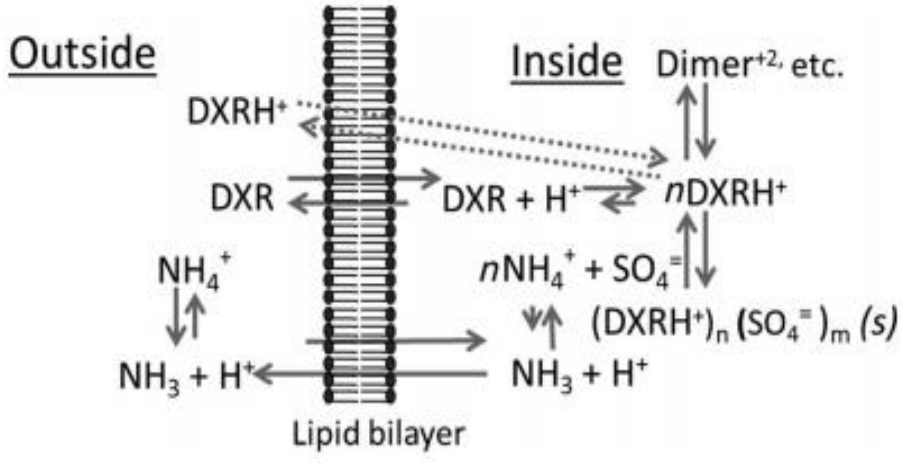


Figure 2.13. Ion gradient method for DOX loading (Source: Csuhai et al., 2015)

2.6. Cellular Uptake Mechanism of LipoDOX

The encapsulated drug within the liposome can be taken by the cell in different ways as shown in Figure 2.14. One of them is that the liposomes are absorbed into the cell membrane. This is where the absorption site disrupts the liposome bilayer by enzymes such as lipase secreted by the cell or by mechanical force. After absorption, the antitumor agent in the liposome passes into the extracellular fluid, where it diffuses with the cytoplasm or cell membrane. Another one is the fusion through cell membrane the release of the drug into the cell. A third one is receptor mediated endocytosis and is the most common. This is suitable for enzymes resistant to the release of lysosomes. Because in this case the cell secretes enzymes with the help of lysosomes and cleaves the liposome membrane.

Liposomes with a size less than 150 nm is suitible for cell uptake. Larger vesicles can cause an immune response and phagocytosis with macrophages (Fanciullino & Ciccolini, 2009; Fanciullino et al., 2005; Torchilin, 2011).

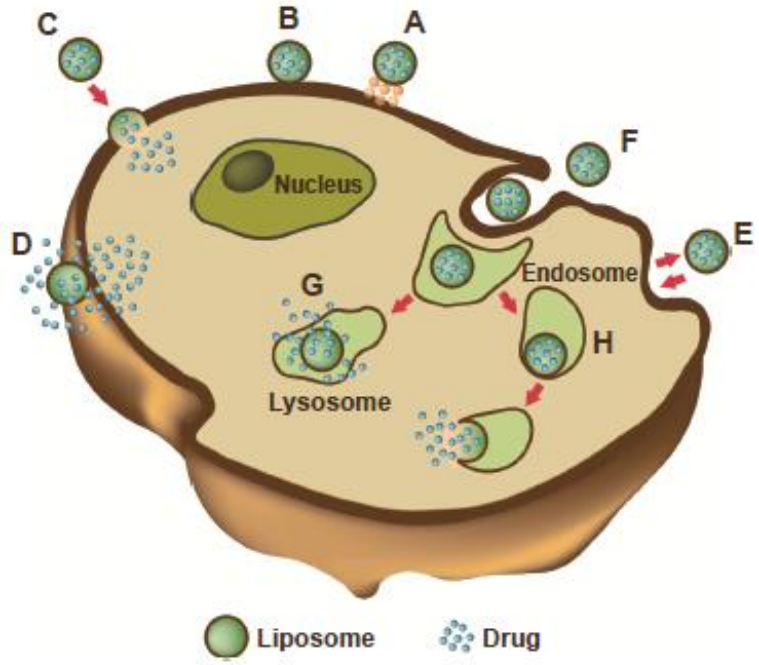


Figure 2.14. Cellular uptake of liposomal drug (Source: Bozzuto & Molinari, 2015) (A). Absorption (B). Fusion (C). After the interaction the structure of the liposome bilayer is affected (D). Exchange of carrier-lipid components with the cell membrane (E). Endocytosis (F). Endosomes fuse with lysosomes (G). Low pH induces thedegradation of the liposome membrane and the drug is released (H). Liposomes release their cargo after fusion.

The extent to which the surfaces of liposomes are electrically charged or the amount of charge are the factors that greatly affect the interactions between the cell and the liposome. The lipid type used in liposome production or the proportion of lipids can be used to adjust the electrical charge and density. Charged liposomes have advantages over neutral liposomes. For example, neutral liposomes interact with each other to cause aggregation, which reduces stability. A negative or positive ζ-potential on the liposome surface prevents aggregation by pushing the liposomes. Furthermore, the possibility of neutral liposomes interacting with the cell is reduced. Therefore, the drug carried by the liposomes can be released outside before it reaches the targeted cells (Harashima et al., 1998; Miller et al., 1998; Cullis at al., 1998).

As shown in Figure 2.15, only a small amount of injected DOX reaches the tumor, while the remaining large amount (about 98%) is distributed to healthy tissues (Zubareva et al., 2014). It was hoped that a more efficient liposomal drug delivery system would be developed if less medication was used and the uptake of liposomes by the cell was

increased. Thus, undesirable side effects such as hand and foot syndrome caused by conventional lipoDOX were expected to be reduced or eliminated.

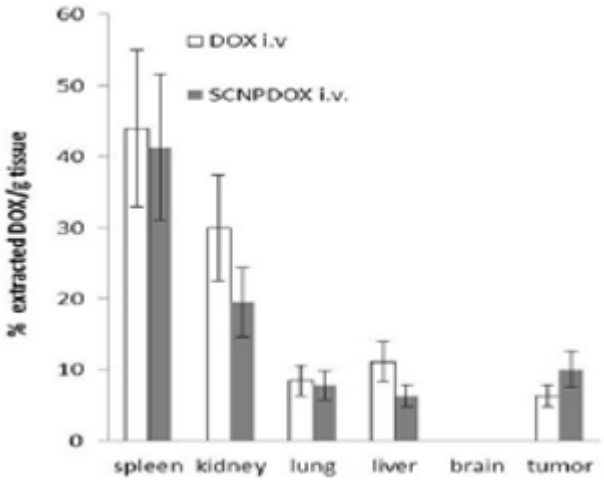


Figure 2.15. In-vivo biodistribution of free DOX to tumor and healthy organs (Source: Zubareva et al., 2014)

As shown in Figure 2.16, uncharged and small molecules can easily pass through the cell membrane while charged and large molecules cannot pass through cell membrane.

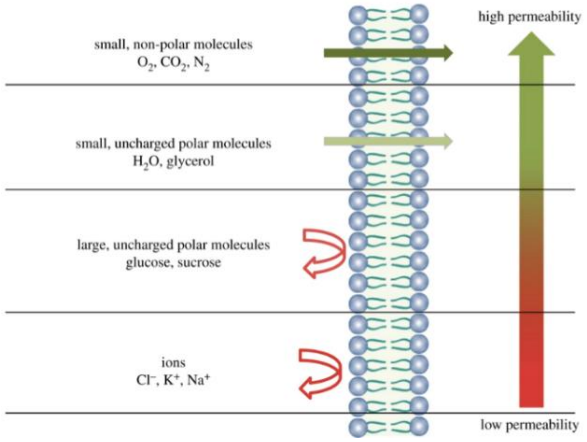


Figure 2.16. Transition of molecules through cell membrane (Source: Monteiro et al., 2014)

The pH of the tumor microenviroment is around 6.8. The DOX-HCl in the liposomes is positively charged because the pH in the liposome is approximately 5.5 and pKa value of DOX-HCl is 8.15. As shown in Figure 2.17, we proposed that if we increase

the pH at the tumor microenvironment in a short time, the drug DOX can easily enter the cell when it is deprotonated and neutral at higher pHs.

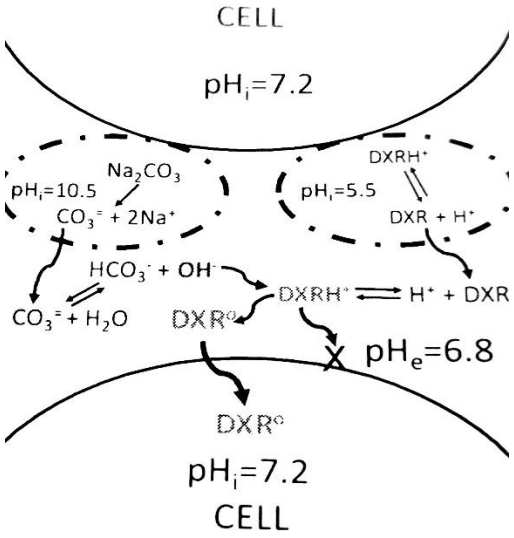


Figure 2.17. Illustration of aim of using alkaline liposomes

It was aimed in this thesis to obtain alkaline liposomes using for instance, the solution of sodium carbonate. A two-component system, one is alkaline liposomes and the other is lipoDOX, was designed and characterized.

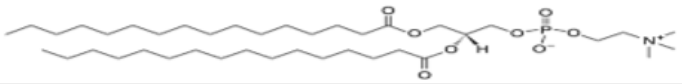
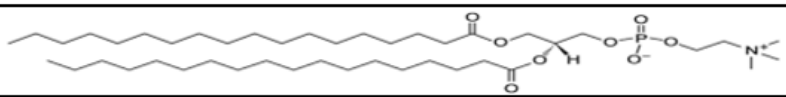
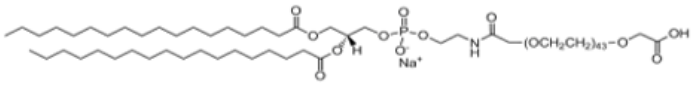
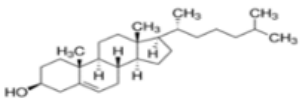
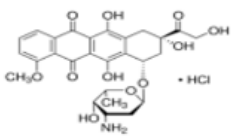
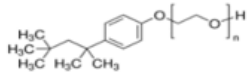
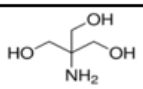
CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Chemicals and their chemical formulas were used in the current studies here shown in Table 3.1. DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG (2000) (amine 1,2-distearoyl-sec-glycero-3-phosphoethanolamine-N [amino (polyethylene glycol)-2000] lipids were purchased from Avanti Polar Lipids. The cholesterol was purchased from Flukan. Chloroform was purchased from Sigma Aldrich and the dialysis membrane was obtained from the RC tubing Spectra/Por 6 Dialysis Membranes (MWCO: 10 kD). DOX-HCl was purchased from European Pharmacopoeia Reference Standard and $Triton_{x\text{-}100}$ was purchased from BioShop. Other chemicals such as tris (hydroxymethyl) aminomethane, sodium carbonate (Na_2CO_3), ammonium chloride (NH_4Cl) were obtained from Merk.

Table 3.1. Chemicals and their chemical formulas used in the current studies (Source: Avanti Polar Lipids, Sigma, 2019)

Material	Chemical Formula
DPPC	
DSPC	
$DSPE\text{-}PEG_{2000}$	
Cholesterol	
DOX-HCl	
Triton X-100	
Tris (hydroxymethyl) aminomethane	

3.2. Methods

3.2.1. Liposome Production

The first step in liposome making is the lipid film formation as shown in Figure 3.1. The liposome components of DSPC, cholesterol and DSPE-PEG_{2000} with 57-38-5% mole were weighted as 9 mg of DSPC, 2.93 mg of cholesterol and 2.8 mg of DSPE-PEG_{2000} and placed into a 20 ml vial. Then 1 ml of chloroform was added to ensure homogeneous mixing. Care was taken not to inhale chloroform. The sample was subjected to nitrogen gas to remove the chloroform and to obtain a homogeneous film. The vial was mixed with an orbital shaker at a speed of 150 rpm. Evaporation took approximately 40 minutes to obtain a dry lipid film. Then, the lipid film bottle was placed in a vacuum oven to dry the film, which was completely free of chloroform. This process took overnight.

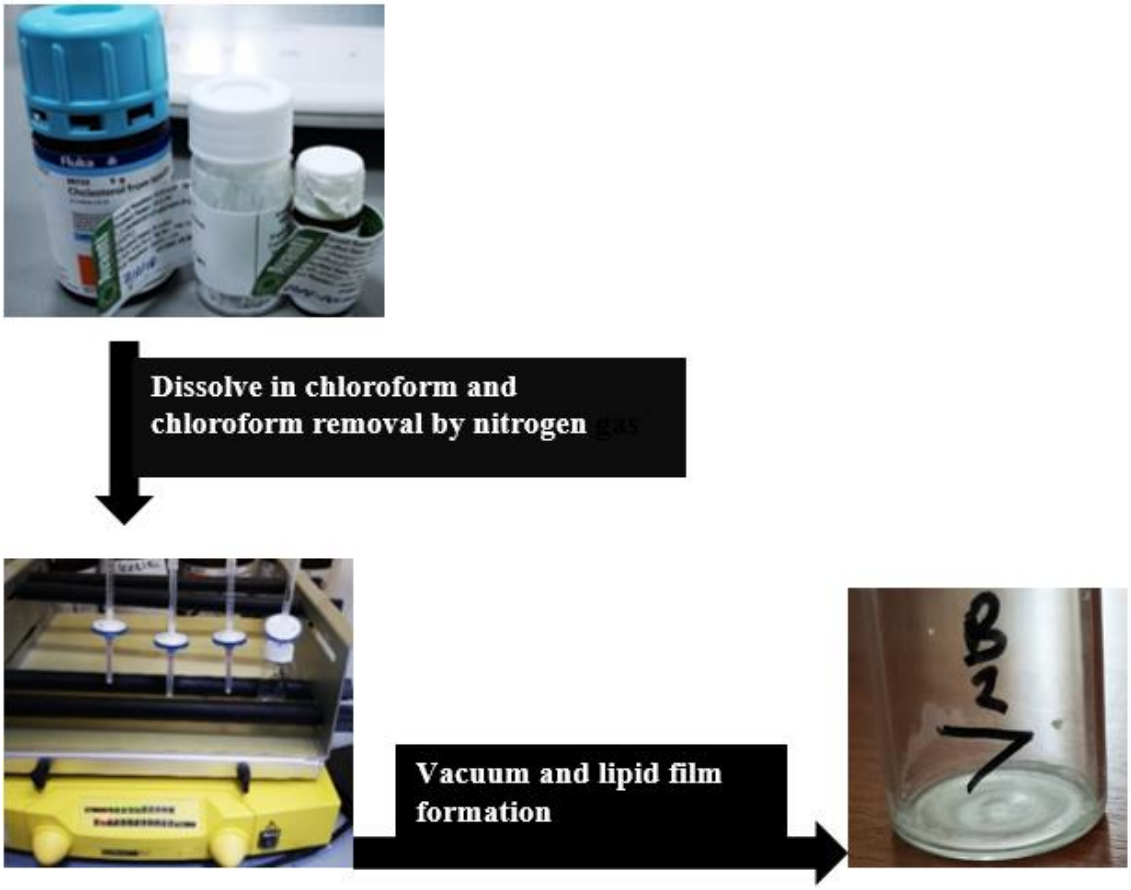


Figure 3.1. Lipid film preparation technique

If liposome formation from the lipid film was not performed on the same day, the film was stored at -20 °C. The next step after forming the lipid film was the hydration. At this stage, the lipid film was treated with the solution loaded, for instance, a 1 ml of ammonium sulfate $(NH_4)_2SO_4$ was added into the lipid film to prepare the medium for DOX-HCl loading. The pH of the buffer solution was 5.5 and its molarity was 250 mM. Hydration took about 1 hour in a shaking water bath at 150 rpm at 65 °C as shown in Figure 3.2. depending on the phase transition temperature of the selected phospholipid (55 °C for DSPC and 41°C for DPPC lipid).

Figure 3.2. Hydration step for liposome formation from lipid film

The third step of liposome preparation was extrusion. The aim of extrusion was to obtain liposomes with appropriate sizes. A mini extruder system was used as the assembly formed with many small parts as shown in Figure 3.3.

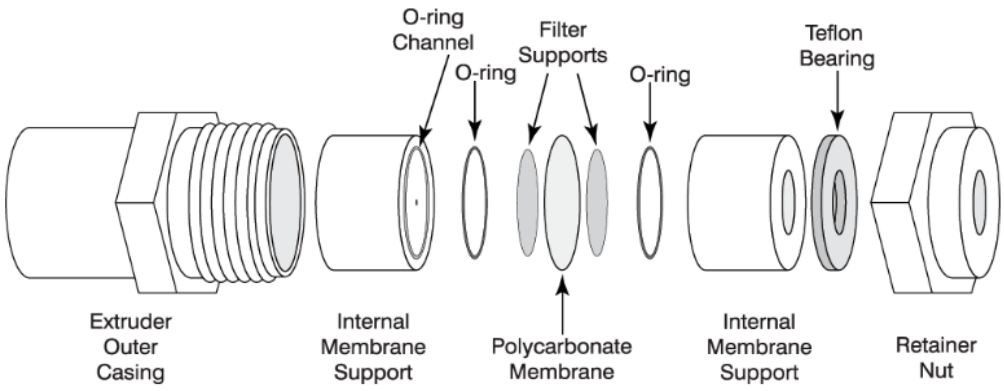


Figure 3.3. Representation of parts of extruder system
(Source: Avanti Polar Lipids, 2019)

The main part adjust the liposome size was the polycarbonate membrane (Whatman ®) with the desired pore size (200 nm). Filter supports made of polytetrafluoroethylene (PTFE) were used for each extrusion process, one at the top and one at the bottom of the membrane. Two O-rings were used for sealing. As shown in the figure, the filtering system was formed in such a way that the membrane would be in the middle as it forms a sandwich. The Swagelok connector holding the system and other parts were supplied from Avanti Polar Lipids. Two glass syringes to be taken into the liposome were purchased from Hamilton.

The membrane and supports were wetted with the buffer ($(NH_4)_2SO_4$) after the extrusion system was installed. In order to install the filtration system, teflon bearing was placed in the retainer nut and one of the internal membrane supports was inserted into this pair. The same nesting process was applied for extruder outer casing and other internal membrane support. The installed mini extruders and lipids were kept on the hot plate for about 10 minutes to allow the system to warm up at 65°. A thermometer for temperature control was placed in the hole on the Swagelok. Then, one of the Hamilton syringes was filled with buffer and the tip of the syringes was placed opposite to the holes in the system. The buffer was passed through the filter several times, passing from a syringe to the other syringe. Transfer was performed using the Longer Syringe Pump to prevent membrane damage and pass at the same velocities. The pump speed was set to 120 mm/min. Then, the buffer in the syringe was emptied and the hydrated lipid mixture in the vial drawn into syringe. 11 passages were performed between two opposing syringes as shown in Figure 3.4.

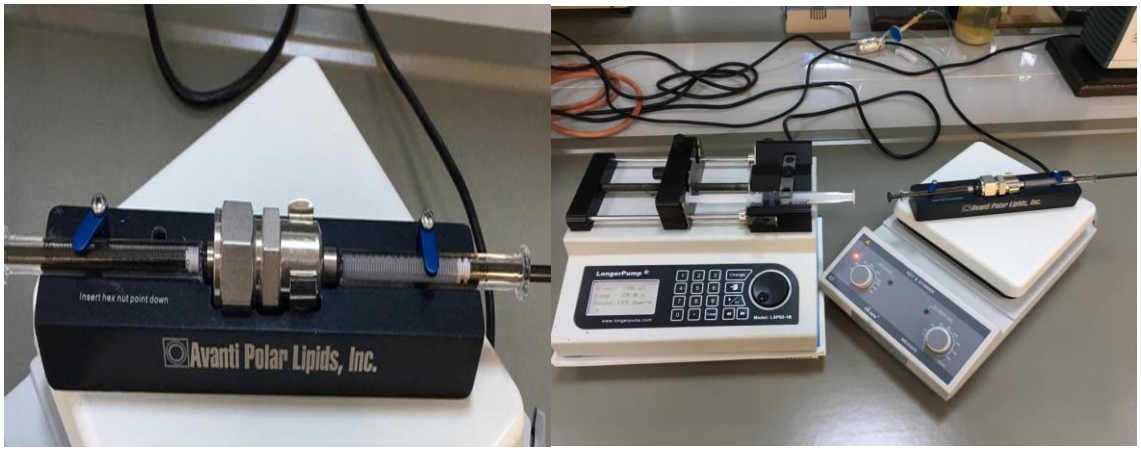


Figure 3.4. Left: empty and liposome filled syringe right: exstrusion system

As shown in the Figure 3.5, about 0.9 ml of liposomes were obtained. The obtained sample was kept in a clean vial and stored at +4 °C. The mean liposome size was measured by Dynamic Light Scattering (DLS) method, which is below 200 nm after extrusion.

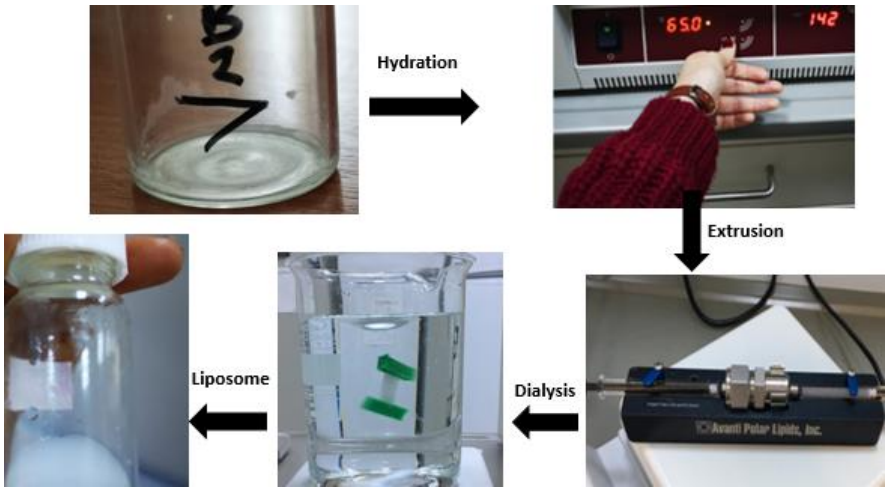


Figure 3.5. Liposome stock formation after all liposome production steps

3.2.2. DOX Loading into Liposomes

Before the DOX loading, dialysis was performed to remove excess ammonium sulphate from the liposomes. 0.9% NaCl solution was prepared for this process. 1 liter of saline was used for approximately each 1 ml liposome dialysis. As shown in Figure 3.6 (a), approximately 7 cm of the dialysis membrane (Spectra / Por 6, Spectrum Laboratories, Inc) was cut for approximately 1 ml of liposome samples and kept in ultra pure water for 10 minutes. The purpose of this procedure was to remove the protective chemical on the dialysis membrane. Approximately 1 cm from one end of the membrane held in water folded. Then, the folded end is tightened and locked with one of the clips seen in the figure. The liposome extracted with the automated pipette was then introduced into the open end of the membrane. The open end was folded and closed with the other clip. Care was taken to avoid air bubbles in the membrane. With osmotic pressure, excess ammonium sulfate in the liposomes was transferred to water. For this, the membrane in which the liposome was placed was placed in the pre-prepared 0.9% NaCl solution beaker. Magnetic stirrer was placed into beaker. The solution in the beaker placed on the

magnetic stirrer plate was replaced after 2 hours. Later, overnight was left on the stirrer as shown in Figure 3.6 (b).

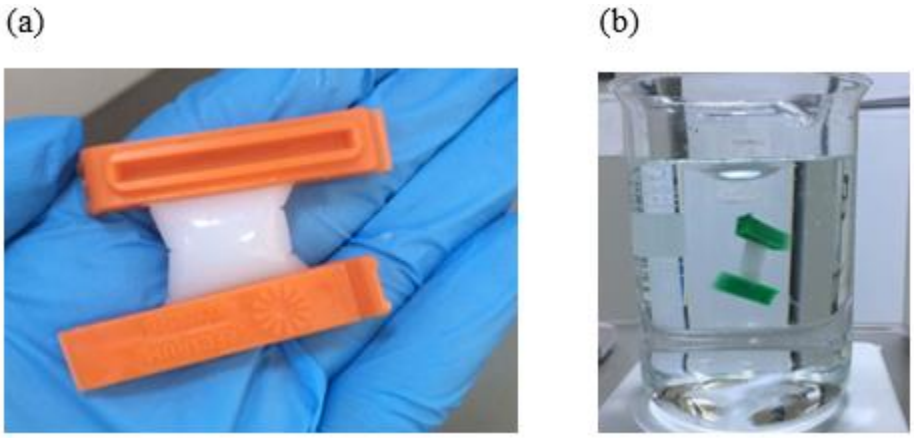


Figure 3.6. (a) Locked liposome sampe into dialysis membrane by two clipsm
(b) Dialysis step of liposome

In general, doxorobicin was dissolved in water or high-pH solution (changed according to test type) for the DOX loading. Liposome and DOX solution were mixed in a vial and incubated at 65 °C for 2 hours using the shaking water bath (150 rpm) as shown in Figure 3.7.

Figure 3.7. Incubation step for DOX loading step into liposomes

3.2.3. Remove of Excess DOX from Liposomes

The liposomal DOX was removed by the same dialysis method used to prepare liposomes. For this purpose, the dialysis membrane was cut to the appropriate size. It was kept in ultra pure water for a while. LipoDOX from the incubation was placed in the membrane which was closed with a single side clip. The other part of the membrane was closed tightly with the other clip as shown in Figure 3.8.

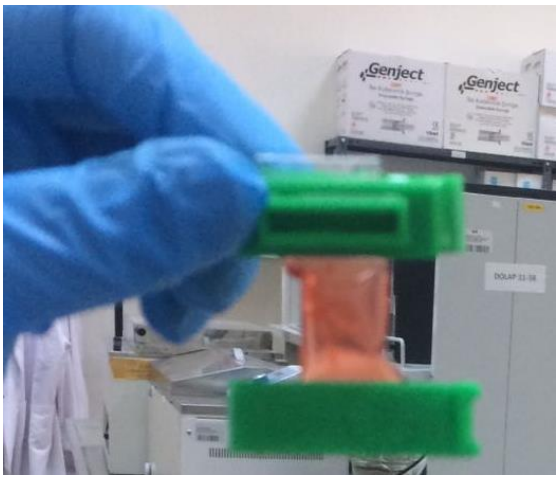


Figure 3.8. Preperation of lipoDOX to dialysis

After checking for leakage, the sample in the membrane was left to a 0.9% NaCl solution (1 liter for 1 ml sample) at room temperature. Magnetic stirrer was used for the mixing. Thus, the free DOX, which is not encapsulated, is allowed to pass into the water. After 2 hours the dialysis solution was replaced with a new one. It was then allowed to stir overnight.

3.2.4. Liposome Size Measurement

The liposome sizes were determined using dynamic light scattering (DLS). The device has an operating principle as shown in Figure 3.9 and Figure 3.10.

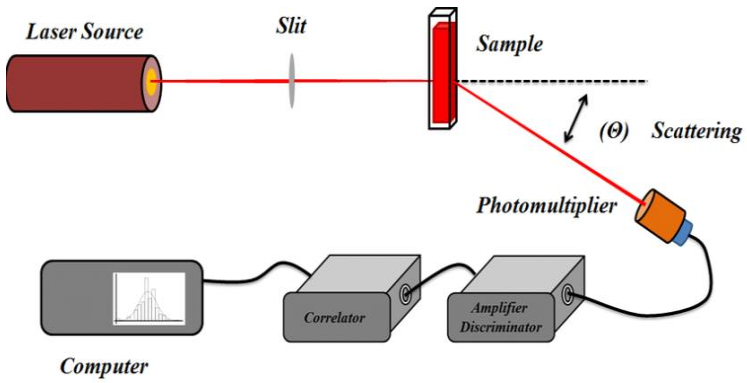


Figure 3.9. Representation of DLS working principle (Source: Kumar, 2012)

The DLS method is used to measure the size distribution and average size of particles. The method of operation is based on Brownian motion of particles dispersed in

a liquid. This movement causes a different distribution of the laser light sent to the cuvette. Thus, the size of the particles is calculated by the software by the computer (Malvern Panalytical, 2019).

Figure 3.10. DLS device using steps

3.2.5. Measurement of Loaded Doxorubicin

The amount of drug loaded was determined by spectrophotometric measurements. Because the drug had spontaneous fluorescence, the amount of drug loaded was measured with fluorescence. In addition, the amount of drug released from the liposomes according to time and temperature was also determined by fluoresence spectrophotometer (BioTek, Synergy HTX Multi-Mode Reader) and this device has a working principle as seen in Figure 3.11.

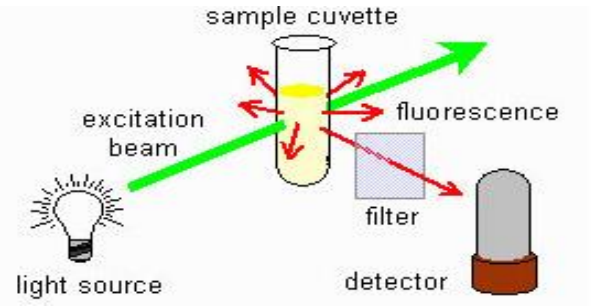


Figure 3.11. Principle of working of fluoresence spectrophotometer
(Source: Notes in Biomedical Science, 2012)

Doxorubicin has 480 nm (excitation) and 590 nm (emmission) for fluorescence wavelengths. Absorbance wavelength is 480 nm. According to this data fluorescence and absorbance graph were obtained according to different DOX concentrations.

3.2.6. Fluorescence Spectrum of DOX at Different pH

Solutions with five different pHs were prepared as 6.96, 7.53, 8.03, 8.51, 9.38. DOX stock solution with a concentration of 1 mg/ml was also prepared. 4 ml of each solution with different pH was mixed with 10 µl of DOX stock solution. The scans between 200 and 800 wavelengths were performed at Em = 630 and Ex = 488 values each. In this way, the effect of pH on DOX fluorescence values was evaluated.

3.2.7. Preparation of High pH Solutions and Application to LipoDOX

Before all pH studies, pH was calibrated with three standard calibration fluids.

3.2.8. Effect of pH and Tris on DOX Loading into DSPC Liposomes

10 mM 500 ml stock tris solution was prepared. The stock tris pH was measured as 9.90. Using HCl the following four different pHs were obtained for tris buffer as 7.2, 7.8, 8.2 and 8.9. DOX weighed approximately 0.5 mg to 4 vials each. 500 µl of tris buffer was added on each vials at different pH obtained. 500 µl of stock DSPC liposome was added to each vial. Incubation and dialysis performed.

3.2.9. DOX Loading to DSPC Liposomes with Different Tris Concentrations

3 lipid films were prepared according to the 57-38-5% formulation. Liposomes were prepared from these films. DOX stock solution with a concentration of 1 mg/ml was prepared using ultra pure water. Tris stock solution was also prepared at a concentration of 10 mM with a pH of 8.0. Tris was diluted to 10 different concentrations with 1/2 dilution factor as shown in Figure 3.12. After dilution was completed, the vials 2,4,6,7 and 9 were separated for use in the experiment. 100 µl of each of different concentrations of tris, 400 µl of the DOX stock with a concentration of 1 mg/ml and 500 µl of the stock liposome were mixed to make a total volume of 1 ml. The vials were placed in the shaking incubator for 1.5 hours. After loading, dialysis was performed for each prepared sample in separate beakers overnight.

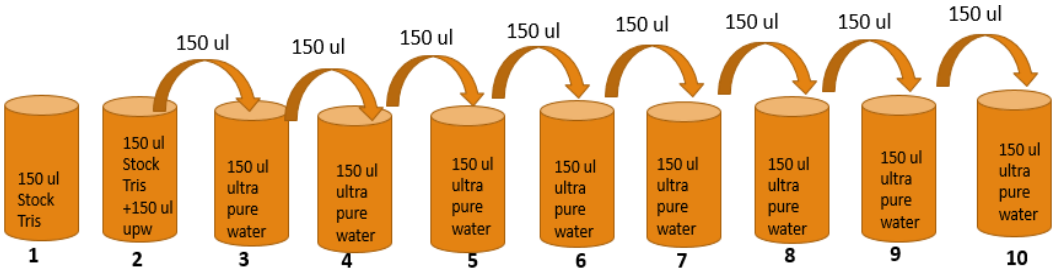


Figure 3.12. Illustration of tris dilution

3.2.10. pH Adjustment with NH_4Cl and NaOH

NaOH (50 mM) and NH_4Cl (50 mM) stock solutions were prepared. Solutions were obtained by mixing different percentages of these stock solutions. The pH of each solution was then measured to obtain a concentration-pH curve.

3.2.11. Effect of pH Prepared with NH_4Cl/NaOH on DOX Loading into DSPC Liposomes

NH_4Cl/NaOH solutions at pH 7.2, 7.6, 8.15, 8.6, 9.2 were prepared from prepared calibration curve. DOX stock solution with a concentration of 1 mg/ml was prepared using ultra pure water. 200 µl of each solution at different pH, 500 µl of DOX solution and 500 µl of DSPC liposomes were mixed. 5 different lipoDOX samples were allowed to incubate for 1.5 hours and dialyzed in separate beakers. Thus, lipoDOX was obtained at 5 different pH (7.25; 7.69; 8.28; 8.70; 9.30) and the DOX amounts loaded according to the pH were calculated (DOX concentration = 0.416 mg/ml). In order to see stability of the liposomes at high pHs, fluorescent values were measured on the spectrophotometer by adding 130 µl of each 5 different pH solutions prepared and 20 µl of the lipoDOX with pH of 7.69. Fluorescence values of each well i.e. each pH, were recorded for different time intervals (0,1,3,5,7,10,20,30,40,50,60 minutes)**.**

3.2.12. Liposome Stability at Higher pH

500 µl of three different solutions with pH of 8.44, 8.69 and 9.15 obtained using NH_4Cl/NaOH calibration curve were mixed with 500 µl of DSPC liposomes and sizes were measured using DLS versus time.

3.2.13. Release of Doxorubicin from DSPC Liposomes at Higher pH

3 different pH solutions were prepared (8.38, 8.51, 9.02). 850 µl of the DOX solution with a concentration of 1 mg/ml was added on 850 µl of DSPC liposome and lipoDOX was obtained. 700 ml of each of three solutions were mixed with 300 µl of obtained lipoDOX. 150 µl of these mixtures were taken and fluorescence and absorbance values were measured in spectrophotometer. Measurements were taken at the 1st, 3rd, 5th, 10th, 20th, 30th, 100th and 120th minutes. 10 µl of $T_{x\text{-}100}$ was added as detergent to degrade the liposomes at 130th minutes. The Triton X-100 was used while examining the release rate. Triton X-100 is a nonionic surfactant having a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon, lipophilic or hydrophobic group. The hydrocarbon group is a 4- phenyl group. Triton X-100 is a surface-active detergent that is used frequently in biochemical applications and dissolves proteins and lipids. It increases the permeability of the cell membrane and liposomes by disrupting the bilayers (Wattiaux & De Duve, 1956; Molina-Bolivar et al., 2002). Time-dependent release percentage was calculated using the following equation. Here F_0 is the fluorescence intensity at the zero, F_{TX} is the fluorescence intensity after disrupting liposomes with $T_{x\text{-}100}$, and F is the fluorescence intensity at different time intervals.

$$\text{Release } \% = (F-F_0) / (F_{TX}-F_0) * 100 \quad (1)$$

3.2.14. Alkali Liposome Preperation and Release from DSPC Liposomes

The liposome prepared with DSPC lipid was hydrated with 1 ml of sodium carbonate (250 mM) for 1 hour (pH of sodium carbonate 11.73). The size of the prepared liposome was measured which were found to be less than 200 nm. 0.9% NaCl solution was added to 20 ml vials for continuous measurement with pH probe. After the first 3 minitues of measurement, 10 µl, 30 µl, 50 µl, 100 µl of alkaline liposomes were added to the vials containing 0.9% NaCl solution at certain time intervals. After the continuous pH measurement, 70 µl of 20% $T_{x\text{-}100}$ was added to break the liposomes. They were then assayed with 10 µl, 30 µl, 50 µl, 100 µl and 150 µl sample, respectively, in different vials at the same time intervals. Continuous measurements were also taken from the vial containing 0.9% NaCl without liposomes for control.

3.2.15. Effect of Ultrasound on Release of Alkaline Solution from DSPC Liposomes

In order to examine the effect of ultrasound on the release from liposomes, ultrasound was sent to 10 ml of 0.9% NaCl solution for about 5 minutes. pH was continuously measured. Then, 30 µl of alkaline liposome was added (with Na_2CO_3) and continued the pH measurement. 70 µl of T_{x-100} was added after approximately 10 minutes to confirm liposomes still contained its contents.

3.2.16. DPPC Liposomes

All of the liposome preparation techniques were performed in the previous procedure. Only the hydration and incubation part of the DPPC lipid were used at 55 °C because the phase transition temperature of the DPPC lipid is 41 °C.

3.2.17. Release of Alkali Solution from DPPC Liposomes with Temperature

After obtaining the DPPC liposomes, the water bath was adjusted to the desired temperature. After equilibrating the water bath, 100 µl of DPPC liposome was added to 10 ml of 0.9% NaCl solution and the sample vials were placed in the shaking water bath. After 20 minutes, the vial was removed and its pH was measured. This study was performed for 25-35-42 and 49 °C. For each temperature, the vials containing only NaCl with and without liposomes (control) was measured.

3.2.18. DOX Release at Different pHs and Temperatures Using NH_4Cl/NaOH Solution

Four different solutions with pH values of 6.88, 7.23, 8.24 and 8.54 were prepared using NH_4Cl/NaOH calibration graph by using 50 mM of NH_4Cl and NaOH stock solutions. 5 ml of each of these solutions was added to each vial. A total of 16 vials were prepared for each pH to be examined at 4 temperatures. 4 vials containing each pH sample were placed in the equilibrated water bath and allowed to mix by the shaker. 50 µl of DPPC lipoDOX sample was added to each vial. 150 µl of sample was taken at certain

time intervals and fluorescence values were measured in 96 well plates. This procedure was performed for 25-37-42 and 50 °C.

3.2.19. pH Adjustment with Na_2CO_3 and $NaHCO_3$

Na_2CO_3 (250 mM) and $NaHCO_3$ (250 mM) stock solutions were prepared. A pH graph was generated according to Na_2CO3 and $NaHCO_3$ by combining these stock solutions with the ratios shown in Table 3.2.

Table 3.2. Ratio for Na_2CO_3 and $NaHCO_3$ stocks

	%				
Na_2CO_3	100	80	50	20	0
$NaHCO_3$	0	20	50	80	100
pH	11	10,70	10,21	9,67	8,56

3.2.20. Effect of Na_2CO_3 on DOX Release

Na_2CO_3 (250 mM) and $NaHCO_3$ (250 mM) were used to obtain solutions with a pH of 7.7, 8.20, 8.58 and 9.24, respectively. 1 ml of each solution was mixed with 10 µl of lipoDOX prepared with DPPC liposome (DOX concentration=0.3 mg/ml). After the shaking water bath equilibrated at 42 °C, the vial with the sample was placed in the water bath. Fluorescence and absorbance values were recorded by taking 150 µl at regular time intervals and placed in the 96 well plate. DOX release was calculated with time. At the end, 10 µl of 10% T_{x-100} was added to release the total DOX in the liposomes. The same study was repeated for 50 °C.

3.2.21. Effect of NH_4Cl on DOX Release

Stock solution with a pH of about 9 was prepared using 50 mM NH_4Cl and NaOH stock solutions. This solution was diluted with 1/2 dilution factor using distilled water to 6 vials. 960 µl of pH solutions were taken from each vials except the third vial and placed into 1.5 ml vials. 40 µl of DPPC lipoDOX was added to each vial. The vials were placed

in a shaking water bath at 42 ° C which reached equilibrium temperature. 150 µl of each sample was placed in 96 wells and measured their fluorescence values with time. DOX release percentages were calculated using release formula. In this experiment, the pH of each vial was checked and found different as a result of dilution. Therefore, in a subsequent study, pHs were adjusted for each vials containing different concentration of NH_4Cl/NaOH and investigated DOX release. For this purpose, solutions with different NH_4Cl concentration were obtained in 5 vials with 1/2 dilution factor. A small amount of NaOH was used to equilibrate the pH. Thus, solutions with the same pH but different NH_4Cl concentrations were obtained. 960 µl of these solutions were mixed with 40 µl of the DPPC lipoDOX sample and NH_4Cl concentration-dependent release was calculated from measured fluorescence values.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Liposomes and Their Characterizations

Liposome size is important for leakage from tumor blood vessels. Normally the spaces between the endothelial cells (which cover the blood vessels) are about 20-25 nm and this range is between 100 and 800 nm at the tumor site (Haley& Frenkel, 2008). Retention by RES is directly proportional to liposome size. The reduction in the size of the liposome is also associated with increased accumulation in the tumor tissue. The reason for this is related to the half-life and circulation of small liposomes. According to the results, the liposomes used to benefit from the EPR effect were determined to be 400 nm and the sizes of liposomes below 200 nm were found to be the highest (Danhier et al., 2010; Sawant & Torchilin, 2012). This size was taken as reference because it was determined that the optimum liposome size was less than 200 nm. In all studies, polycarbonate membranes with 200 nm pore size were used to adjust the liposome sizes. In all experiments, liposome size was measured with DLS after extrusion. Figure 4.1 shows the DLS result for one liposome sample. Others were found similar. As shown in the figure liposome below 200 nm was obtained and result indicates a homogeneous distribution. It was also good to have a single peak.

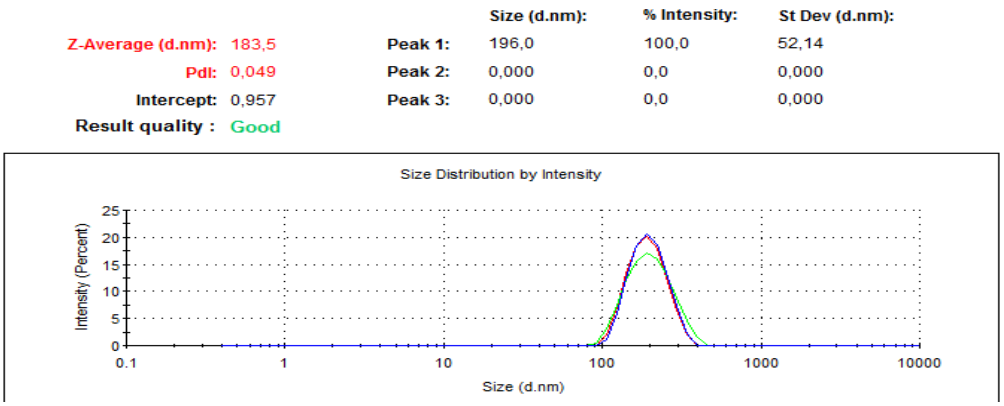


Figure 4.1. DLS result of liposome

A value greater than 200 nm meant that the polycarbonate membrane was damaged and its pores were torn. The reasons for this situation may be the excessive adjustment of the longer pump speed or the manual pumping of the extrusion step.

The sizes of liposomes obtained in the first step were also measured and found that they formed multi-lamellar vesicles because the sizes were very large. This multilayered vesicles (MLVs) were transformed into unilamellar vesicles (LUVs) with 11 times passing through the extrusion step as determined by the optimum passing number.

4.2. Calibration of Doxorubicin-HCl

The calibration curve was prepared to measure the amount of the DOX solution prepared in any concentration encapsulated in the liposome. All loaded amounts were calculated with the inclined slope and prepared a table. For this, a stock DOX solution at a concentration of 1 mg/ml was prepared and diluted to obtain different concentrations. The absorbance and fluorescence values at 480 nm were then recorded and a slope graph was generated. According to this result, the absorbance values increased almost linearly as the DOX concentration increased as seen in Figure 4.2. 590 nm emission and 480 nm excitation values were used to calculate the fluorescence values.

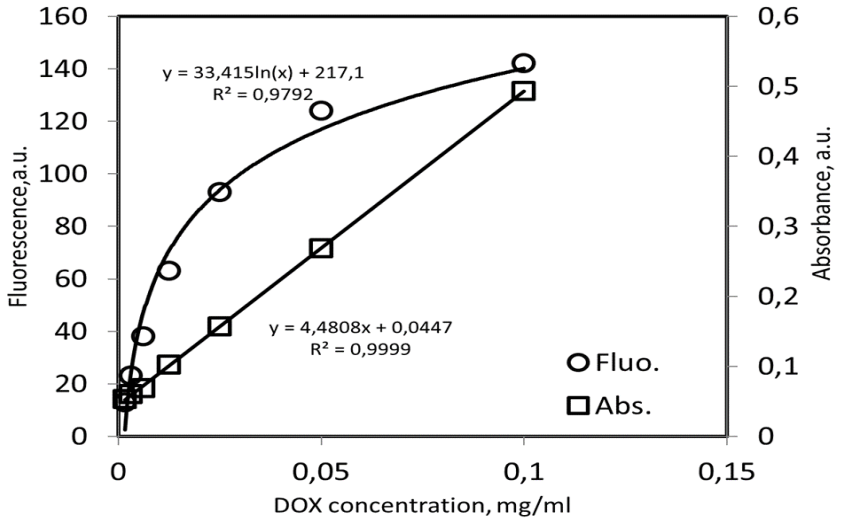


Figure 4.2. Absorbance and fluorescence calibration curve of DOX
(Source: Coşkun, S., 2017)

The main goal in measuring the absorbance and fluorescence values of DOX at these different concentrations was to obtain a linear curve.

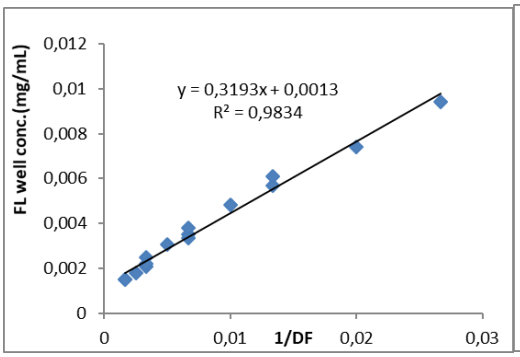
Diluted to DOX until a slope was obtained as shown in the figure. DOX calibration curve was used to determine the amount of DOX loaded in the liposome. For this purpose, a calibration graph was obtained by recording the fluorescence values of DOX solutions at different concentrations. The slope here was used to calculate loaded DOX in the samples.

4.3. Encapsulation Efficiency (EE%)

The following formula, Eq (2), is used to calculate the percentage of DOX loaded into the liposomes. For this purpose, spectrophotometric measurements were made twice before and after dialysis as shown in Figure 4.3. The slope gives DOX concentration. Over 90% loading was achieved in the studies.

$$\text{Encapsulation efficiency (EE)\%} = \frac{weight\ of\ actual\ drug\ content}{weight\ of\ theoretical\ drug\ concent} \qquad (2)$$

before dialysis



after dialysis

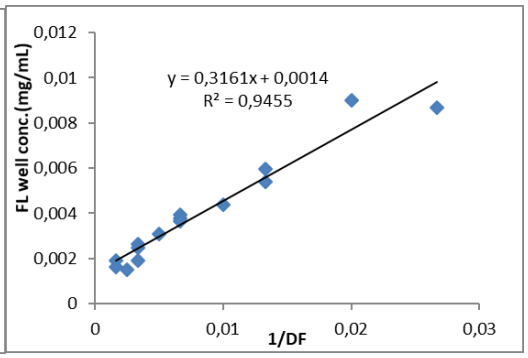


Figure 4.3. Amount of DOX loaded into liposomes before and after dialysis

4.4. DOX Scan at Different Alkaline Solutions

Doxorubicin is a popular chemotherapeutic drug that uses anthracycline antibiotic derivative to treat many types of cancer, such as breast, prostate, and stomach. One of the reasons why it is popular is that it has its own specific fluorescence. When excited with laser, it gives an emission at 595 nm against an excitation signal at 480 nm (Shah et al., 2017).

It was aimed to see whether these basic solutions would affect the amount of DOX fluorescence since higher pH solutions were used in studies. For this purpose, a stock DOX solution was prepared (600 µl) using ultra pure water and powdered doxorubicin-hydrochloride, with a concentration of 1 mg/ml. Solutions of 5 different pHs were prepared with NH_4Cl/NaOH solutions. These pH values were between 6.96 and 9.38. And then 4 ml of these different alkaline solutions were mixed with 10 µl of DOX. Then, 3 ml of this mixture was taken and fluorescence scan measurements were performed with fluorescence spectrophotometer.

As shown in Figure 4.4 and Figure 4.5, different pHs did not affect the DOX fluorescence.

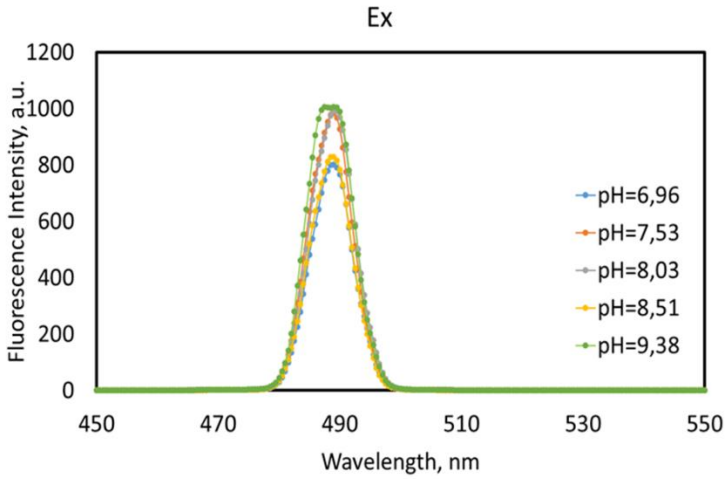


Figure 4.4. Excitation values of DOX at different pH solutions

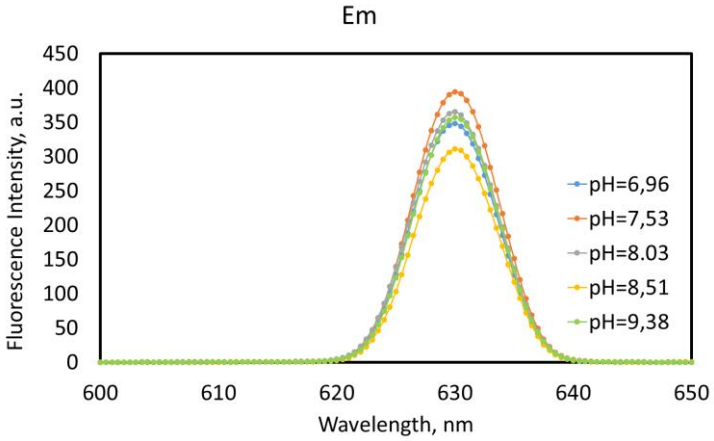


Figure 4.5. Emission values of DOX at different pH solutions

As shown in the figures, doxorubicin gave an emission at 488 nm and an excitation peak at 630 nm. The effect of different pHs on these values was negligible. Given the emission and excitation peaks that free DOX normally gives, the emission value is slightly higher than normal (630 nm), while the excitation value (480 nm) is almost the same.

This was associated with the pKa values of DOX. Because two of the three DOX pKa values are higher than all these pHs, one of the pKa values (8.15) is lower than the two pH values used (8.51 and 9.38). And it was observed that the fluorescence value of DOX is not affected by pH.

4.5. Effect of Tris on DOX Loading into DSPC Liposomes

In this study, the powder DOX-HCl was dissolved in tris solutions to prepare DOX solution. The prepared stock tris pH was measured as 9.90. HCl was used to obtain 4 different pHs from this stock and tris solutions with 4 different pH were obtained. 4 different DOX solutions were obtained with 1 mg/ml concentration with tris solutions with different pH. As shown in Figure 4.6, except for the solution with physiological pH (pH = 7.2), DOX particles were observed to form precipitate in the tris buffer in solutions. However, after a period of vortex mixing, the precipitates disappeared. In most studies, tris was used as the buffer to stabilize the pH of the liposomes in storage conditions. It is also known that tris has a much higher ionic strength than many buffers (Kimoto et al., 2017). However, it was not found in the literature that the pH was adjusted with HCl and mixed with DOX. In addition, some studies have shown that tris increases the release of doxorubicin through liposomes. In other words, tris is a basic buffer for the release of DOX from liposomes.

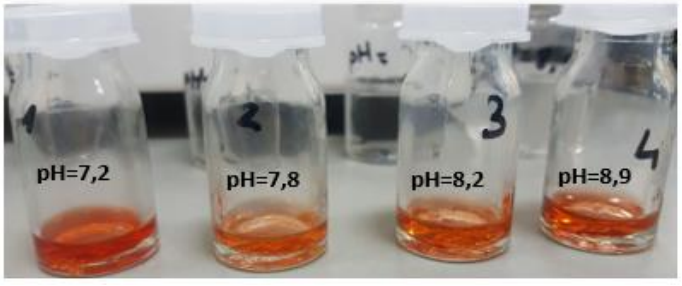


Figure 4.6. Before incubation preparation DOX solutions with different pH values

The pH of the tris solution is 7.8 at room temperature homogenous lipoDOX were obtained. These lipoDOX had different pHs as shown in Figure 4.7. After incubation, precipitates disappeared and a homogeneous mixture was obtained. After the DOX loading into liposomes, the amount of DOX loaded into liposomes was determined based on the previously obtained DOX calibration curve. For this purpose, 96 well plates were used.

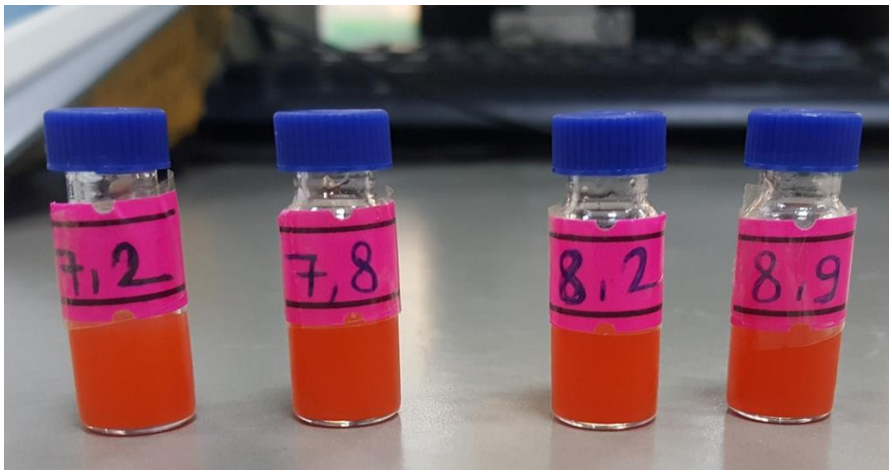


Figure 4.7. LipoDOX samples after incubation with different pHs

As shown in Figure 4.8, measurements were performed for the loaded DOX amounts. Dilution was performed if fluorescence values were higher than 80. The total volume in each well was adjusted to 150 µl. 4 wells were processed for each sample.

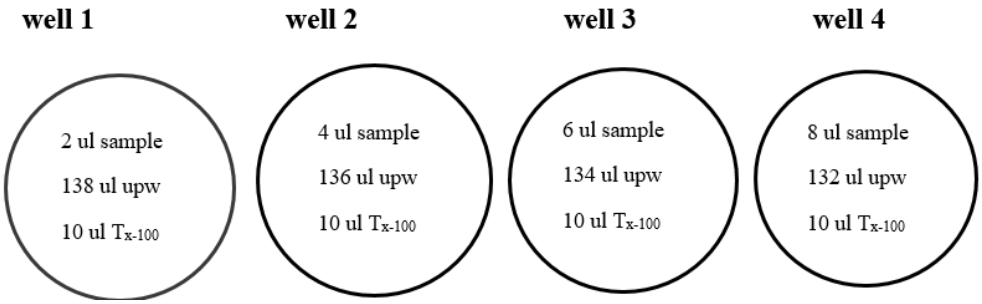


Figure 4.8. Representation of amount of liquids for spectrophotometer measurement

Before loading, the concentration of DOX was 0.5 mg/ml and after the loading, the DOX concentrations were estimated and given in Figure 4.9. As shown in the figure, a higher amount of DOX could be loaded into liposomes in tris buffer. This was

encouraged us if there is any tris effect on DOX loading. In order to test any tris effect, different concentrations of tris buffer were prepared and DOX was loaded into liposomes.

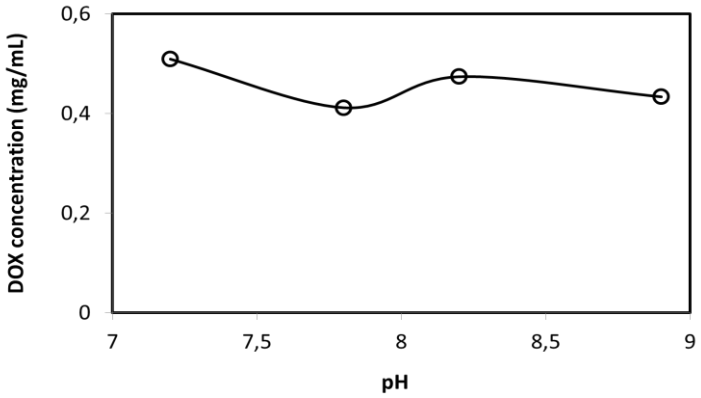


Figure 4.9. Loaded DOX concentrations in tris buffer at different pH values

According to this result, the loading efficiency of DOX dissolved in high pH tris in liposomes is around 80%. At the pH between 7.2 and 8.9, the difference between the loading amounts were negligible. According to the result, a high loading rate was observed even when the pH was about 9. So the question arises: "Does tris have an effect on DOX loading? "

4.6. DOX Loading to Liposomes with Different Tris Concentrations

In this study, only the effect of tris on the loading of DOX on liposomes at the same pH was examined. The same amount of DOX solution was added to liposomes containing different tris concentrations as shown in Figure 4.10. LipoDOX samples were obtained.

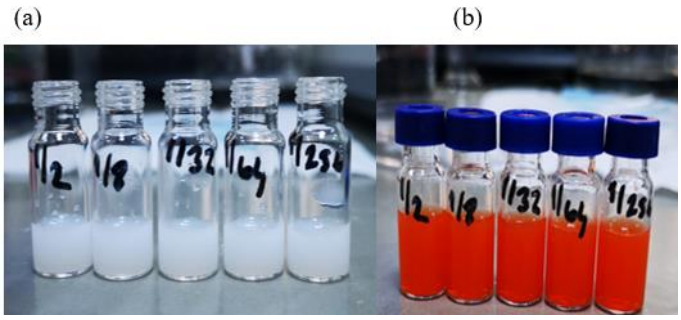


Figure 4.10. (a) Liposomes with different tris concentrations (b) LipoDOX samples before incubation

As shown in Figure 4.11, a significant trend was not obtained to confirm the tris effect hypothesis.

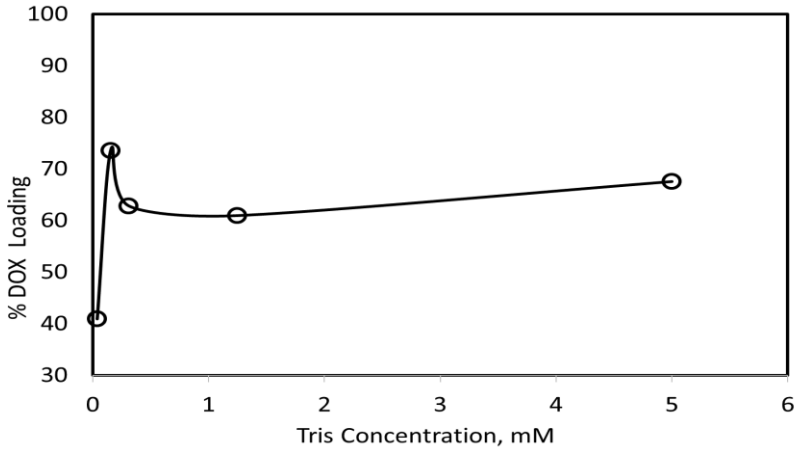


Figure 4.11. Efficiency of DOX loading according to different tris concentrations

4.7. Effect of Ammonium Chloride on DOX Loading into DSPC Liposomes

Firstly, solutions having many different concentrations was prepared and pH measurements were performed using ammonium chloride and sodium hydroxide to obtain a calibration graph. A pH calibration chart was obtained in the LipoDOX assay with NH_4Cl and NaOH as shown in Figure 4.12. This graph was used on further studies for the amount of each component needed to obtain the desired pHs.

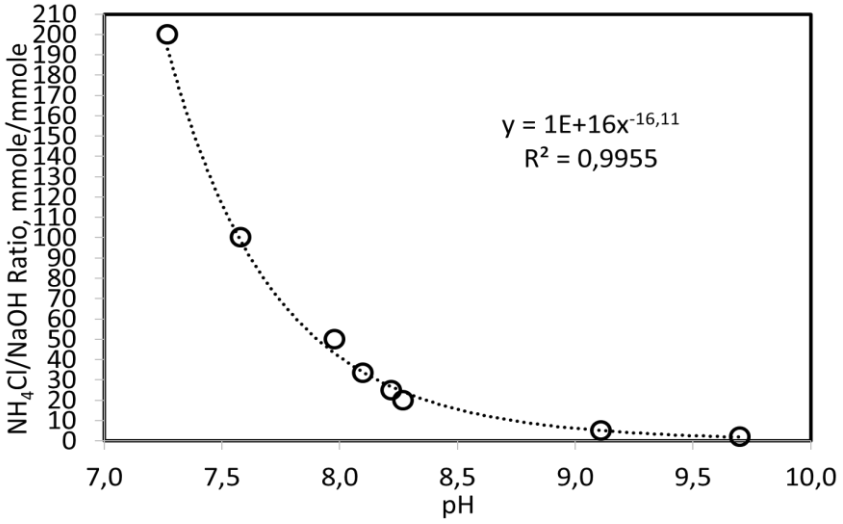


Figure 4.12. pH calibration chart from NH_4Cl and NaOH

In later studies, this graph was used to obtain the desired solution. In this way, the desired pH was obtained by using only ammonium chloride and sodium hydroxide without acid. This saves time and workload.

Then, at the desired pH value of 5 (with a high degree of basicity) solutions were prepared according to this data. In this study, 500 µl of liposome, 500 µl of DOX (1 mg/ml) solution and 200 µl of prepared pH solutions were used, with a total volume of 1200 µl for each sample as shown in Figure 4.13. In this case, the concentration of DOX before loading was 0.416 mg/ml.

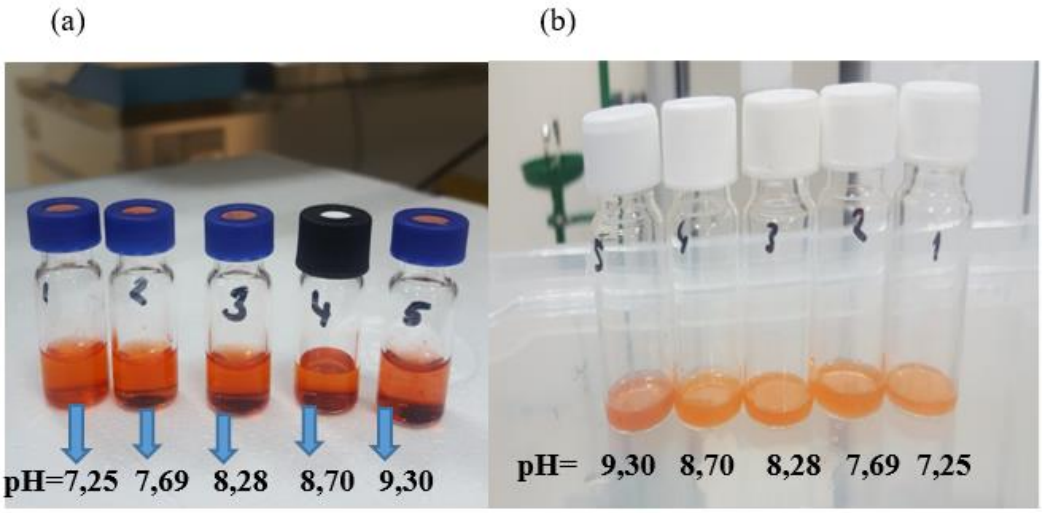


Figure 4.13. (a) DOX stocks with different pH solutions (b) Combining DOX solutions and liposomes prepared at different pHs before incubation

This situation was in parallel with the literature (Abraham et al., 2005). According to this information, the DOX solution changes from physiological pH to alkaline pH and turns from orange to a slight purple.

DOX was loaded at different pHs in NH_4Cl/NaOH solutions and the results was shown in Figure 4.14, the maximum load was seen at pH 7.69 and decreased as pH increased. Even though the rate of increase in pH decreased, the total amount of drug loaded was quite high.

It was observed that the loading decreased as the pH increased using ammonium chloride and the maximum loading was obtained with a pH of 7.69. The reason for this was that the ammonia (NH_3) concentration is higher outside as pH increases. This causes a net NH_3 exchange from liposomes and caused lower DOX loading at higher pHs.

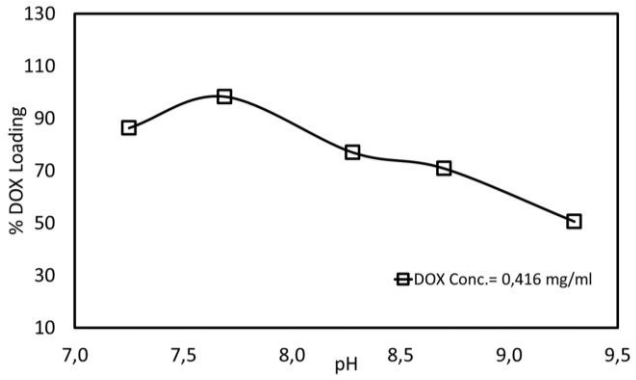


Figure 4.14. DOX Loading percentage with alkaline solutions (NH_4Cl and NaOH)

4.8. Effect of High pH on Liposome Stability

A study was performed to determine the stability of liposomes at higher pHs that were not loaded with doxorubicin with time. For this, 3 basic solutions were prepared according to the previously obtained calibration chart. Lipid films prepared earlier were stored at -20 °C and used to prepare DSPC liposomes. From the resulting liposome stock, 500 µl were added to 3 different vials and 500 µl was added to each vial with a different pH solution. Added to the DLS cuvette from each sample. The first two days two measurements were made at mornings and evenings. The mouths of the tubs were closed with parafilm and stored at +4 °C for the next measurement. 900 µl of ultra pure water and 100 µl of sample were used for the measurement. The pHs obtained were 8.44, 8.69 and 9.15. After 2nd day, each measurements were done once a day. The size change of liposome in alkaline environments was recorded for 63 days. The stability of liposomes were shown in Figure 4.15. As seen in the graph, liposomes in higher pHs are stable up to 2 months in size.

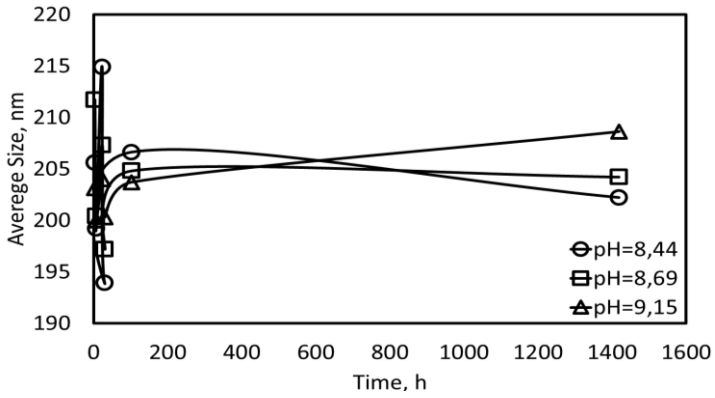


Figure 4.15. Liposome size stability in different pH solutions against time

4.9. Release of DOX from DSPC Liposomes at Higher pHs

The release of DOX from lipoDOX with 3 different pHs were examined. The absorbance and fluorescence values was recorded and the Triton X-100 was added at 130 min. The release rates were calculated according to the fluorescence values using Eq (1). As shown in Figure 4.16, the liposomes were very rigid and did not release the DOX into the medium at room temperature until Triton X-100 was added.

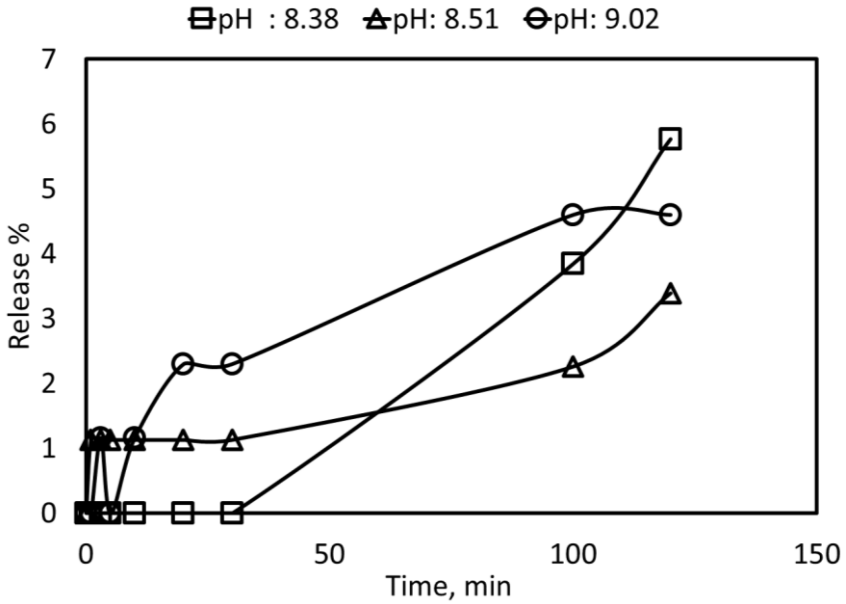


Figure 4.16. DOX release-pH relation

4.10. Release from Alkaline DSPC Liposomes

As shown in Figure 4.17, in a single vial, the pH of only 10 ml of 0.9% NaCl solution was measured in a continuous mode. Then, 10-30-50 and 100 µl of alkaline liposome containing 250 mM Na_2CO_3 (pH= 11.73) were added subsequently into the same vial at certain time interval. Before completion of the experiment, 70 µl of 20% $T_{x\text{-}100}$ was added to allow the liposomes to disrupt and release its contents. It was observed that pH slightly increased with the amount of alkali liposome. This indicated that liposomes are rigid and did not release their contents. After disrupting the liposomes, a sudden increase in pH indicated that liposomes contained alkaline solution.

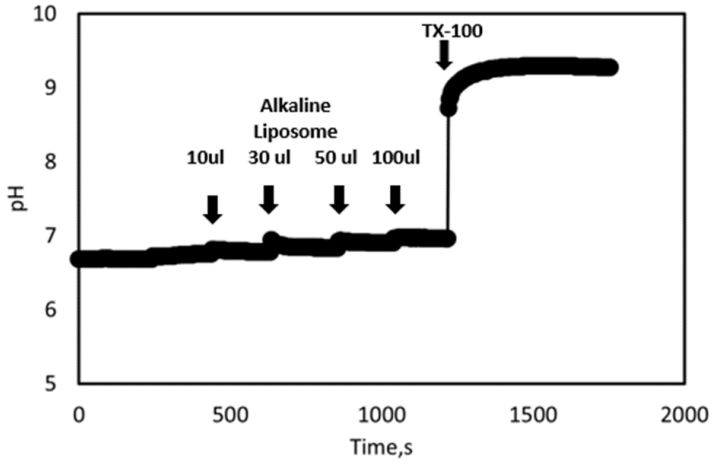


Figure 4.17. pH change by adding different amounts of alkaline liposomes

A similar study was performed as shown in Figure 4.18, but different vials were used for each quantity of sample. 10 ml of 0.9% NaCl was added to each vial. For the first vial, after only the pH of the saline solution was continuously measured for the first 5 minutes, 10 µl of alkaline liposomes were added. After 300 seconds, 70 µl of $T_{x\text{-}100}$ was added. The same procedure was repeated for 30-50-100 and 150 µl of alkaline liposome. As shown in the figure, when liposomes were added negligible pH changes were seen. When $T_{x\text{-}100}$ was added a sudden increase in pH were imminent indicating that liposomes contained alkaline solution and they are rigid. It can also be noted that the increase in pH is liposome amount dependent, that is, when liposome content increased, pH change also increased, releasing higher alkaline solution content.

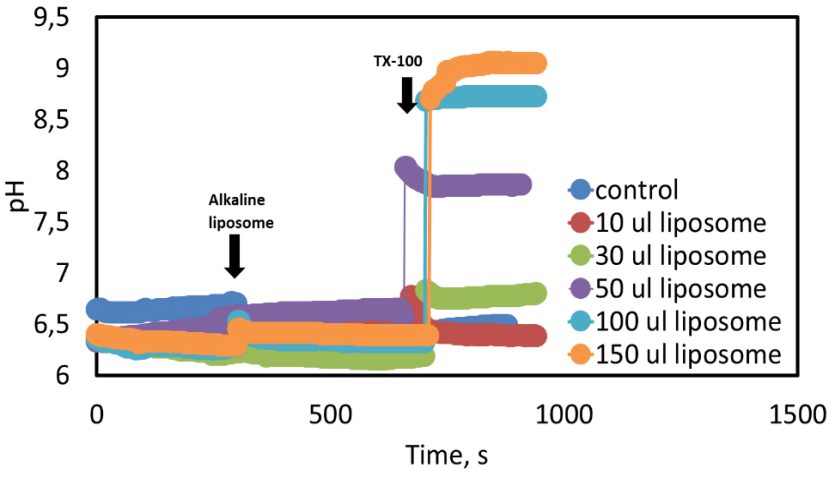


Figure 4.18. pH solution release from alkaline liposomes at different concentration

4.11. Ultrasound Effect on Release

Effect of ultrasound on release from alkaline liposomes were studied. A 2 MHz probe was used in measurements. pH was measured in case of many release. Initially 0.9% NaCl solution was added into a 30 ml cuvette and base pH was obtained. Then, 30 µl, 50 µl and 100 µl of alkaline liposomes were added while ultrasound intensity was applied at 2 MHz using GamPI ultrasound apparatus. As shown in Figure 4.19, no release was observed from liposomes. When $T_{x\text{-}100}$ was added, a sudden increase in pH was observed indicating that liposomes contained alkaline solution and they are highly rigid.

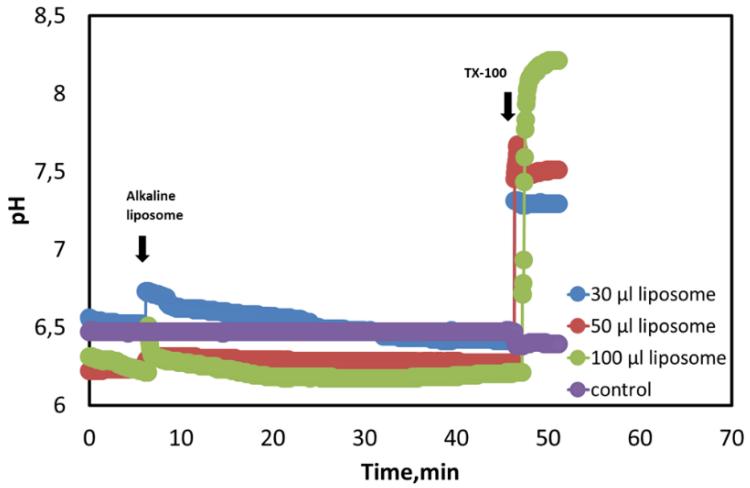


Figure 4.19. Ultrasound effect on alkaline liposomes at different concentrations

Release studies with DSPC liposomes showed that DSPC liposomes are very rigid. Therefore, we decided that use DPPC liposomes with lower phase transition temperature of 41 °C.

4.12. Release of Alkali Solution from DPPC Liposomes with Temperature

DPPC liposomes containing Na_2CO_3 were prepared and tested for their release with temperature. DPPC lipid has a phase transition temperature of 41°C and it was expected that the bilayer structure of liposomes would transform from solid phase at low temperatures and liquid phase at higher temperatures causing release from liposomes. For the study, a 10 ml of 0.9% NaCl was added to 20 ml vial and put into the shaking water

bath adjusted previously to the desired temperature. After equilibration, 100 µl of DPPC alkaline liposome (Na_2CO_3) were added.

After 20 minutes, the vial was removed from the water bath and its pH was measured. The same procedure was performed for 4 temperatures of 25-35-42 and 49 °C. Control experiments without containing liposomes were also applied. Figure 4.20 shows the pH readings at different temperatures indicating that there is no release at 25 °C for which DPPC liposomes are rigid. When temperature increased to 37 °C and higher, pH increased significantly indicating that release from DPPC liposomes is remarkable. The DPPC liposomes containing alkaline solution released the alkaline solution with increasing temperature and this was observed as pH change.

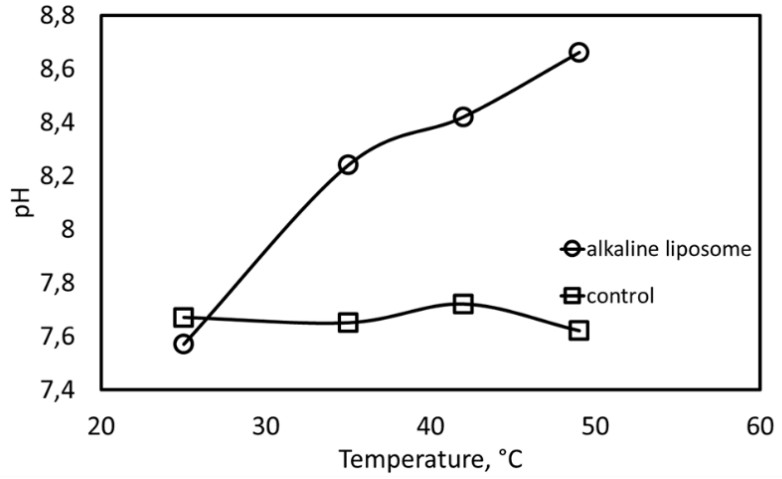


Figure 4.20. pH change in alkali liposomes with DPPC according to different temperatures

4.13. DOX Release from DPPC Liposomes

Release of DOX from DPPC liposomes were studied at 42 °C and 50 °C, which are higher than the phase transition temperature of 41 °C for DPPC. pHs of external medium were adjusted using $Na_2CO_3/NaHCO_3$ buffer. For this purpose, sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) were mixed in different proportions. The pHs of the mixtures are shown in Table 4.1.

Solutions were prepared for the desired pH values. After temperature equilibration, DPPC liposomes containing DOX was added to each vials. Samples were taken from the vials and measured fluorescence intensities.

Table 4.1. pH adjustment with sodium carbonate and sodium bicarbonate

	ml				
Na_2CO_3	15	12	7,5	3	0
$NaHCO_3$	0	3	7,5	12	15
pH	11	10,70	10,21	9,67	8,56

Release rate was calculated using Eq (1). As shown in Figure 4.21, no significant DOX release was observed at 42 °C and 50 °C at different pH. We concluded that DOX could not be released from DPPC liposomes at either higher temperatures or higher pHs.

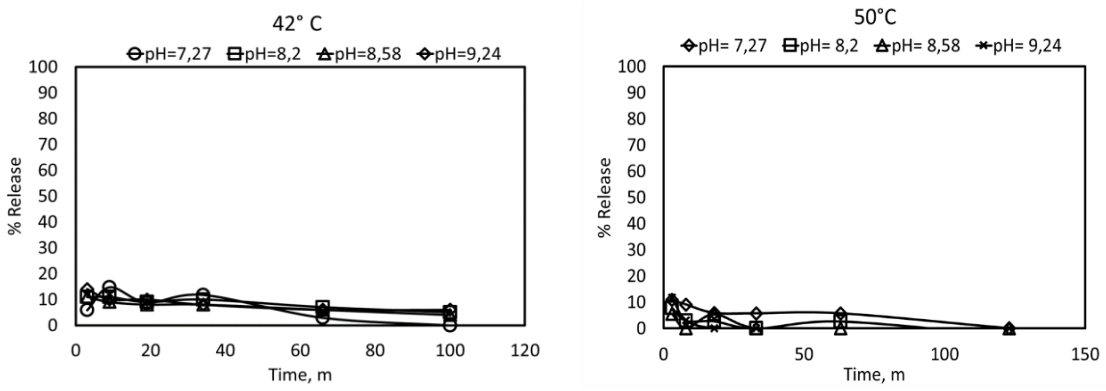


Figure 4.21. The effect of mixing sodium carbonate and sodium bicarbonat on release

4.14. DOX Release from DPPC Liposomes in NH_4Cl/NaOH Solution

Release of DOX from DPPC liposomes were studied in NH_4Cl/NaOH solutions at different pH and temperatures. Desired pH solutions of NH_4Cl/NaOH were prepared by mixing NH_4Cl solution and NaOH solution at raitos previously determined from its calibration curve. A total of 16 vials were prepared for 4 different temperatures and 4 different pH values. 4 vials were used for each temperature. 5 ml of pH solutions were added to each vial. The vials were inserted into constant temperature water bath. After thermal equilibrium 50 µl of lipoDOX (DPPC) were added to each vial. Samples were taken at certain time interval to measure fluorescence intensities. At the end of the release study, a 10 µl of $T_{x\text{-}100}$ (20%) was added in order to disrupt liposomes and determine the total DOX contentration. Thus, the percentages of DOX release were calculated. 150 µl

of the mixtures were taken and fluorescence values were measured with 96 well plate at certain time intervals. Figure 4.22 shows DOX release from DPPC liposomes in NH_4Cl/NaOH solution at different pH and different temperatures.

As shown in the figure, when pH is lower than tha pKa value of DOX which is 8.15, release of DOX increased as temperature increased. At higher pHs almost 80% of DOX was released at temperatures higer than 37 °C. There is no release at temperature of 25 °C.

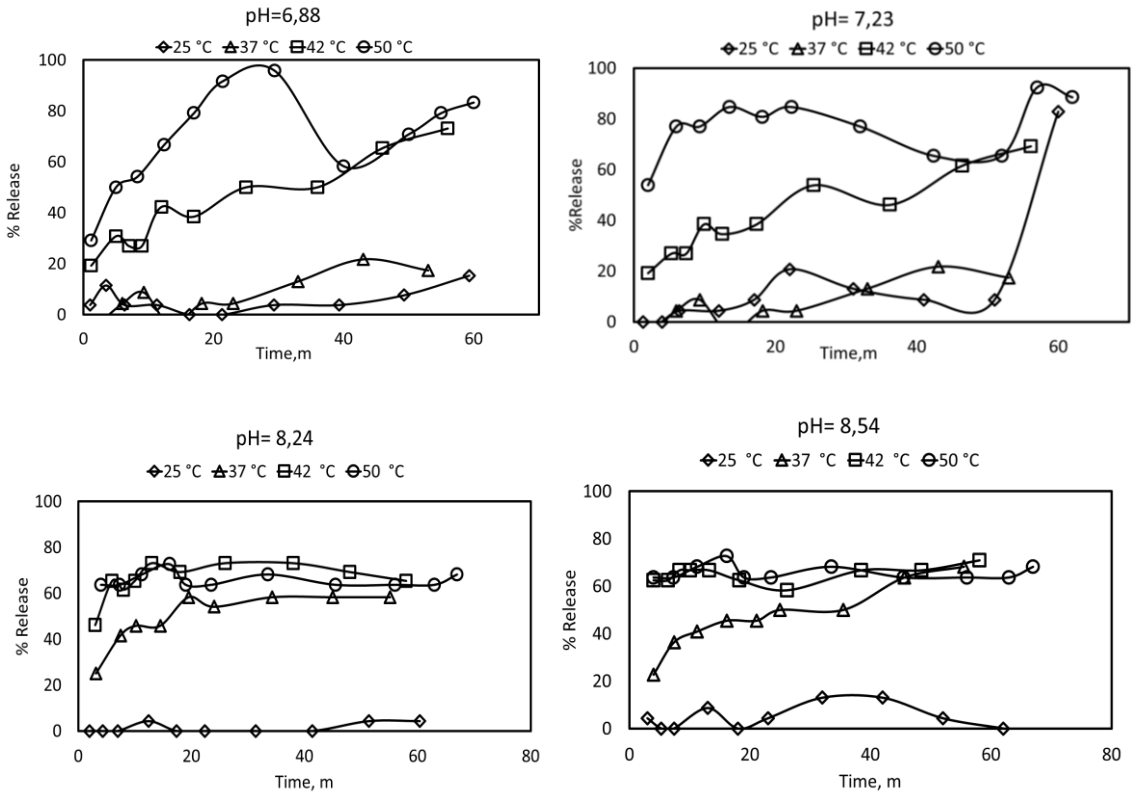


Figure 4.22. Release results according to different temperatures at the same pH

The DOX release can be seen in Figure 4.23 clearly at different temperature and pHs. As shown in the figure, there is no DOX release at 25 °C irrespective to any pHs indicating that DPPC liposomes are highly rigid at temperature lower than phase transition temperature of 41 °C. At body temperature of 37 °C, DOX release is pH dependent, that is, almost negligible release was observed at pHs lower than pKa value of DOX, 8.15, and higher DOX release were observed at higher pH values. Similar trend was seen at 42 °C irrespective of pHs. At 50 °C which is higher than the phase transition temperature of 41 °C for DPPC, almost 80-100% of DOX released. It seems that is an

ammonia effect on DOX release from DPPC liposomes. To ensure this, it was decided to investigate the effect of different concentrations of ammonium on release.

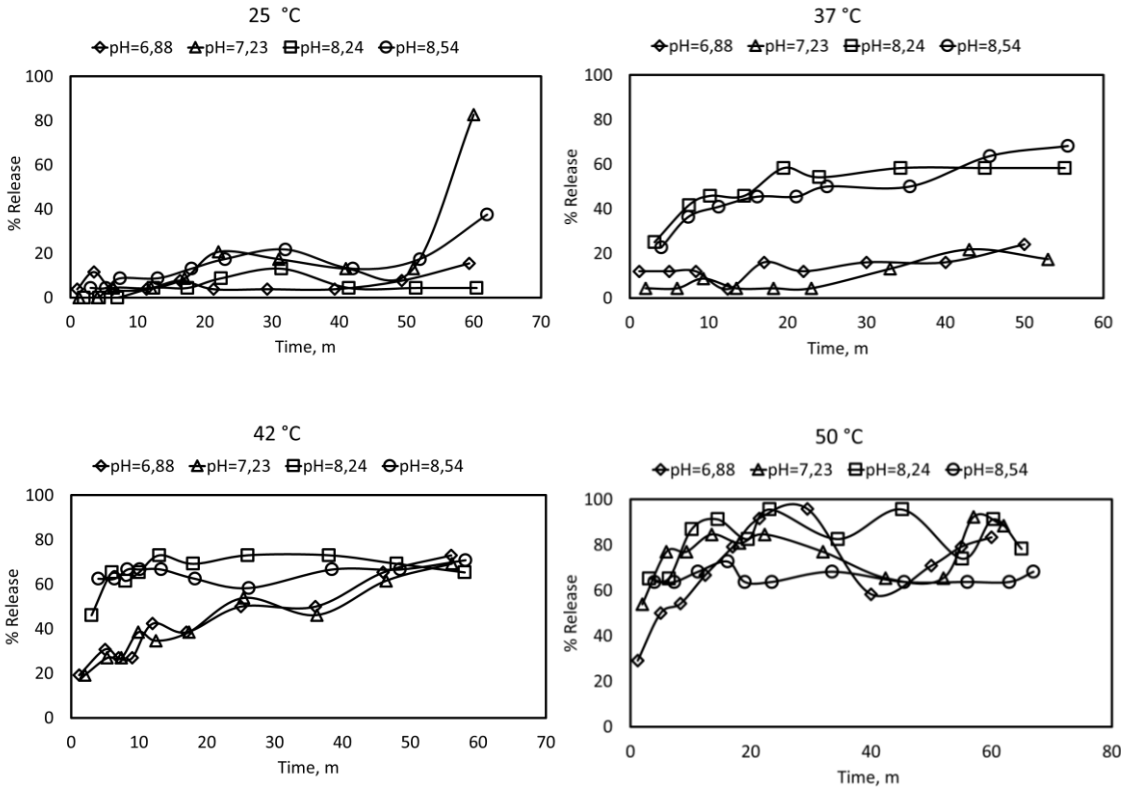


Figure 4.23. Release results according to different pH values at the same temperatures

4.15. Effect of NH_4Cl Concentration on DOX Release

A stock solution with a pH of about 9 was prepared using 50 mM of NH_4Cl and 50 mM of NaOH stock solutions. This stock solution was diluted with 1/2 dilution factor using distilled water to 6 vials. 960 µl of solution was taken from each vial and placed into 1.5 ml vials. The vials were placed in a shaking water bath at 42 °C. 40 µl of DPPC lipoDOX was added to each vials. 150 µl of samples were taken from each vials and measured fluorescence intensity in a 96 well plate. DOX release percentages were calculated using Eq (1) according to fluorescence values. Because the pH decreased for subsequent dilitions, an additional experiment was conducted by adjusting the pH to initial value.

Figure 4.24 shows the DOX release percentages at different NH_4Cl/NaOH concentrations. It is clear that DOX release rate is NH_4Cl dependent. There is no DOX

release during the absence of NH_4Cl, and release rate increased as NH_4Cl concentration increased. Also it was seen that release rate is pH dependent. Release rate is higher when pH is higher for each NH_4Cl concentrations. We think that the pH effect shows ionic sperciation of NH_4Cl as shown in Eq (3).

$$NH_4Cl \longrightarrow NH_4^+ + Cl^- \qquad \text{Eq (3)}$$

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \quad pK_a = 9.3$$

Here we understood that DOX release is depended on NH_4Cl concentration and pH. That is, the neutral form of NH_3 is the main component in DOX release from DPPC liposomes.

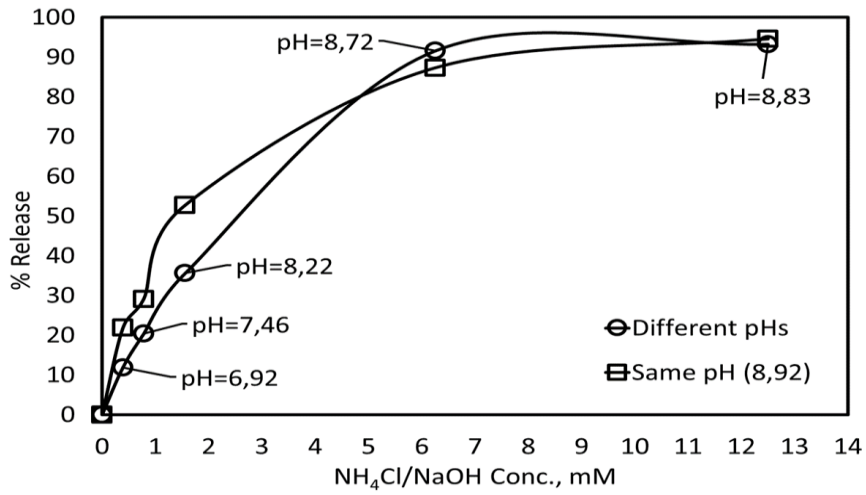


Figure 4.24. Effect of NH_4Cl concentration on DOX release according to different and same pH values

4.16 Comparison of NH_4Cl and Na_2CO_3 Effect on Release

Effects of NH_4Cl and Na_2CO_3 buffers on DOX release from DPPC liposomes were compared at 42 °C as shown in Figure 4.25.

As shown in the figure, almost no or negligible DOX release was seen in the presence of Na_2CO_3 solution and significant release was seen in the presence of NH_4Cl solution. It was understood that NH_3 is required in order to release DOX from DPPC liposomes.

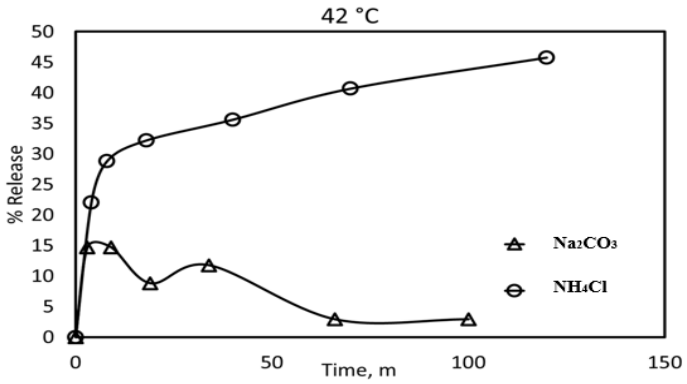


Figure 4.25. Comparison of NH_4Cl and Na_2CO_3 effect on release

4.17. A Two-Component System for DOX Release

In this study, a new pH chart was obtained using ammonium sulfate and NaOH as shown in Figure 4.26. With this graph, a solution of pH 8.97 was prepared. Alkali liposome was obtained using this solution and DPPC film. It was shown that NH_3 is required to release DOX from liposomes. Here, we designed a two-component liposomal system, one contained NH_3 and the other contained DOX. When the two liposomes were combined, release of NH_3 was expected to enhance DOX release from lipoDOXes containing DOX. In order to increase the NH_3 amount, $(NH_4)_2SO_4$ was used instead of NH_4Cl since $(NH_4)_2SO_4$ has 2 moles of NH_3 compared to 1 mol of NH_3 in NH_4Cl. Equal mole of $(NH_4)_2SO_4$ and NaOH solutions were mixed at different ratios and pH of the mixtures were recorded. Figure 4.26 shows pH of solutions with respect to $(NH_4)_2SO_4$/NaOH mole ratios. For a desired pH value, the indicated $(NH_4)_2SO_4$/NaOH molar ratio were mixed from this calibration curve.

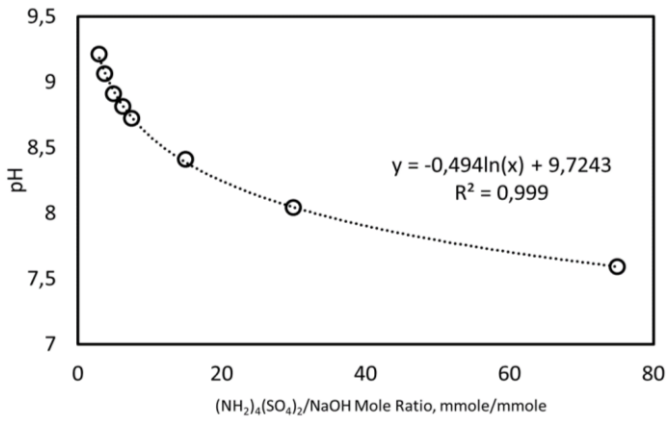


Figure 4.26. pH adjustment chart using $(NH_4)_2SO_4$ and NaOH

In order to test our hypothesis, DPPC liposomes containing $(NH_4)_2SO_4$/NaOH were prepared at pH 8.97. Another DPPC liposomes were also produced containing DOX. In a 0.9% NaCl solution, containing lipoDOX, different amount of alkaline liposomes were added and DOX release were measured with time. Figure 4.27 shows the effect of alkaline liposomes on DOX release for two-component system. As shown in the figure, DOX release from liposomes increased as the amount of alkaline liposome increased. It is most likely that ammonia (NH_3) is released from alkaline liposomes at temperature higher than its phase transition temperature and enhances the DOX release from DOX-loaded DPPC liposomes.

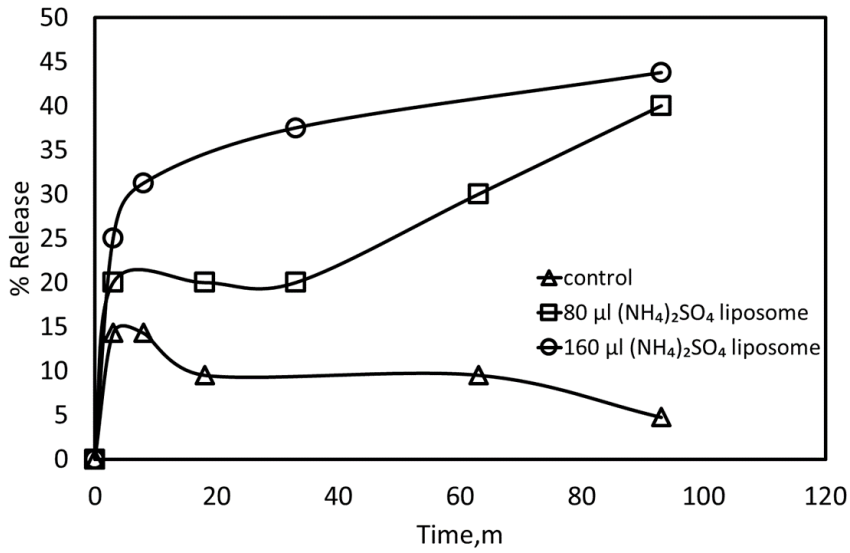


Figure 4.27. Effect of alkaline liposome concentration on DOX release

CHAPTER 5

CONCLUSIONS

The doxorubicin used as chemotherapeutic agent in many cancer treatments was encapsulated into liposomes. However, liposomes were found to be rigid and did not release their concents at room temperature. In order to enhance release from liposomes, various approaches such as pH of external liquid, temperature and ultrasound were applied. It was shown that small neutral molecules such as ammonia (NH_3) are needed to release DOX from liposomes. Higher pH is needed to convert NH_4^+ to NH_3 and enhance DOX release. Temperatures higher than the phase transition temperature of lipids are needed to enhance DOX release. Loading of DOX into liposomes were studied in alkali solutions including tris and ammonium chloride. Normally in non-alkali liposomes, an encapsulation efficiency of 90% was achieved. In tris buffer, the loading efficiency was achieved at about 85%. In alkaline solution prepared with a mixture of ammonium chloride and sodium hydroxide, the loading efficiency was found to be approximately 70%. Liposomes were found to be stable since there was no change in size of up to 2 months in high pH environments. These findings can be used in future studies to develop a more efficient drug delivery system.

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