MENADIONE (VITAMIN K3) AND DOXORUBICIN LOADED LIPOSOMES FOR ENHANCED ANTICANCER EFFECT BY DUAL TREATMENT

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ABSTRACT

MENADIONE (VITAMIN K3) AND DOXORUBICIN LOADED LIPOSOMES FOR ENHANCED ANTICANCER EFFECT BY DUAL TREATMENT

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Chemotherapy is the first line of cancer treatment, but due to its adverse effects, it is set to be limited. Therefore, liposomes are mainly extensively studied to be employed as nanocarriers as they are more advantageous than traditional therapy. They overcome the obstacle of cellular and tissue uptake and improve drugs' bioavailability, biodistribution and pharmacokinetics. Doxorubicin, an anthracycline drug, is widely used to treat hematological malignancies and solid tumors, to ameliorate its usage a liposomal formulation DOXIL, is commercially available and used in clinical settings. Vitamin K3, also known as menadione, have shown to possess anticancer effects through the production of reactive oxygen species (ROS) and mitochondrial damage. To enhance the anticancer effect of both agents, liposomal formulations of the drugs were synthesized. Both drugs are hydrophilic, they were encapsulated within the core of PEGylated liposomes to improve their anticancer effects. Doxorubicin was loaded within liposomes using ammonium sulfate method, achieving a high encapsulation efficiency (96.2 ±1.1 %), vitamin K3 was loaded passively by dissolving it in the hydrating solution its encapsulation efficiency was around 53.81±14 %. Liposomal formulations produced were ranging

between the sizes 128-150 nm. *In vitro* release profiles of the liposomal formulations showed controlled release of the drugs the release of DOX was faster than that of the VitK3. In vitro cell cytotoxicity was done to evaluate the potency of both drugs on human breast cancer cells (MCF-7) and mouse fibroblasts (L929), firstly free drugs were administered alone and then after that in a dual manner. DOX IC50 on MCF-7 cells was lower than that for L929 cells, when Vitamin K3 was administered with doxorubicin, it inhibited the cytotoxicity of doxorubicin, therefore coloaded liposomes were not produced. For the anticancer enhancement cells were pretreated with vitamin K3 for 72 hours prior the treatment with doxorubicin for 24 hours there was no effect on the cytotoxicity of the drugs. Pretreatment was also done with liposomal vitamin K3 for 3 days followed by liposomal DOX for another 3 days, the results were unanticipated, and it showed a decrease in the cytotoxicity. As the liposomal formulations did not perform as expected, future works might be paved for trial of simultaneous administration of the liposomal drug formulations.

Keywords: Liposomes, Vitamin K3, Doxorubicin, Anticancer effect, Breast cancer

ÖZ

İKİLİ TEDAVİ İLE ANTİKANSER ETKİSİYİ ARTIRMAK İÇİN MENADİONE (K3 VİTAMİNİ) VE DOKSORUBİSİN YÜKLÜ LİPOZOMLAR

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Kemoterapi, kanser tedavisinin ilk basamağıdır, ancak yan etkilerinden dolayı sınırlamaları vardır. Bu sorunun üstesinden gelmek için lipozomlar, geleneksel terapiden daha avantajlı oldukları için nanotaşıyıcı olarak kullanılmak üzere kapsamlı bir şekilde araştırılmaktadır. Lipozomlar hücresel ve doku alımı engelinin üstesinden gelirler ve ilaçların biyoyararlanımını, biyodağılımını ve farmakokinetiğini geliştirirler. Bir antrasiklin ilacı olan doksorubisin, hematolojik maligniteleri ve katı tümörleri tedavi etmek için yaygın olarak kullanılır. Kullanımını iyileştirmek için bir lipozomal formülasyon olan DOXIL, ticari olarak temin edilebilir ve klinik ortamlarda kullanılabilmektedir. Menadion olarak da bilinen K3 vitamininin, reaktif oksijen türlerinin (ROS) üretimi ve mitokondriyal hasar yoluyla antikanser etkilere sahip olduğu gösterilmiştir. Bu çalışmada her iki ajanın antikanser etkisini arttırmak için ilaçların lipozomal formülasyonları sentezlendi. Her iki ilaç da hidrofiliktir, antikanser etkilerini iyileştirmek için PEGillenmiş lipozomların çekirdeği içinde kapsüllenmiştir. Doksorubisin, amonyum sülfat yöntemi kullanılarak lipozomlara yüklenerek yüksek bir kapsülleme etkinliği elde edildi (%96,2 ±1,1 %), K3 vitamini hidratasyon çözeltisi içinde çözülerek pasif olarak

yüklendi, kapsülleme etkinliği yaklaşık %53,81±14'tü. Üretilen lipozomal formülasyonlar 128-150 nm boyutlarındaydı. Lipozomal formülasyonların in vitro salım profilleri, ilaçların kontrollü salımını gösterdi. Sonuçlara göre DOX salımının Vit K3'ünkinden daha hızlı olduğunu gözlemlendi. Her iki ilacın insan meme kanseri hücreleri (MCF-7) ve fare fibroblastları (L929) üzerindeki etkisini değerlendirmek için in vitro hücre sitotoksisitesi yapıldı. Önce serbest ilaçlar tek başına, ardından ikili şekilde uygulandı. MCF-7 hücreleri üzerindeki DOX IC50, L929 hücreleri için olandan daha düşüktü, Vitamin K3 doksorubisin ile birlikte uygulandığında, doksorubisinin sitotoksisitesini inhibe ettiği için birlikte yüklenmiş lipozomlar üretilmedi. Antikanser güçlendirme hücreleri için, 72 saat süreyle K3 vitamini ile ön işleme tabi tutulup daha sonra 24 saat doksorubisine maruz bırakılmasına rağmen sitotoksisitesi değerleri üzerinde herhangi bir etki görülmedi. 3 gün boyunca lipozomal vitamin K3 ile ön tedavi yapılıp ardından 3 gün daha lipozomal DOX maruz bırakılma sitotoksisitede bir azalmaya sebep oldu. Lipozomal formülasyonlar beklendiği gibi performans göstermediği için, lipozomal ilaç formülasyonlarının aynı anda uygulanmasının denenmesi için gelecekteki çalışmalar yapılabilir.

Anahtar Kelimeler: Lipozomlar, K3 Vitamini, Doksorubisin, Antikanser etkisi, Meme kanseri

To my mum, family, and the memory of dad.

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ABBREVIATIONS

DMEM: Dulbecco's Modified Eagle Medium

DDS: Drug Delivery System

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DOX: Doxorubicin

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

MLV: Multilamellar Vesicles

MSB: Menadione Sodium Bisulfite

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW: Molecular Weight

NaCl: Sodium Chloride

PBS: Phosphate Buffer Saline

PBS: Phosphate Buffer Saline

TEM: Transmission Electron Microscope

UV: Ultraviolet

EE: Encapsulation Efficiency

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

NP: Nanoparticle

EPR: Enhanced Permeability and Retention

PEG: Polyethylene Glycol

MLV: Multilamellar Vesicles

SUV: Small Unilamellar Vesicles

LUV: Large Unilamellar Vesicles

EMA: European Medicines Agency

UV-Vis: Ultraviolet Visible

VitK3: Vitamin K3

CHAPTER 1

INTRODUCTION

1.1 Drug Delivery Systems

Drug delivery systems (DDS) are formulations or devices that act as vehicles for delivering different therapeutic molecules for treatment and/or regeneration purposes in a disease or another medical situation.

These molecules can be therapeutic substances such as drugs, genes, and proteins and can also be diagnostic structures such as quantum dots and radioactive molecules for imaging (Pati et al., 2018; Xingyong Wu et al., 2003).

These systems improve the pharmacological properties of free drugs by enhancing their efficacy by controlling the molecule's release rate, time, and location. The activity of conventional drugs is hindered by their own pharmacology, as a drug must accumulate at the diseased site to a specific dosage for a medicine to be effectively functional (Figure 1). Rather than organ accumulation of drugs, systemic drug administration distributes the therapeutics evenly within the body, limiting its accumulation at the site of action (Mainardes & Silva, 2004). Throughout the pharmacokinetic journey of drugs, it passes through different

organs and cell membranes and enters within the cells, which might lead to multiple undesired effects. Primarily the administered drug might become inactivated by some organs, or the use of the drug might lower the dose reaching the aimed site. Secondly, the drug distribution all over the organs means that healthy organs are exposed to these drugs; for example, a chemotherapeutic will affect them negatively. Given these conditions, higher dosages of drugs will be required to reach the therapeutic index, leading to higher toxicity in healthy cells (Torchilin, 2000).

The main aims of drug delivery systems are to protect the drug from the host

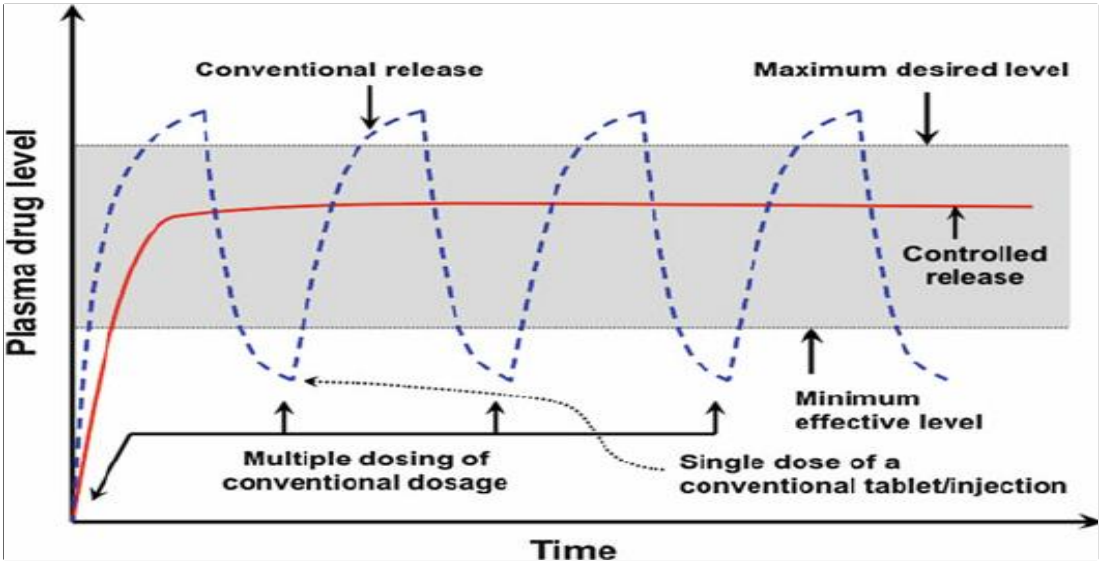

Figure 1: The desired level of drug in the plasma, and the behaviors of drug released by tradition system, single and multiple dosages (blue curve) and the plasma drug level in controlled release systems (red curve) (Huynh et al., 2020)

environment, clearance/detoxication mechanisms, and to protect the host from the drug's side effects. The former enables extending the drug's bioavailability, increasing its half-life, while to achieve the latter, the drug should accumulate more at the target site avoiding its widespread, systemic distribution (Torchilin, 2008).

For treating diseases with conventional (free administered) drugs, some of the properties of these molecules impediment their optimal therapeutic effects. Drug delivery systems alter these agents' pharmacokinetics and biodistributions, improving the pharmacological characteristics of direct administration. Free drugs have poor solubilities, affecting their bioavailability and the usage of the suitable

solvent is usually toxic, which lowers the molecule's efficiency, leading to higher toxicity. Nanoparticles with lipid basis, such as liposomes, have the capacity to encapsulate hydrophobic and lipophilic drugs ameliorating the drug's poor solubility (Mohammadabadi & Mozafari, 2018). Unlike DDS, the distribution of free drugs is not controlled; in return, this leads to the extravasation of the medications into surrounding tissues leading to damage and even necrosis. The usage of DDS regulates drug release and eradicates accidental tissue extravasation. When drugs are administered freely, they face *in vivo* degradation and might lose their activity, but encapsulating them within a carrier would protect them from rapid breakdown, which means less dosage of the therapeutic agent is needed, which lowers the toxicity to healthy tissues. Healthy tissues are also affected by the poor biodistribution of conventional therapeutics, as they are administered freely in the blood where they circulate in the body, affecting all tissues, diseased and healthy. This biodistribution requires the providence of higher amounts of drug to reach the needed concentration in the diseased site, which means healthy cells will be exposed to more damage or side effects. The utilization of drug carriers is beneficial as they have the mechanism of selective targeting; these cargos employ the physiology of diseased tissues. They may passively target them, like accumulation of nanoparticles in cancerous tissue due to EPR (enhanced permeation and retention) effect of capillaries in most cancer tissues characteristics of specifically solid tumors such as breast cancer, are high vascular permeability. Their lymphatic drainage is insufficient to filter out nanoparticles due to EPR (Figure 2). Another form of passive targeting is using a pH-labile chemical around nanoparticles or as the carrier for the drug, and these chemicals tend to degrade in low pH environments such as the microenvironment of tumors (Taurin et al., 2012). They can also be actively targeted using ligand-mediated recognition, which is specific to the site of disease, for example, antibody to an antigen, antigen to a receptor for example: targeting Epidermal growth factor receptor (EGFR) (Master & Sen Gupta, 2012). Targeting the carriers has helped solve the problem of adverse side effects on healthy tissues (Peer et al., 2007).

The functionality of drug delivery systems is due to their ability to alter the pharmacokinetics of free drugs. The efficiency of conventional drugs is limited due to their unfavorable pharmacokinetics that is expressed in the rapid renal clearance and lowering of the concentration of available medicine in the bloodstream. To compensate for this drawback, high drug dosage is administered, or the drug is repeatedly infused. The introduction of DDS reduces the renal clearance of the drug molecules (therapeutic agents) to avoid being cleared by the kidneys; these systems have a size larger than 15nm (Choi et al., 2009) (Allen & Cullis, 2004).

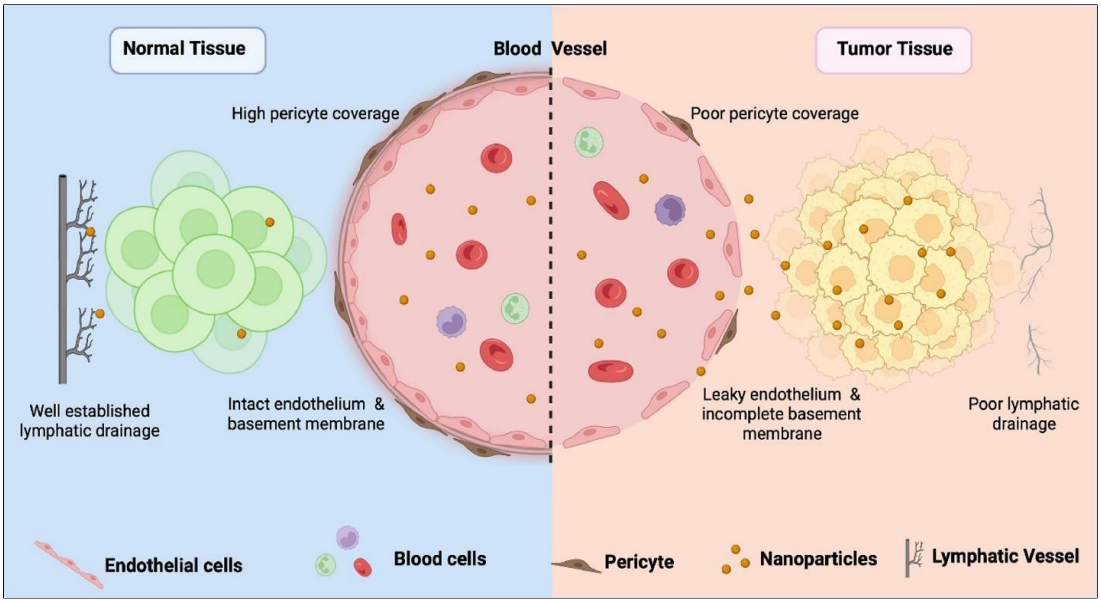

Figure 2: Tumor microenviroment and nanoparticle distribution (Shinde et al., 2022)

The main specificity of the drug delivery system is that it can also have features for site-specific release properties; it can respond to the pathological site anatomy, such as the intrinsic abnormalities, or extrinsic ones, such as the exertion of stimuli such as magnetic field, heat to the site, or ultrasound effect. The drug in these carries will be released upon these stimulations, or some drug delivery system can have a protective layer around the drug, which would be deleted as a response to the stimuli. Medical advances have allowed the usage of more sensitive molecules such as RNA as therapeutic agents (Figure 3); a complete drug delivery system would allow intracellularly delivery of these molecules (Kamaly et al., 2016).

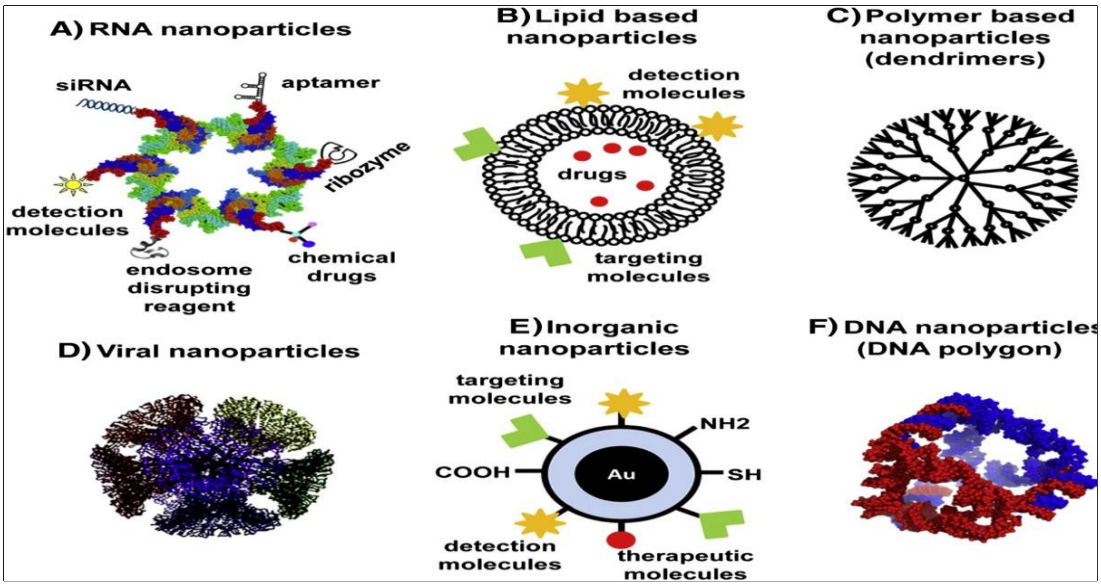

Figure 3: Different types of nanoparticles used in drug delivery systems (Shu et al., 2014)

1.1.1 Nanoparticles as nanomedicine devices

Nanoparticles are particulates within 1-100nm in size as defined by the International Union of Pure and Applied Chemistry (Shi et al., 2017). These particles are ubiquitously implemented in various fields of life due to their properties; they are used in electronics and optical fields due to their conductivity and optical assets. But mostly, the interest in these nanotechnological products is due to their unique features that offer significant benefits in medical fields such as cancer therapy and diagnosis, tissue engineering, therapeutics, gene therapy, and vaccine delivery. In the medical sense, especially in chemotherapy, the favorable use of NPs is attributed to their loading capacity, where it's possible to incorporate different types of molecules and agents into those nanocarriers, such as chemotherapy drugs, imaging diagnostic agents, siRNA, DNA, proteins, and peptides (Aghebati-Maleki et al., 2019). Due to their targeting capacity, NPs are extensively researched as an alternative to conventional chemotherapy, with adverse side effects lowering its

efficiency. As nanoparticles are used to enhance the *in vivo* efficiency of traditional drugs, they are expected to solve their limitations. One of the limitations is the rapid renal clearance, and it has been revealed that molecules with a diameter smaller than 7nm are filtered out of the kidney (Choi et al., 2009). Also, nanoparticles act on increasing the longevity of drugs in the bloodstream; therefore, these carriers should escape from reticuloendothelial system (RES) recognition; molecules larger than 300nm are considered foreign bodies and captured by RES. Thus, nanoparticles used in nanomedicine exist in sizes ranging from 7nm to 100nm (Decuzzi et al., 2009). Other than size modifications, nanoparticles can be bound or covered with other molecules that can avoid RES recognition, such as PEG polymer. Nanoparticles in nanomedicine are advantageous in the sense that they are small in size, biodegradable, biocompatible, have high encapsulation capacity, and are stable in the body, offering a high bioavailability (Contini et al., 2018). These nanoparticles act as a vehicle for the delivery of therapeutics and medical agents. They can be produced by a plethora of materials, such as polymers, hydrogels, metal particles, ceramics, and lipids. Liposome, lipid-based nanoparticles and polymeric nanoparticles are primarily used in nanomedicine due to the ease of their surface functionalization (Shi et al., 2017).

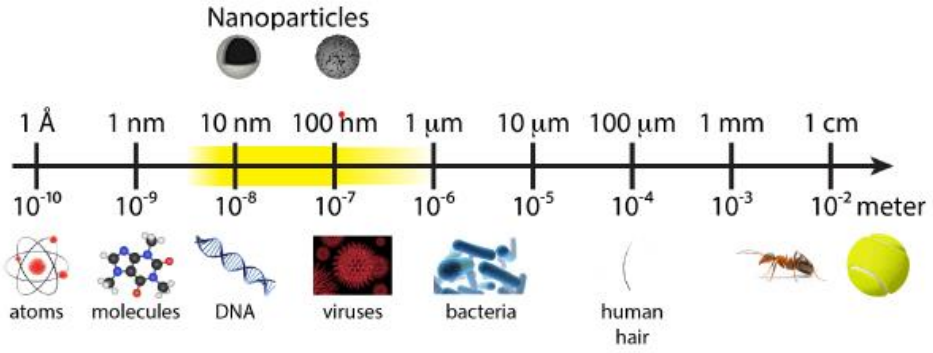

Figure 4: Nanoparticle size compared to different objects (Bloemen, 2015)

Cancer cells differ from healthy cells in many aspects. Some receptors are unique for cancerous cells, while other membrane proteins can be over-expressed on tumors

compared to normal cells. (Bazak et al., 2015) Actively targeting NPs to these molecules using ligands or antibodies such as anti-CD20mAbs (Bisker et al., 2012) allows the specific targeting of cancer cells and lowers the exposure of healthy cells to cytotoxic agents. One of the well-known examples of membrane receptors that are over-expressed in breast cancer but minimally expressed in healthy cells is HER2 (human epidermal growth factor receptor). It has been found to be a suitable target for NPs in HER2-positive breast cancer (Wartlick et al., 2004).

Many types of nanoparticulate have been accepted from different kinds of polymer nanoparticles, nanocrystals, lipid-based NPs, and dendrimer-based and inorganic NPs for various applications such as chemotherapy, infections, nutrients deficiencies (Table 1).

Table 1: Nanoparticles approved by FDA and EMA for clinical use (Halwani, 2022) .

Type	Trade name	Active Pharmaceutical Ingredient	Usages	Approval agency	Production company
Nanocrystalsc	Ostim	Calcium Hydroxyapatite	Bone grafting material	FDA 2004	Osartis GmbH & Co.
	Rapamune®	Rapamycin	Immunosuppressant	EMA 2001, FDA 2010	Wyeth Pharmaceuticals Inc.
	Ritalin LX®	Methylphenidate	Attention deficit hyperactivity disorder (ADHD) in children	FDA 2002	Novartis
Polymer-based	Genexol-PM®	Paclitaxel	Breast cancer	FDA 2007	Lupin Ltd.
	Cimzia®	IgG Fab' fragment that recognizes and binds to TNF-α	Rheumatoid arthritis, Crohn's disease	FDA 2008, EMA 2009	UCB

	PegIntron®	Alpha interferon (INF)	Hepatitis C	EMA 2000, FDA 2001	Merk & Co. Inc.
	Restasis®	Cyclosporine	Chronic dry eye	FDA 2003	Allergan
Lipid-based nanoparticles	Doxil®	Doxorubicin	Metastatic ovarian cancer, HIV-associated Kaposi's sarcoma	FDA 1995, EMA 1996	Johnson & Johnson
	Marqibo®	Vincristine	Philadelphia chromosome-negative chronic myelogenous leukemia	FDA 2012	Talon Therapeutics
	Vyxeos®	Daunorubicin and Cytarabine	Acute myeloid leukemia	FDA 2017, EMA 2018	Jazz Pharmaceutics
Protein-based Nanoparticles	Abraxane®	Paclitaxel	Metastatic breast cancer 2005, lung cancer 2012, metastatic pancreatic Adenocarcinoma 2013	FDA 2005, 2012, 2013, EMA 2008	Celgene Pharmaceutical Co. Ltd
Inorganic Nanoparticles	Ferinject®	Iron carboxymaltose colloid	Iron deficiency anemia	FDA, EMA 2013	Vifor
	Hensify®	Hafnium oxide nanoparticles	Locally advanced squamous cell carcinoma	EMA 2019	Nanobiotix

1.1.1.1 Liposomes

With the extensive need for a drug delivery system to facilitate the treatment of some diseases, liposomes offered a breakthrough in nanomedicine. Liposomes are artificial lipid-based vesicles, first discovered by the British scientist Alec Bangham in 1963 (Bangham, 1963). These vesicles can be produced using natural or synthetic

phospholipids. Phospholipids are amphiphilic molecules having a hydrophilic head and a hydrophobic tail; when in contact with aqueous mediums, they self-assemble, forming a bilayer of phospholipids around a hydrophilic core (Figure 5).

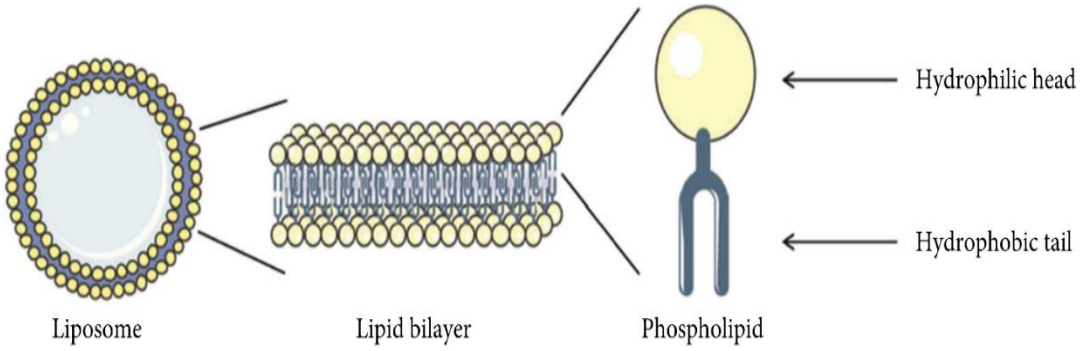

Figure 5: Liposome made from a double bilayer of phospholipids (Allahou et al., 2021).

Liposomes are widely used in various fields like pharmaceutical, biomedical, food, and cosmetics (Kapoor et al., 2018; W. Liu et al., 2020; Yadav et al., 2017). One of their strengths is that due to the structure of the liposome, it can encapsulate both hydrophobic molecules within the lipid bilayer and hydrophilic molecules within the aqueous core or on the hydrophilic membrane layer (Figure 6).

As these vesicles have different usages, they are also administered in different routes to improve the efficiency of the molecules, such as parenteral, oral, transdermal, topical, and pulmonary (Bozzuto & Molinari, 2015). They were found to have application in drug delivery by Gregory Gregoriadis in the 1970s; before that, they were investigated to understand the behavior of phospholipids as mimicking human cellular membranes (Perrie, 2008).

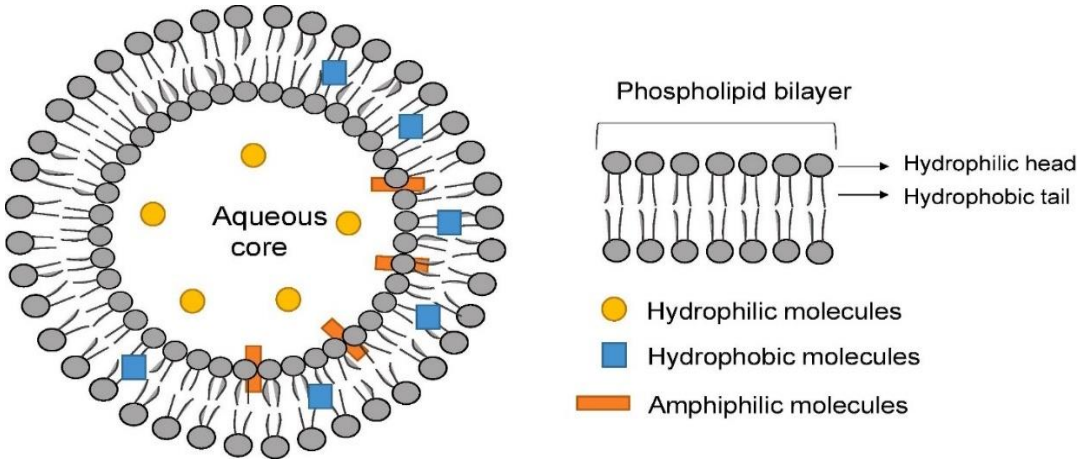

Figure 6: Encapsulation compartments of hydrophilic, hydrophobic, and amphiphilic molecules (Guimarães et al., 2021).

Liposomes provide most requirements of nanomedicine; they are small in size, made out of lipids, are biocompatible, biodegradable, and do not evoke adverse reactions such as antigenic, pyrogenic, allergic, etc. As mentioned before, the advantage of drug delivery systems is the ability to alter the pharmacokinetics and biodistribution of drugs. Therefore, liposomes would be a great fit as a drug delivery system as they can encapsulate hydrophobic drugs, enhance their solubility, and make them more readily available to be used by the cells. Compared to conventional drugs, liposomes have lower cytotoxicity to healthy cells due to the sustained and controlled release property and the targeted drug delivery opportunity liposomal systems offer. When the drug is not consumed by healthy cells but instead consumed by the targeted, diseased cells, its blood circulation increases, allowing more dosage to reach the aimed site. Thus, the half-life of the active pharmaceutical ingredients (APIs) increases, and less dosage is required to achieve the therapeutic index (Allen et al., 2008).

Liposomes are categorized according to i) lipid composition, ii) the number of bilayers, also known as lamellarity, ii) their size, and iv) surface charge. Unilamellar vesicles (ULV) consist of a singular phospholipid bilayer and can be further classified by their size as small unilamellar vesicles (SUV) that are in the range of 20-100 nm or large unilamellar vesicles (LUV) which are larger than 100 nm.

Multilamellar vesicles (MLV) have multiple concentric lipid bilayers and are separated by a hydrophilic layer; due to the various layers of phospholipids, MLVs are suitable for encapsulating lipid-soluble molecules. There is also a mixture of vesicles, multivesicular vesicles (MVV), and one large unilamellar vesicle (LUV) encapsulating multiple SUVs (Figure 6) (Pattni et al., 2015). Altering the surface charge of liposomes protects them from being opsonized by the reticuloendothelial system (RES). Opsonin binds on the surface of the liposomes, eliminating it from circulation after intravenous administration, thus producing liposomes in the range of 80nm and 200nm, as well as having a neutral or negative charge that protects it from RES recognition. SUVs have a longer half-life in blood circulation than MLVs, due to their smaller size, which makes them not recognized by the mononuclear phagocytic system (Taurin et al., 2012). Using a phosphatidylcholine mixture of phospholipids forms neutral liposomes; thus, it is more favorable for the structure of liposomes. Liposome stability, however, is challenging; their size changes with time, the molecule entrapped leaks, and vesicle degradation occurs. Adding cholesterol to the lipid mixture has been shown to increase its stability and provide better control over the drug release profiles (Nakhaei et al., 2021). Besides that, cholesterol controls the structure of liposomes, increasing the packing of the phospholipid molecules (Briuglia et al., 2015).

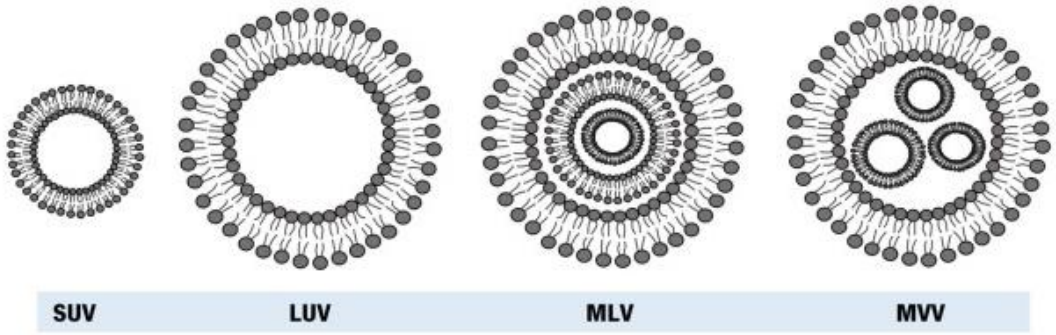

Figure 7: Classification of liposomes based on size and lamellarity (Guimarães et al., 2021)

Conventional liposomes can be considered plain liposomes and easily opsonized and eliminated by the immune system as the body finds it a foreign body. Therefore, to

enhance the efficiency of these therapeutic nanoparticles, the need to increase the longevity of the particles rises, producing long-circulating liposomes. Steric stabilization, such as coating molecules with polyethylene glycol (PEG), a hydrophilic polymer, has been revealed to protect nanoparticles from interacting with blood cells and lowering plasma protein binding (Milton Harris & Chess, 2003). PEG's advantages are due to its antifouling property, allowing the resistance of drug delivery carriers to protein adsorption and cell adhesion (Chen et al., 2010). PEGylation is the process of attaching PEG to nanocarriers that, in return, hinders complement activation. PEGylation was first researched as a method to decrease proteins' destruction *in vivo*, and then studies have shown that PEG utilization improves the pharmacokinetics and pharmacodynamics of therapeutic agents and nanocarriers by making them more hydrophilic due to the steric interactions. Another property of PEG is that it makes molecules larger by binding to two-three water molecules, which slows down renal filtration. PEGylated liposomes thus have more prolonged blood circulation and longer half-life than unmodified liposomes; PEG reduces their plasma clearance and has better biodistribution toward tissues (Gabizon et al., 2003).

Another advantage of liposomes is the possibility of targeting the drug carriers to specific sites by attaching molecules to the surface of liposomes. Liposomes are targeted either by using antibodies or ligand-mediated approaches. Such liposomes are defined as immunoliposomes. By binding antibodies from the IgG class to their membranes, increasing the moiety of liposomes at the specific diseased site and accumulation of the drug at the site is achieved. As immunoliposomes are beneficial in terms of controlling drug release at the site, there are also stimuli-responsive liposomes, where the drug is released at the desired location due to a stimulus such as pH or temperature changes, that create a difference in the microenvironment between healthy and diseased cells (Figure 8) (Cao et al., 2022).

To have a better therapeutic index of drugs, it is essential to observe the effect of the agents selectively on the diseased cells without causing high toxicity to healthy cells. This enhancement is done by attempting to produce drugs or medical devices that

can achieve this selectivity. The tumor microenvironment differs from the normal cellular microenvironment by having an acidic pH, redox potential (glutathione), extensive vascular permeability, and insufficient lymphatic drainage. Normal tissues' blood vessels are composed of a single layer of endothelial cells, and the junction within these cells is tightly closed. When cancerous cells multiply and start clustering, angiogenesis is initiated, yet the newly formed blood vessels differ from the healthy ones surrounding normal tissues.

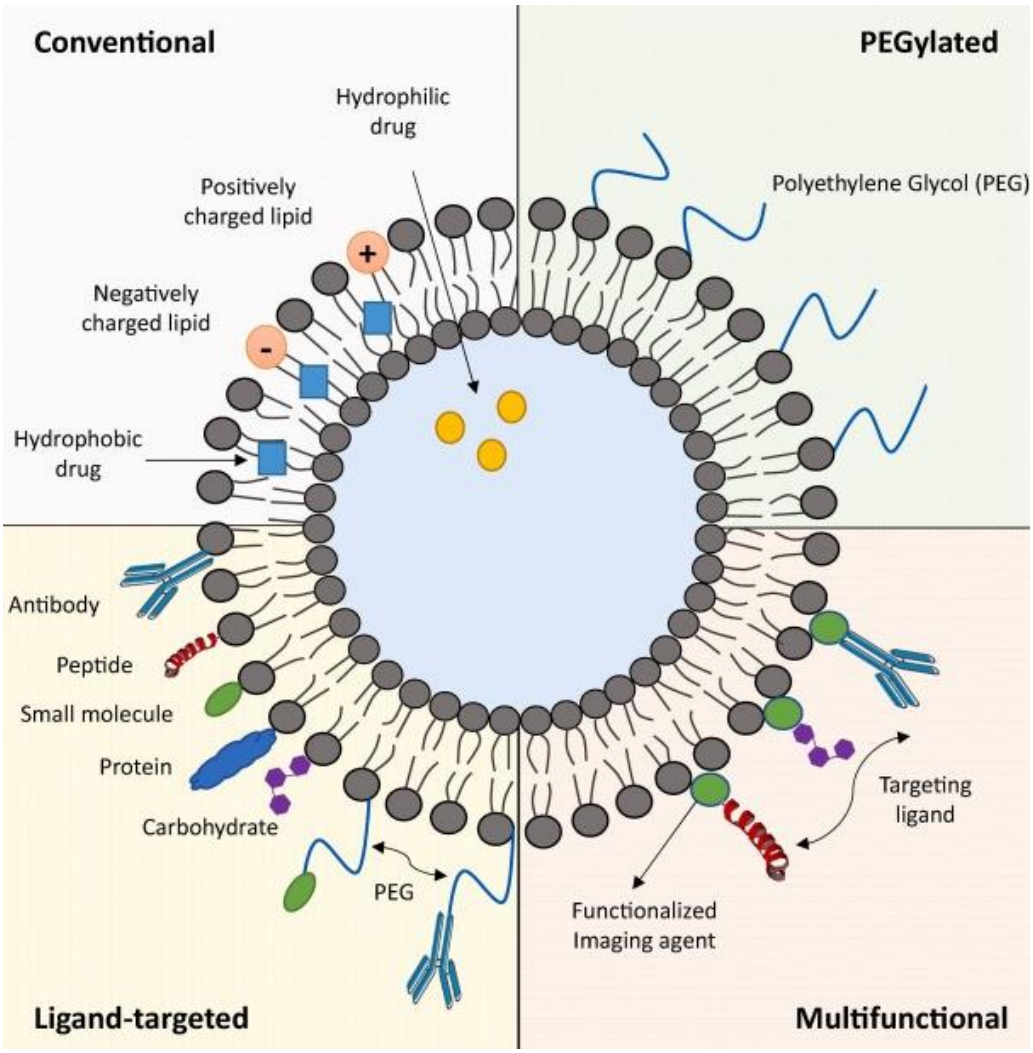

Figure 8: Liposome types based on their functionality (Guimarães et al., 2021)

The neovasculatures are leaky and defective and are poorly aligned and loose, causing the formation of fenestrations within the abnormal endothelial cell

alignment. Besides the leaky blood vessels, tumors have insufficient lymphatic drainage, meaning that macromolecules and nanoparticles such as liposomes are retained within the tumor after leaking from the blood circulation. This process is called enhanced permeability and retention, and it is exploited by liposomal formulas of the drugs and is considered as passive targeting of the nanocarriers (Maruyama, 2011).

The liposomal formulations of drugs are currently holding the top on the clinically approved nanomedicine to be administered, and multiple medications are approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA). These liposomes are used to treat various types of cancers such as leukemia, ovarian cancer, pancreatic, and Kaposi's sarcoma, as well as several types of infections such as fungal infections and meningitis (P. Liu et al., 2022).

Liposomal doxorubicin was formulated to increase its efficacy, prolong its blood circulation, enhance the tumor site's accumulation, and escape phagocyte uptake (Gill et al., 1996). Previous studies showed that PEGylated liposomal doxorubicin provided a more efficient antitumor effect, prolonged circulation time, and was more stable. Stealth liposomes do not interact with plasma proteins, making them escape from mononuclear phagocytes (Northfelt et al., 1996). Liposomal formulation of doxorubicin is commercially available under the name of DOXIL®, PEGylated formulation, and it is one of the first nano-drug approved by the FDA in 1995, named Caelyx® outside the US (Ventola, 2012).

1.2 Cancer and its therapeutic treatment approaches

Cancer is referred to the uncontrollable growth of abnormal cells that can spread to other organs. Breast cancer is one of the most prevalent cancers among women; annually, 2.3 million women worldwide are diagnosed (World Health Organization, 2021). The high incidence of mortality caused by this cancer is due to its metastatic feature, which transfers the cells to different organs such as the liver, lung, and brain

by lymphatic or hematological spread leading to distant metastasis and poor prognosis. Chemotherapy is currently the primary option to manage breast cancer; its advantage over surgery and irradiation is that chemotherapy kills both primary cancer cells and metastatic tumors. On the other hand, chemotherapy's disadvantages are evident in its high dose toxicity and nonselective biodistribution (Wang et al., 2014).

Breast cancer is classified based on histopathological morphology and hormonal and growth factor receptors such as estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2). Some lifestyle factors such as high body index and physical inactivity increase the risk of breast cancer incidence, as some other non-genetic risk factors as having early menarche, late menopause, late childbearing, and short breastfeeding period. Certain people are at higher risk of developing this cancer, such as having a predisposed mutation in some high-risk genes such as BRCA1 and BRCA2 (Britt et al., 2020). There are two approaches to treating breast cancer: systemic therapy, such as endocrine therapy and chemotherapy, and local treatment, such as surgery and radiation. Surgery can either be total mastectomy or removal of the cancerous tumor followed by radiation; surgery is a high-risk operation, has side effects physically and psychologically, and is not efficient to be used on its own. Radiation is another local approach used to eradicate cancerous cells but kills healthy tissues surrounding the tumor (Waks & Winer, 2019). In cases where hormonal receptors are expressed, endocrine therapy includes using drugs such as tamoxifen, an estrogen receptor modulator, as estrogen receptors are found in multiple systems; usage of tamoxifen has shown to cause widespread toxicity, showing adverse symptoms in reproductive, vasomotor, and musculoskeletal systems (Condorelli & Vaz-Luis, 2018). Chemotherapy is currently the primary option to manage breast cancer; its advantage over surgery and irradiation is that chemotherapy kills both primary cancer cells and metastatic tumors. On the other hand, chemotherapy's disadvantages are evident in its high dose toxicity and nonselective biodistribution (Wang et al., 2014). Mostly used chemotherapeutic drugs belong to the anthracycline family, natural antibiotics, other

drugs used in breast cancer are cisplatin, paclitaxel, epirubicin, 5-fluorouracil, and so on (Jain et al., 2020).

1.2.1 Synthetic drugs used in cancer treatment

Cancer drugs have different mechanisms of action; some are antimetabolites such as 5-Fluorouracil, and others perform their activity on DNA such as anthracyclines (doxorubicin, epirubicin, daunorubicin…). Monoclonal antibodies are also used in treating some cancers, such as trastuzumab, a human monoclonal antibody that targets breast cancer cells biologically. Another mechanism of antineoplastic drugs is interference with microtubulin dynamics; in breast cancer, taxanes are used (Nussbaumer et al., 2011).

1.2.1.1 Doxorubicin

Doxorubicin (DOX), also known as Adriamycin, is an anthracycline drug first found in 1960; DOX was initially isolated from the bacteria *Streptomyces peucetius var. caesius* as a bright red pigment. These bacteria also produce another anthracycline, Daunorubicin (Minotti et al., 2004).

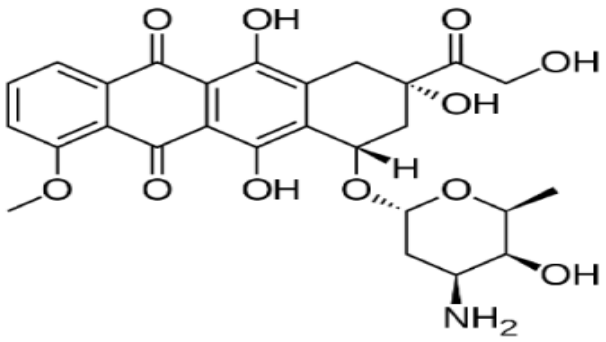

Figure 9: Doxorubicin structure (Singh & Kaur, 2019).

Anthracyclines are one of the most widely used chemotherapy drugs; they are also known as antitumor antibiotics, as they are used in the treatment of various cancer

types (Figure 9). These wide-spectrum anticancer drugs have numerous undesirable effects, such as kidney and bone marrow damage and, most importantly, cardiotoxicity strongly connected with Doxorubicin (Waterhouse et al., 2001). To overcome these drawbacks that limit the usage of doxorubicin, researchers have formulated a liposomal formulation of doxorubicin that improves the drug's pharmacokinetics and lowers the undesired effects (Lyseng-Williamson et al., 2013).

Doxorubicin has the molecular formula of C27H29NO11 and a molecular weight of 543.5 g/mol (Figure 9). Nowadays, doxorubicin is used in its synthetic form, doxorubicin hydrochloride (S. A. Abraham et al., 2005). Studies have shown that doxorubicin is highly sensitive to light, and it is unstable and subjected to biodegradation and loss of bioactivity when kept under direct light (Wood et al., 1990).

Doxorubicin is stable mainly at pH=4.8 and neutral pH=7.4; it decomposes after 3 hours (D. C. Wu & Ofner, 2013). The primary action of these drugs is their ability to intercalate within the DNA base pairs, which leads to breakage in the DNA strands, and inhibits the biosynthesis of DNA/RNA. It also interacts with the topoisomerase II enzyme, which leads to cell apoptosis (Figure 10) (Rabbani et al., 2005).

Three pathways metabolize doxorubicin; the primary metabolic pathway is a two-electron reduction which yields secondary alcohol, doxorubicinol (Mordente et al., 2009). A one-electron reduction mechanism also metabolizes doxorubicin by being oxidized into an unstable semiquinone, which is then converted back into doxorubicin. When doxorubicin accumulates in the mitochondria, it induces mitochondrial complex I of the mitochondrial respiratory chain to initiate redox cycling, which transfers single electrons to DOX, which reacts with mitochondrial complex I to form an unstable semiquinone, a short-lived, toxic metabolite. During this metabolism, reactive oxygen species (ROS) are produced. ROS cause oxidative stress, leading to cellular and DNA damage, lipid peroxidation, and membrane damage, leading to apoptosis (Carvalho et al., 2009). The generation of the free

radical molecules is proposed to be one of the anticancer mechanisms of doxorubicin (Doroshow, 1986). The third and minor metabolism pathway in the myocardium is deglycosidation which forms DOXol hydroxyaglycone (Licata et al., 2000).

Another DOX anticancer mechanism is the interference with topoisomerase. Topoisomerase is an enzyme that manages the topological state of the DNA; the enzyme binds to the DNA and cleaves the phosphate backbone of one or both DNA strands (Figure 10). This cleavage relieves the tangling and allows the resealing of the DNA strands, maintaining transcription and replication, leading to the biosynthesis of macromolecules. Topoisomerases are used as a drug target for treating cancer; these antitumor drugs disrupt the topoisomerase-II-mediated DNA repair that permanently breaks the DNA strands, thereby leading to cell death. Doxorubicin is a topoisomerase type II inhibitor that causes a double-strand break in DNA, leading to an arrest in the cell cycle at the G2 stage (Buchholz et al., 2002; Buzdar, 2006). Another proposed mechanism of this drug is that DOX intercalates itself within the strands, inhibiting DNA and RNA polymerase and halting DNA replication and RNA transcription (Tacar et al., 2013).

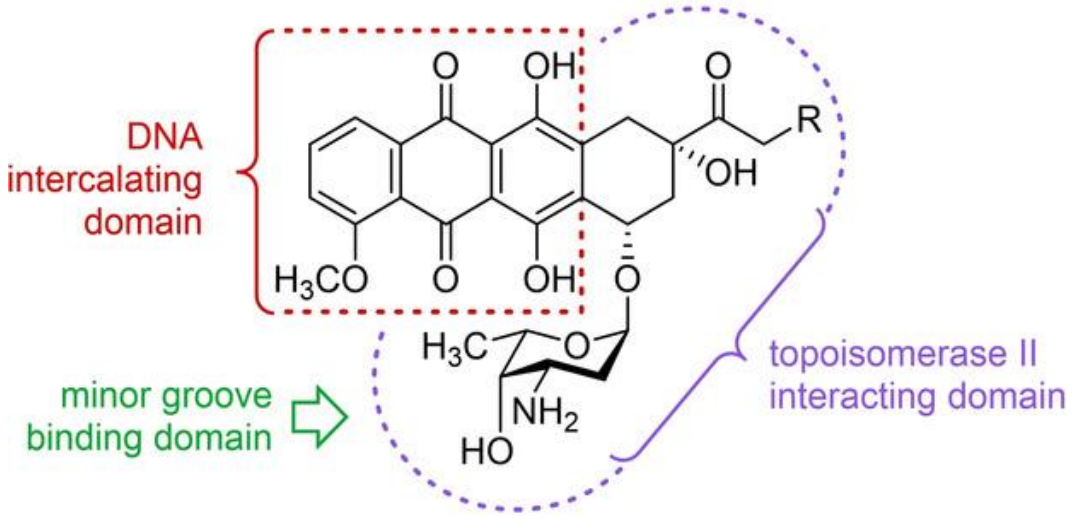

Figure 10: Anthracycline anticancer action by binding to DNA and topoisomerase II (Martins-Teixeira & Carvalho, 2020).

As anthracyclines are broad-spectrum antitumor antibiotics, they are therapeutically used to treat a variety of cancers; for example, it is used for solid tumors and

hematological cancers, breast carcinoma, ovarian carcinoma, acute leukemia, lung cancer, and multiple types of lymphomas (Hodgkin's and non-Hodgkin's) (Cortés-Funes & Coronado, 2007). DOX also showed antitumor action against bladder cancer and stomach cancer. Besides these, it has efficiency against multiple myeloma and AIDS-related Kaposi sarcoma (S. A. Abraham et al., 2005).

Chemotherapy generally has many side effects; they usually manifest as nausea, vomiting, disturbance to the gastrointestinal system and the neurological system as dizziness, hallucination, and headaches. Like other chemotherapy drugs, anthracyclines, and doxorubicin cause general cell toxicity since they are not explicitly targeted for neoplasms. These anticancer drugs cause nonspecific cell death and hence halting the growth of different healthy cell types. The hinder of cell growth would lead to inhibition of bone marrow cell renewal, leading to immunosuppression in the patient. Also, myelosuppression will lead to extra bleeding and slower healing of injuries (Tacar et al., 2013). The most prominent side effect associated with doxorubicin is cardiotoxicity, as the drug causes nonspecific cell deaths. Cardiotoxicity, alongside other side effects, lead to dose limitation, thus hindering the treatment of the disease. Eleven % of patients treated with doxorubicin suffer acute cardiotoxicity within a few days after exposure. Cardiotoxicity is manifested by chest pain, sinus tachycardia, and myopericarditis. On the other hand, chronic cardiotoxicity, which is dose-dependent, is only in 1.7% of the patients; it can occur within a month or after ten years (Chatterjee et al., 2010). This manifestation is due to the general release of free radicals and reactive species that are toxic to the cardiac system during drug metabolism and to the interference of doxorubicin with mitochondrial function (Gewirtz, 1999).

Clinical liposomal formulations of DOX

The first approved NP goes back to 1995, a PEGylated liposomal formulation of Doxorubicin HCl traded under the name DOXIL by the company Johnson & Johnson, used for the treatment of metastatic ovarian cancer and HIV-associated Kaposi's sarcoma (Bobo et al., 2016). Due to doxorubicin's nonspecific toxicity, it

causes damage to healthy cells, which guided the development of new formulations of the drug that aims to increase the specificity and decrease its toxicity to other organs. These efforts focus on solving the dose limitations of this drug by developing delivery systems, such as hydrogels, nanotubes, nanogels, nanospheres, and lipid base formulations (liposomes) (Kanwal et al., 2018). Liposomes are recorded as the most successful formulation of doxorubicin encapsulation. The development of doxorubicin liposome was based on the mechanism of liposome circulation in the blood. Liposomes cannot diffuse into tight blood capillaries like those in the heart muscles; thus, it does not diffuse into the heart, lowering the drug's cardiotoxicity. Besides that, liposomes cannot leave the circulation to enter other organs with tight vascular structures. Still, they can penetrate organs where the vessels are loose, like the ones in tumor tissues, therefore passively targeting tumor cells. Lipid nanocarriers also employ the defective lymphatic drainage of the tumors to target them and lead to specific toxicity (Rivankar, 2014). The utilization of liposomes for these unique solid tumor characterizations is known to be enhanced by permeation and retention (EPR) (Iyer et al., 2006). Liposomes are also biocompatible, biodegradable, and nonimmunogenic; therefore, they can be ideal for drug delivery systems.

PEGylated liposomal doxorubicin formulations are used in clinical treatments of some solid tumors for ovarian cancer, multiple myeloma, AIDS-related Kaposi's sarcoma, and metastatic breast cancer. DOXIL is the first-line treatment for metastatic breast cancer, but with the development of a novel formulation of doxorubicin, new side effects have risen. These were non-hematological and hematological side effects. The most prevalent side effects associated with the liposomal doxorubicin were skin toxicity, mucositis, anemia, neutropenia, and palmar-plantar erythrodysesthesia syndrome (PPE), also known as Hand-Foot syndrome (HFS). These problems occur at high doses, with short time intervals (Ansari et al., 2017). These nanocarriers face some challenges as some cancers are found in body parts surrounded by physiological barriers that make it hard for the liposome to diffuse into those organs, such as the brain, and peritoneum, causing low

diffusion into the organs as well as non-uniform drug distribution. Another challenge liposomal formulation faces the ability to sustain the release of the drug at tumor sites and prevent the nonspecific toxicity on healthy cells around cancer cells (Niu et al., 2010).

1.2.2 Vitamins used in cancer treatment

1.2.2.1 Vitamin E

Vitamin E is a fat-soluble nutrient known as a potent antioxidant and anti-inflammatory molecule. This vitamin has multiple benefits on the cardiovascular and immune systems, as well as on the skin and bones. Its anti-inflammatory is exerted by regulating inflammation, mediating molecules such as COX-2, and suppressing pro-inflammatory pathways, showing promising effects on diseases such as inflammatory arthritis. Vitamin E is also used in dermatological and cosmological products as antioxidant protecting skin from harmful UV rays. This vitamin has also been shown to induce osteoblast formation, hence having a benefit on bone formation. *In vivo*, vitamin E has shown anticancer effects on many cancers by inhibiting cell proliferation, promoting apoptosis and cell cycle arrest, and suppressing angiogenesis (A. Abraham et al., 2019). Preclinical studies have shown promising antineoplastic activity, making it an attractive molecule to study. The solubility of this molecule attenuates its benefits. Therefore, drug delivery systems were needed to enhance its effects and bioavailability. Multiple formulas of vitamin E have been produced, such as solid-lipid nanoparticles, polymer nanoparticles, and nanoemulsions. These formulas are under preclinical studies (Mohd Zaffarin et al., 2020). Due to its promising anticancer effects, vitamin E has been studied with other molecules to enhance its activity, such as vitamin C (Yiang et al., 2021) and in combination with gemcitabine (Husain et al., 2011).

1.2.2.2 Vitamin C

Ascorbic acid, also known as vitamin C, is vital in regulating the immune system and inflammation. It is also an essential cofactor for some enzymes in different bodily processes (Ang et al., 2018). Vitamin C is also a potent antioxidant, which facilitates its anticancer effects, allowing it to be a candidate for cancer treatment (Pawlowska et al., 2019),(Bos, 2019).

1.2.2.3 Vitamin D

Vitamin D, a steroidal hormone, is synthesized in the skin by the induction of ultraviolet (UV) radiation. This vitamin is essential for the maintenance of healthy bones by the regulation of calcium and phosphate homeostasis. Vitamin D increases calcium serum levels by stimulating intestinal absorption and bone resorption. It also absorbs phosphate from the gut, by these two mechanisms, it facilitates keeping the skeleton mineralized. Vitamin D deficiency leads to osteoporosis and osteomalacia, and healthy dietary requirements enhance bone growth through the action of osteoblasts and osteoclasts (DeLuca, 2004). Many organs express vitamin D receptors (VDR), including immune system cells, T and B cells, monocytes, and neutrophils; thus, vitamin D also affects the modulation of the immune system and has antimicrobial and anti-inflammatory activity (Chun et al., 2014). Other novel functions have been found for vitamin D, which was evident due to the expression of VDR on a broad type of cells such as pancreatic islet cells, keratinocytes, ovarian cells... It has been found that vitamin D has a function in regulating cell proliferation and differentiation and that high serum levels of it have both chemoprotective and chemotherapeutic effects. The crucial role of vitamin D is also due to regulating of genetic expression of cell proliferation and differentiation, and induction of cell cycle arrest and apoptosis, alongside suppression of inflammation, angiogenesis, metastasis, and angiogenesis. *In vivo* and *in vitro* studies have revealed that vitamin D administration has reduced the risk of cancer, favorable prognosis, and tumor

suppression in pancreatic and breast cancer (Gil et al., 2018). Clinical studies combining vitamin D with other drugs have shown promising anticancer effects; these combinations included dexamethasone in prostate cancer and docetaxel in pancreatic cancer (Xu Wu et al., 2019). Vitamin D is a potential anticancer molecule; nanoparticles formulation of this vitamin has been produced, such as PLGA nanoparticles, quantum dots, and liposomes (Ramalho et al., 2017). As vitamin D has shown positive results in combinations of drugs, it has also shown enhanced effectiveness when coloaded with doxorubicin in liposomal formulations (Dalgic et al., 2016).

1.2.2.4 Vitamin K

The coagulation vitamin, vitamin K, was discovered by Henrik Dam in the early 1930s as the antihemorrhagic vitamin, which is an essential factor in maintaining homeostasis (DAM, 1935). Vitamin K refers to a lipophilic, hydrophobic family that belongs to the 2-methyl-1,4-naphthoquinone group. A methylated naphthoquinone ring characterizes the different derivates of this vitamin, and this family consists of naturally occurring phylloquinone (vitamin K1) and menaquinone (vitamin K2), and a chemically synthetic derivative menadione (vitamin K3). Phylloquinone is a naturally occurring vitamin found in plants and green leafy vegetables. Menaquinone is also a naturally occurring vitamin originating from a microbial source, as animal and human gut bacteria produce menaquinone by converting other forms of vitamin K (McKee, R. W. et al., 1939). Menadione, the synthetic form of vitamin K3, is much simpler in form than other vitamin K compounds. Vitamin K3 is a provitamin, and Billeter et al. found that mammalian intestinal bacteria could cleave phylloquinone into menadione that the body will later use; vitamin K3 can be converted to an active form of vitamin K2 (menaquinone) (Okano et al., 2008).

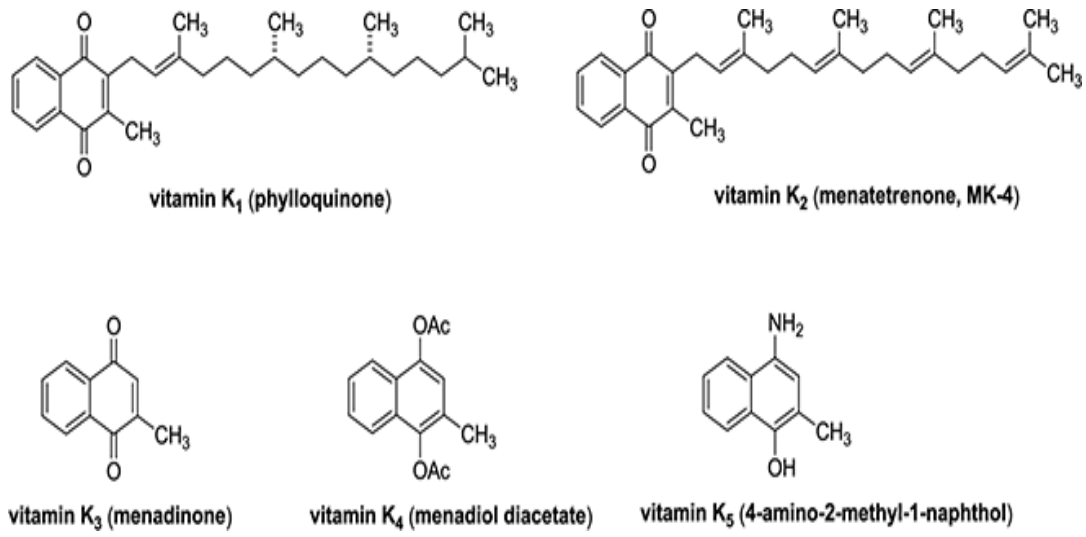

Figure 11: Structure of different vitamin K analogs (Kurosu, 2017)

Vitamin K is essential for synthesizing hepatic blood-coagulating proteins, as it is a cofactor in the post-translational modification of these proteins. These proteins are also called vitamin K-dependent (VKD) proteins, and they are factors II (prothrombin), VII, IX, and X (Rannels et al., 1987). Vitamin K acts as an essential cofactor in the enzymatic carboxylation of glutamic acid residues into gamma-carboxyglutamate (GLA) by γ-carboxyglutamyl carboxylase. This conversion is crucial for the biological activity of VKD proteins; therefore, they can also be called Gla proteins (Furie & Furie, 1990). Proteins C and S belong to the Gla proteins, which have a role in the negative feedback control of coagulation; hence they are anticoagulants. Besides being a cofactor in the coagulation cascade, vitamin K plays a role in bone metabolism, reduces bone loss, and regulates the calcification of blood vessels. It also acts on cardiovascular mineralization and apoptosis. The recommended dietary allowance (RDA) is 1ug/kg/day; this RDA is enough for its coagulation function, but it is not sufficient for its other extrahepatic operations. Deficiency in Vitamin K is rare, but it is not only manifested by bleeding complications but also by an increased risk of bone fractures, osteoporosis, postmenopausal bone loss, and increased vascular calcification (Berkner & Runge, 2004; Booth, 2012; Chlebowski et al., 1985; Cranenburg et al., 2007; Sweatt et al., 2003).

Besides the previously mentioned roles of vitamin K, it has also been observed to possess anticancer effects and inhibit cancerous growth *in vivo* and *in vitro* (Chlebowski et al., 1985; Ngo et al., 1991; Prasad et al., 1981). (Okayasu et al.,) have shown that within the family of Vitamin K group, menadione showed the highest cytotoxicity against tumor cell lines (Dasari et al., 2017), (Okayasu, Ishihara, Satoh, & Sakagami, 2001).

Anticancer mechanisms of Vitamin K3:

Vitamin K3 (menadione) was found to have anticancer effects against various cell lines *in vitro*, such as breast, mammary, bladder, hepatic, lung, pharyngeal, and blood cancers (Lamson & Plaza, 2003)

Menadione is also named 2-methyl-1-4naphthoquinone; it belongs to the quinone family. Due to its quinoid structure, vitamin K3 undergoes redox cycling, producing toxic oxygen species (Smith, 1985). Induction of apoptosis by oxidative stress due to menadione is phenotypically characterized by cell shrinkage, DNA fragmentation, and activation of caspase cascades (Lamson & Plaza, 2003). The anticancer action of vitamin K3 is due to the oxidative stress that occurs during its cellular metabolism, which leads to the generation of reactive oxygen species (ROS). Redox recycling is utilized in cancer therapy, as the metabolism of vitamin K3 is through quinone reductases 1 and 2 (NQO1, NQO2). QR1 leads to a two-electron reduction of menadione to produce vitamin K hydroquinone, while QR2 lead to a one-electron reduction, producing semiquinone and ROS as a byproduct (Gong et al., 2008).

This oxidative stress is dose-dependent as menadione can cause apoptosis at low concentrations and necrosis at higher concentrations (Sata et al., 1997).

The generation of ROS mediated by vitamin K3 treatment has led to single and double-strand DNA breaks. DNA damage that was demonstrated on MCF-7 treated cells was topoisomerase-independent DNA breaks based on hydroxyl radical production (Nutter et al., 1992).

Another mechanism related to the quinoid structure of vitamin K3 is the direct arylation of cellular thiols, which results in glutathione depletion. When introduced to molecules like glutathione (GSH), menadione leads to the alkylation of intracellular macromolecules, which become inactivated, ultimately resulting in cell death (Ross et al., 1985).

Menadione specifically binds to peptides at cysteine sulfur residues; some proteins, such as cdc2A phosphatase, depend on the sulfhydryl domain. Therefore, when vitamin K3 binds to Cdc25 phosphatase, it leads to the inactivation of cyclin-dependent kinases, specifically Cdk1, which hinders the cell cycle continuation (F. Y. H. Wu & Sun, 1999)

Akiyoshi et al. have shown that vitamin K3 cause apoptosis in MCF-7 cell line via mitochondrial dysfunction in breast cancer cells. Menadione activates the caspase 9 pathway, which causes irreversible apoptosis through direct mitochondrial damage; then caspase 7 is commenced, which is the conclusive apoptotic factor in MCF-7 cells. Activating caspase 7 and 9 pathways results in mitochondrial damage, thus cell death. Vitamin K3 also generates ROS due to the activation of the caspase 7 pathway and causes a decrease in intracellular ATP levels, leading to mitochondria-related cytotoxicity (Akiyoshi et al., 2009).

An additional antiproliferative action of menadione is through binding to tubulin and inhibiting the polymerization of microtubules, which are essential for regulating cell growth and modulating fundamental cellular processes. This interference hinders cell proliferation and suppresses normal cell miotic progression in human cervical epithelial cell line (HeLa) and human oral epithelial cell line (KB). (Acharya et al., 2009).

Another antineoplastic activity of vitamin K3 is through the inhibition of DNA polymerase gamma activity, which is responsible for mitochondrial DNA replication and repair. This study has shown that vitamin K3 suppresses angiogenesis in rat models (Matsubara et al., 2008). DNA polymerases are an essential target for

anticancer agents as DNA Polymerase is responsible for replicating and repairing DNA in cells and is the basic need for cell growth.

Chemically synthesized Vitamin K3 (menadione) lead to mitochondrial dysfunction and produces reactive oxygen species (ROS), which leads to apoptosis and tumor inhibition. Thus, proving its anticancer effect against hepatic, breast, bladder, and blood cancers. Vitamin K3, alongside other vitamin K subtypes, vitamins K2 and K5, hindered the proliferation of colorectal cancer by suppressing cyclin-dependent kinase 4 (CDK-4), leading to cellular arrest at G1 phase of the cell cycle (Ogawa et al., 2007).

Previous *in vitro* and *in vivo* experiments showed synergistic effects of combining vitamin K with different chemotherapeutic molecules. Such combinations included vinblastine (VBL), 5-fluorouracil, mitoxanthine, mitomycin C, ascorbic acid, and doxorubicin. These studies were successful and progressed into *in vivo* studies. The study by (Semkova et al.) showed successfully *in vivo* results by combining menadione and ascorbate, and this assembly can be used as a conventional chemotherapeutic agent (Liao et al., 2000; Semkova et al., 2020). A clinical study assessing the efficacy of Apatone (Vitamin C and Vitamin K3) has shown promising results and synergistic effects on prostate cancer patients by lowering cancer biomarkers (Tareen et al., 2008).

One study showed, pretreated cells with 10 μg/mLof menadione lowered the concentration of doxorubicin needed to kill 50% of cells from 22.3μg/mL to 5.3 μg/mL, showing the resistance of cells was inversely proportional to menadione concentration. The action of vitamin K3 on resistant cells was by depleting cellular glutathione, thus increasing the cells' susceptibility to Doxorubicin (Xu, Zhang, Wang, & Zhang, 1998).

Natural forms of vitamin K are safe to consume, but its synthetic form of it vitamin K3 has shown some adverse effects when administered in high doses. It has been observed to cause allergic reactions, hemolytic anemia, and liver cytotoxicity

(Hassan, 2013). Therefore, it is necessary to co-administer it with another chemotherapeutic drug to lower the dosage further to achieve its therapeutic index.

1.3 Aim of the study

Breast cancer is leading as the second reason of death after lung cancer in women. The first-line treatment for cancer is chemotherapy, yet chemotherapy is clinically dose limited due to its typical side effects of cytotoxicity of healthy cells, which lowers its therapeutic index. As an alternative, drug delivery systems such as nanocarriers are extensively studied to be used clinically. Liposomes are an example of nanocarriers, which have advantages over traditional chemotherapy with better solubility, improved bioavailability, pharmacokinetics properties, and lower cytotoxicity as it passively targets tumor cells. This study aims to develop a drug delivery system based on liposomes for use in the treatment of cancer. As doxorubicin and Vitamin K3 have both been seen to have anticancer activity, it is expected that the liposomal formulation of each of these drugs would have enhanced activity, lowering the needed dosage for a potent antitumor effect. As combinational treatments have an advantage over monotherapy, in this study, cancer cell line MCF-7 will be exposed to the sequential treatment of liposomal vitamin K3, followed by liposomal doxorubicin, which is expected to increase the effectiveness of the drugs as well as decrease their side effects. The novelty of this study is that it is the first-time development and investigation of liposomal formulation of Vitamin K3, and combination treatment of liposomal vitamin K3 and liposomal doxorubicin, the pretreatment of L929 by vitamin K3 followed by treatment with doxorubicin was done for the first time.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

For liposomal synthesis and experiments: L-a-Phosphatidylcholine, hydrogenated (Soy) (HSPC), mini extruder set, polycarbonate filter membranes, and membrane supports were purchased from Avanti Lipids (USA). mPEG-DSPE MW:2000 was bought from Nanocs (USA). Cholesterol (99%) was bought from Sigma Aldrich (USA). Sephadex G-75, PD-10 Disposable Columns were purchased from GE Healthcare (UK). Ammonium sulfate, iron (III) chloride hexahydrate, ammonium thiocyanate, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, chloroform, methanol, and absolute ethanol were bought from Merck (Germany). Sulfuric Acid (95-98%) was purchased from ISOLAB (Germany).

Menadione Sodium Bisulfite was kindly provided by Oxyvit (Turkey), and Doxorubicin was kindly provided by Murat Akıncı from Deva company.

For cell culture studies: Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L) with L-glutamine, heat-inactivated fetal bovine serum (FBS), trypsin EDTA, 1%- penicillin/streptomycin, trypan blue (0.5%) and sodium pyruvate were purchased from Biological Industries (Israel). Thiazolyl Blue Tetrazolium Bromide (MTT reagent) (3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) was bought from Sigma Aldrich (USA). Dimethyl sulfoxide (molecular grade) was bought from Serva (Germany).

2.2 Methods

2.2.1 Preparation of liposomes

L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) based liposomes were prepared by thin film hydration method. The liposome formulations were composed of HSPC, cholesterol, and DSPE-mPEG2000, maintaining a molar ratio of 2:1:0.2, respectively, as done previously by (Dalgic et al., 2016). Prior to weighing, the lipids and the needed chemicals were incubated at room temperature for 15 mins. The liposome components were weighted and added to a round bottom eppendorf tube and dissolved in 100 mL chloroform. The lipid solution was flushed with nitrogen gas for three hours to evaporate the chloroform. The formed lipid film was kept in a vacuum oven (Nüve EV 018, Turkey) at room temperature overnight to remove any chloroform residues. After this step, a thin white lipid film was obtained at the bottom of the tube, which was then rehydrated by 1mL of phosphate buffer solution (PBS, pH=7.4) to prepare empty liposomes. In order to prepare loaded liposomes, two different methods were applied (passive and active method) in the hydration step, as explained in the following sections. Hydration was done by altering the hydrating solution and lipid film in cycles of vortex (CAT VM3, France) and heating for 2 minutes each, for approximately 45 minutes. For heating, a water bath was used at 65°C (transition temperature of HSPC). Thin film hydration produces heterogenous multilamellar vesicles (MLVs). For homogenization, extrusion was performed using a mini extruder by pressing the liposomal suspension through polycarbonate membranes with defined pore sizes. Reduction of MLVs was achieved by extrusion through 400 nm, 200 nm, and 100 nm membrane filters 11 times for each size, and the heated block was kept at 65°C temperature. Extrusion controls the size homogeneity and forms unilamellar liposomes in the final suspension; after extrusion, Sephadex column chromatography was applied to remove unencapsulated drugs.

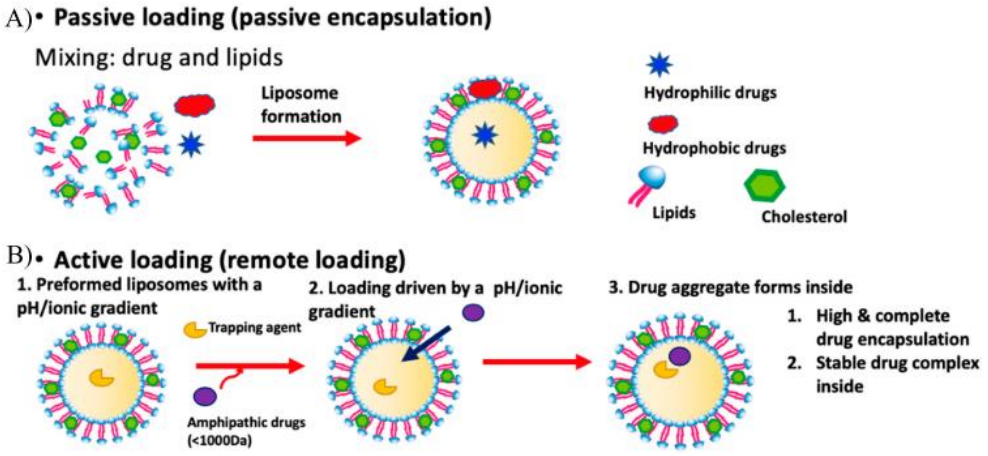

Figure 12: Liposome drug loading methods. (A): Passive loading, (B): Active loading (Pauli et al., 2019)

2.2.2 Preparation of Doxorubicin loaded liposomes

For doxorubicin loading, an ion gradient method was used (Haran et al., 1993); an ion gradient was formed by hydrating the lipid film with ammonium sulfate (120 mM). After hydration of the lipid film using ammonium sulfate (pH=5.5) forms a pH gradient between the intra and extra liposomal environment when dialyzed in NaCl (pH=7), creating a higher concentration of hydrogen ions inside the liposomes. Doxorubicin was dissolved in NaCl (pH=7). When added to the liposome environment, driven by the ionic gradient, doxorubicin accumulates within the core of the liposome. After dialysis for 20 hours, liposome solution and doxorubicin were heated to 65°C (HSPC's transition temperature), then they were mixed and incubated for 10 mins at 65°C then quickly dipped in an ice bath.

Size extrusion chromatography is the method of choice to separate the encapsulated liposomes from the free drug molecules. Therefore, for the removal of unencapsulated doxorubicin Sephadex G-75 chromatography column was used. In brief, Sephadex G-75 beads were swollen overnight by 0.9% NaCl and then kept under vacuum for 2 hours before using to remove air bubbles. Then, the column was saturated with Sephadex gel with 0.9% NaCl. Liposome solution was introduced to

the column, and aliquots were collected in fractions of 1mL. The turbidity of the aliquots was read at 410 nm using a UV-visible spectrophotometer. According to the turbidity reading, liposome samples were collected and stored at 4°C for further studies.

Due to doxorubicin photosensitivity, all the doxorubicin steps were carried out in the dark and covered with aluminum foil.

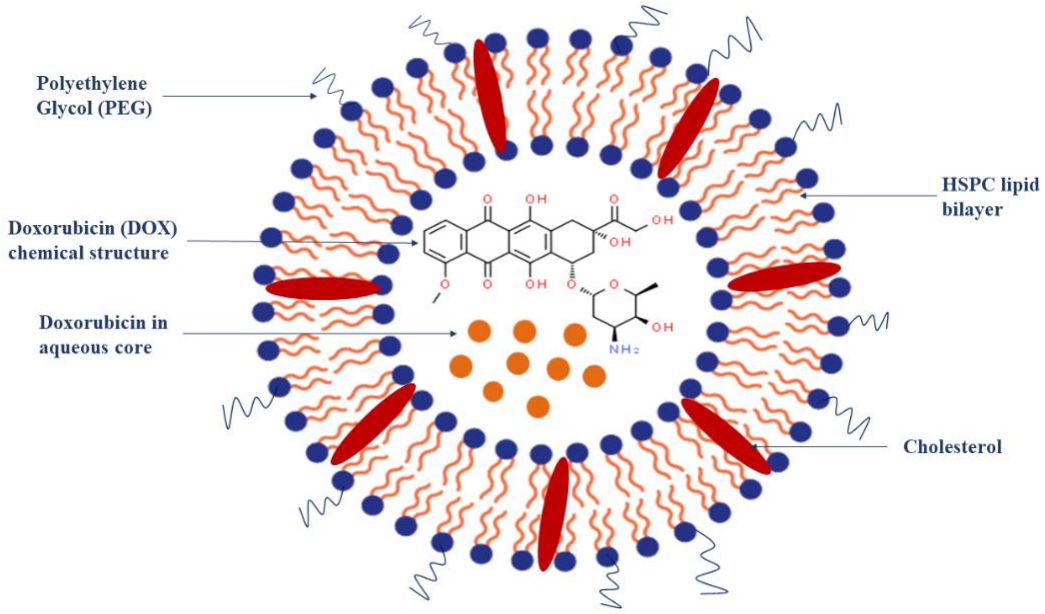

Figure 13: Doxorubicin loaded liposome

2.2.3 Preparation of Vitamin K3 loaded liposomes

Menadione sodium bisulfite (MSB) is the water-soluble form of Vitamin K3. Therefore, using MSB, Vitamin K3 could be loaded into the hydrophilic core of liposomes. First, 5 mg of VitK3 was dissolved in PBS, which was used as the hydration solution during the hydration step. Then liposomes, steps described above in section 2.2.1 were applied to form VitK3-loaded liposomes.

Size chromatography was prepared as described in section 2.2.2. and applied for the removal of unencapsulated VitK3; briefly, Sephadex G-75 beads were swollen overnight by PBS, then kept under vacuum for 2 hours prior to usage; the column

was then saturated with the swollen beads. Then the liposomal VitK3 solution was introduced to the column, and aliquots were collected in fractions of 1 mL. The turbidity of the aliquots was read at 410 nm using a UV-visible spectrophotometer. According to the turbidity reading, liposome samples were collected and stored at 4°C for further studies. The unencapsulated VitK3 solution was also collected for the measurement of encapsulation efficiency.

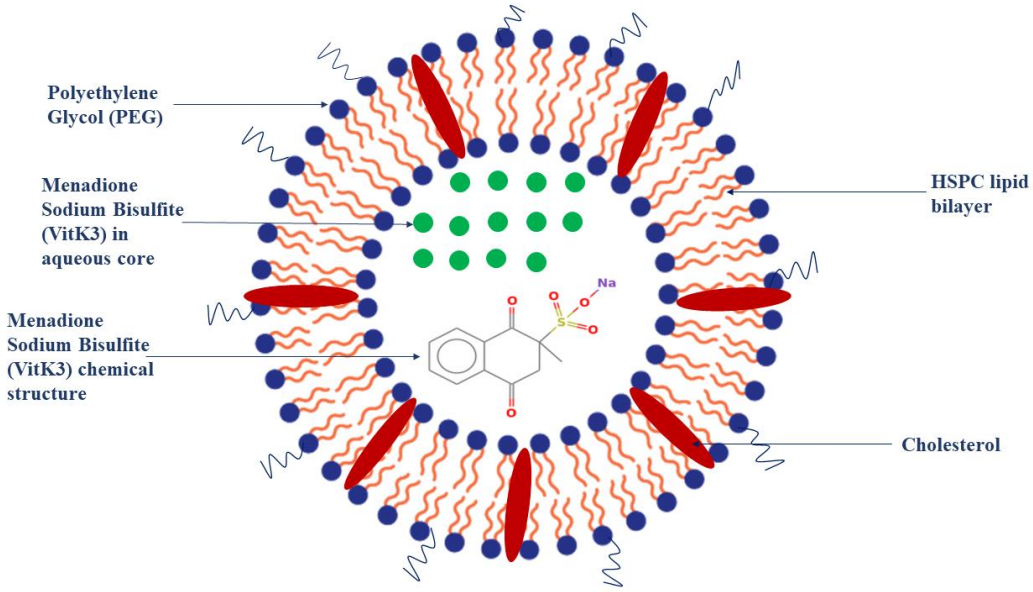

Figure 14: Vitamin K3 loaded liposome

2.3 Detection methods

Spectrophotometric method was utilized to find the absorbance peak by wavelength scanning of the molecules to be quantified, HSPC, Doxorubicin, and MSB. Wavelength scan was measured in UV–Vis spectra range was 190–1200 nm on UV-spectrophotometer (Hitachi U-2800A, Japan). The absorbance peaks for HSPC, doxorubicin, and vitamin K3 were found at 488 nm, 480 nm, and 520 nm, respectively, against corresponding solvents.

2.3.1 Quantification of doxorubicin

Doxorubicin was quantified at the wavelength of 480 nm by UV-Vis spectrophotometer (Hitachi U-2800A, Japan). DOX calibration curves (Appendix A) were constructed by triple measurements between 1-100 μg/mL in methanol for encapsulation efficiency and PBS for release studies. The concentrations of doxorubicin were determined by optical density measurements with UV-spectrophotometer (Hitachi U-2800A, Japan) using lambda max = 480nm.

2.3.2 Quantification of vitamin K3

Quantifying Vitamin K3 was done by a colorimetric method (Nagaraja et al., 2002), briefly in a 25 mL volumetric flask, and 2.5mL of vitamin K3 solution was added. In the next step, 0.2% alcoholic resorcinol (1mL) was put into the Vitamin solution. After adding 5mL sulfuric acid (95-98%), the solution was waited for 5 minutes for the reaction to cool down. After cooling down, distilled water was added to a complete volume of 25mL. A red complex was obtained, and optical density measurements were applied at a wavelength of 520 nm with a spectrophotometer (Hitachi U-2800A, Japan). The formed color complex is stable for up to 3 hours in the temperature range 5- 65°C. A calibration curve was constructed by triple measurements between 10 – 50 μg/mL concentrations. The concentrations of MSB (Vit K3) in loading, encapsulation efficiency, and release experiments were determined by resorcinol colorimetry at optical density measurements with UV-spectrophotometer (Hitachi U-2800A, Japan) using lambda max = 520nm.

The Resorcinol colorimetric method of MSB detection is based on, in the presence of concentrated sulfuric acid, the oxygen atom within the MSB structure undergoes protonation to produce a carbocation. This carbocation undergoes electrophilic addition with resorcinol (an electron-rich molecule), then the loss of water molecule from the product produces a red-colored complex that will be spectrophotometrically measured at wavelength= 520 nm (Figure 15). Using the MSB constructed

calibration curve (Appendix A), the concentration of encapsulated MSB was determined.

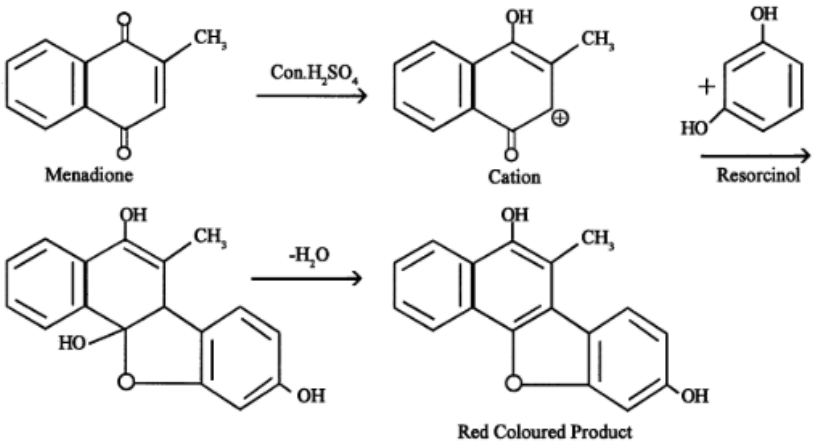

Figure 15: Reaction of MSB (VitK3) with resorcinol and sulphuric acid producing red color (Nagaraja et al., 2002)

2.3.3 Quantification of phospholipids (HSPC)

The colorimetric method, Stewart assay, was used to calculate the amount of lipids in the liposome formulations prepared (Stewart, 1980). The principle of the Stewart assay is based on the ability of phospholipids within the liposomes to form a colored complex with ammonium thiocyanate. Stewart solution, ferrothiocyanate reagent was prepared in 100mL of dH20 with 2.7 g of ferric chloride (FeCl3 6H2O) and 3g of ammonium thiocyanate (NH4SCN). In glass tubes, 100uL of liposome solution was added to 2mL of chloroform and mixed well until liposomes were dissolved. Then, 2 mL of Stewart reagent was added, vortexed for 20 s, and spun in a centrifuge for 10 mins at 1000 rpm. After centrifugation, there were two aqueous phases, the lower phase containing chloroform was removed with a syringe, and its absorbance at 488 nm was measured on UV-Vis spectrophotometer (Hitachi U-2800A, Japan). Using the HSPC constructed calibration curve (Appendix A), the concentration of lipids was measured.

2.4 Characterization of the liposomal formulations

2.4.1 Encapsulation efficiency

Encapsulation efficiency is the parameter that identifies the amount of compound encapsulated within the liposomes and the amount of DOX and VitK3 to be delivered to the target site. Encapsulation efficiency is measured in the form of percentage and expressed as the ratio of encapsulated drug amounts (doxorubicin orVitK3) to the initial added amount of drug (doxorubicin or VitK3) in triplicate measurement (n=3). The actual encapsulated amount of the compounds is calculated by the following equation:

$$Encapsulation\ Efficiency\ (\%) = \frac{Amount\ of\ encapsulated\ DOX\ or\ Vit\ K3}{Initial\ added\ amount\ of\ DOX\ or\ Vit\ K3} x100$$

2.4.1.1 Encapsulation efficiency of doxorubicin

For the measurements of encapsulated doxorubicin,50 μL of liposomal doxorubicin was ruptured using 950 uL methanol, and the absorbance was calculated by spectrophotometer against methanol as a blank. The absorbance of lipids was checked not to interfere with the absorbance of doxorubicin. The utilization of the calibration curve calculated the encapsulated concentration. Then the encapsulation efficiency was calculated in percentage by the ratio of the encapsulated concentration of doxorubicin to the initial concentration of the drug using the equation in section 2.4.1.

2.4.1.2 Encapsulation efficiency of vitamin K3

For quantification of Vit K3 indirect method, using the unencapsulated drug, the aliquots from chromatography were collected. Then the method for detection of VitK3 concentration was applied as explained in section 2.3.2. The constructed

calibration curve calculated the concentration. The encapsulation efficiency of Vit K3 was calculated by the ratio of encapsulated Vit K3 to the initially added Vit K3 using the equation in section 2.4.1.

2.4.2 Drug loading measurement

Drug loading is the actual capacity of the liposomes to encapsulate and deliver the drug. It is expressed as a drug to lipid ratio, as drug loading affects the therapeutic index of the liposomes. It indicates the actual dosage of the drug that will reach the target according to amount of liposome that is delivered to the target site. It is estimated by calculating the ratio of the actual encapsulated drug (DOX, VitK3) to the amount of present lipids (HSPC) in the formulation. The encapsulated drug is quantified by the previous spectrophotometric measurements, and lipids are calculated by Stewart assay. For quantification of Vit K3 indirect method, using the unencapsulated drug, the aliquots from chromatography were collected. Then the method for detection of VitK3 concentration was applied as explained in section 2.3.2. The constructed calibration curve calculated the concentration. The encapsulation efficiency of Vit K3 was calculated by the ratio of encapsulated Vit K3 to the initially added Vit K3 using the equation in section 2.4.1.

$$Drug\ Loading(\%) = \frac{Amount\ of\ encapsulated\ \ DOX\ or\ Vit\ K3}{Amount\ of\ liposomal\ lipid} x100$$

2.4.3 *In Vitro* release studies

The release profile of liposomes was studied by the dialysis method. Liposomes were put in a semi permeable cellulose dialysis bag (12000MW cut-off) in fractions of 1 mL in 15 mL polypropylene tubes containing 10mL phosphate buffer saline PBS. The setup was placed in an automated water bath shaker maintained at 100 rpm and 37°C (NUVE, ST 30, Turkey). At specified time intervals of 6, 24, 48, and 72 h, 1 mL aliquot was collected from the tube medium. The total media was changed with

fresh PBS (0.1 M, pH 7.4) at all collection intervals. The released drugs were measured spectrophotometrically for doxorubicin at 480nm and with a specific colorimetric method for VitK3 at 520 nm. Cumulative release amounts vs. time graphs were plotted for each formulation release studies were done in triplicates.

2.4.4 Particle size measurement

The particle size and polydispersity index (PDI) were measured using dynamic light scattering (DLS) by the refractive index of the light by the liposomes. DLS measures liposome size by measuring the average hydrodynamic diameter. Besides that, it provides the polydispersity and homogeneity information for the liposome size distribution. Prior to measurement, liposomes were diluted using NaCl (0.9%) at 1:10 (v:v) ratio. The size distribution of the solution was measured by a particle size analyzer (Malvern Mastersizer 2000) in Central Laboratory, Middle East Technical University, METU.

2.4.5 Surface charge measurement

Zeta potential analysis is applied to measure liposomal vesicles' surface charges in a given suspension medium. Using 0.9% NaCl, liposomal doxorubicin solution was diluted in a 1:2 (v:v) ratio, and VitK3 and empty liposomes were diluted by PBS at a ratio of 1:2 (v:v), then measured by Malvern Zetasizer 2000 in Central Laboratory, Middle East Technical University, METU.

2.4.6 Morphology characterization

Transmission electron microscopy (TEM) analysis was used to investigate the liposomal morphology. A drop of liposomal solution was placed on a mesh copper grid, left to air dry, and absorbed overnight. Then it was negatively stained by 2% uranyl acetate. The samples were characterized at 80 kV under a transmission

electron microscope (Philips, JEM-100CX) in Central Laboratory, Middle East Technical University, METU.

2.4.7 Liposomes stability studies

Liposome formulations were stored at 4°C for up to 45 days to study the stability of doxorubicin, and vitamin K3 loaded liposomes. Then particle size determination, zeta potential, and drug encapsulation efficiencies were determined as a function of storage time.

2.5 Cell culture studies

2.5.1 Cell culture conditions

Cell culture studies were done to evaluate the cytotoxicity of the drugs, liposomes, and drug liposomal formulations. *In vitro* studies were performed on mouse fibroblast cell lines (L929 cells) as a control cell type and on human breast adenocarcinoma cell lines (MCF-7 cells). L929 and MCF-7 cells were grown on tissue culture plates using cell culture growth media consisting of high glucose Dulbecco's Modified Eagle Medium (DMEM), phenol red with 10% (v/v) Fetal Bovine Serum (FBS), and 1% (v/v) Penicillin/Streptomycin antibiotic. The cells were incubated in a 5% CO2 – 95% air humidified atmosphere incubator at 37°C (Panasonic, InCu safe, Japan). Cells' growth media was replaced with fresh media every two days. When 80% confluency is reached, the cells are passaged onto a new plate.

2.5.2 Cytotoxicity assay

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay was used for cell proliferation inhibition experiments to assess the cells' viability. The

principle of this assay is based on an MTT reagent, a positively charged tetrazolium dye capable of penetrating metabolically active cells. These viable cells convert MTT into formazan crystals, a purple insoluble product (Mosmann, 1983). Then, the absorbance of this product is measured spectrophotometrically at wavelength= 570nm. The intensity of the purple color produced reflects the amount of cell metabolic activity as an indicator of the viability of cells. L929 and MCF-7 cell lines were used to evaluate the cytotoxicity of VitK3 and DOX liposomal formulations and free forms of VitK3 and DOX separately and together.

MCF-7 and L929 cells were seeded at a density of $4x10^3$ cells/well on 96 wells and incubated for 24 hours to allow attachment and grow plate in a 5% CO2 – 95% air humidified atmosphere incubator (Panasonic, InCu safe, Japan) at 37°C. For cytotoxicity experiments, the media is removed and treated with the different experimental groups at the end of 24 h. Different concentrations of free drugs and liposomal formulas were added to the wells, and the cells were treated with these dug/formulation groups for 24, 48, and 72 h periods. The treatment was followed by discarding the media. The experimental and control group wells were washed with sterile PBS (0.01 M, pH 7.4). MTT solution was added to the wells (100 μL/well) and incubated for 4 hours in darkness. After incubation, the MTT dye solution was removed, and DMSO was added (100 μL/well) to solubilize the formazan crystals while shaking the plate for 15 minutes continuously. Then the optical density of the wells was measured by spectrophotometry with a microplate reader (Molecular Devices, SpectraMax ID3, USA) at 570 nm.

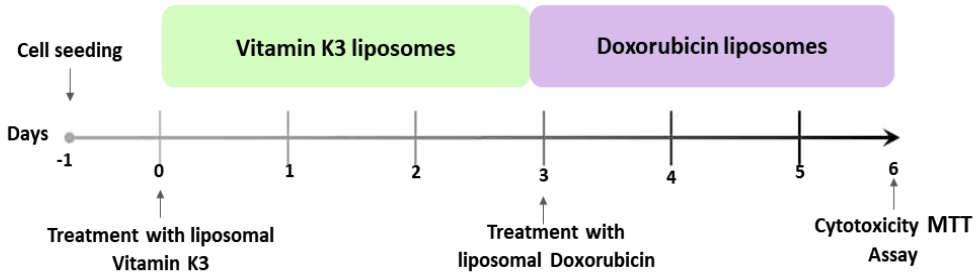

Figure 16: Liposomal pretreatment scheme for cell culture studies

2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used as a statistical analysis to compare different groups for a single parameter. IBM SPSS 28 software program was used for Tukey post-hoc pairwise comparisons. Minimum confidence level that was considered as statistically significant result was 95%, with p-value<0.05.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Characterization of liposomal formulations

3.1.1 Encapsulation efficiency, drug loading and lipid recovery

Encapsulation efficiency (EE) is affected by the choice of phospholipids, cholesterol amounts, method of synthesis, the encapsulated agent (charge, polarity, size…), and method of encapsulation (Gonzalez Gomez et al., 2019). Encapsulation of drugs into liposomes can be done in two ways, passively or actively. In passive methods drug is added at different steps while producing the liposomes, such as adding it to the lipid mixture prior to the formation of the lipid film. This method is done mainly for hydrophobic drugs which get entrapped in the liposomal bilayer. In a study by (Zhang et al. (2012)), Liposomal docetaxel formulation was produced passively, and incorporated into the phospholipid bilayer, when mixed with the lipid mixture before the lipid film formation. They achieved 36.4% encapsulation efficiency. Active loading of drugs or bioactive agents is another method, and it is used mainly for hydrophilic drugs. This method is based on diffusion, driven by the formation of an ionized gradient between the intra and extra environment of liposomes. An example of such loading is doxorubicin-loaded liposomes by Fritze and co-researchers; the encapsulation efficiency in their study ranged from 16% to 100% with the usage of various salt gradients, having the highest EE with ammonium sulfate (98.1%) and ammonium citrate (100%) (Fritze et al., 2006).

Unencapsulated agents are removed after the production of liposomes by chromatography, and the obtained eluted solution can be used for the measurement of EE indirectly by subtracting this concentration from the initial amount of added drug. Alternatively, measurements can be done directly by disrupting the liposomes

using organic solvents, and the drug (agent) gets released in the extraction medium and quantified.

Table 2: Encapsulation efficiency, drug loading and lipid recovery of empty and drug-loaded liposomal formulations (n=2)

Liposome formulation	Encapsulation Efficiency (%)	Drug Loading (%)	Lipid Recovery (%)
DOX-loaded liposomes	96.75 ±1.1	2.82±0.85	72.64
VitK3-loaded liposomes	53.81±14	13.47±3.27	68.48
Empty liposomes	-	-	81.13

In this study, DOX was encapsulated using an ammonium sulfate ion gradient, and the EE (%) was obtained by the direct rupture of liposomes. The advantage of active loading of drugs is the high EE (%), as seen in Table 2. Here, DOX was encapsulated at 96.75 ±1.1% values. Menadione sodium bisulfite, the hydrophilic form of vitamin K3, was loaded passively during the hydration step after initial trials and optimization experiments. For optimization of VitK3 loaded liposomes preparation, vitamin K3 was dissolved in ammonium sulfate as a rehydration solution and then continued liposome preparation by film hydration. Upon dialysis in NaCl (0.9%), chromatography was done, followed by quantification of vitamin K3; encapsulation efficiency was found to be very low (below measurable units). Another trial was preparing vitamin K3 liposomes, using ammonium sulfate gradient as DOX-loading, which also resulted in 0% encapsulation efficiency.

A passive method was used for dual encapsulation of DOX and VitK3; initially before cell culture studies using DPPC lipids. Both drugs were dissolved in PB solution (pH=5) and then hydrated the lipid film using the solution containing the drugs. The encapsulation efficiency of DOX was 58.77% and of VitK3 was 33.75%. Co-loading of DOX and VitK3 was not continued; after cell culture findings, it was

revealed that Vitamin K3 inhibits the cytotoxicity effect of doxorubicin; thus, the liposomal formulation of the coloaded drug is expected to show lower cytotoxicity than individual liposomal formulations.

Quantifying encapsulated menadione sodium bisulfite, a hydrophilic form of Vitamin K3 was done indirectly by collecting the eluted solution after chromatography. EE (%) of Vitamin K3 was found to be around 53.81±14%. In passive encapsulation methods, hydrophilic molecules are not loaded in the liposome with high efficiency; due to the exchange surface limitations, the liposomal core is not as hydrated as the outside medium (Pattni et al., 2015). Passively loading of hydrophilic antibiotic ciprofloxacin during hydration of the lipid film was 68.5-70.2% efficient (Feghhi et al., 2020).

Drug loading efficiency was higher for VitK3 liposomes than DOX liposomes. This might be due to the higher number of added drugs initially; VitK3 was added ten times more than doxorubicin.

Table 2 shows that the lipid recovery was 72.64 and 68.48 % for Dox and VitK3 loaded liposomes, respectively. So, an 8.5% and 13.13% decrease in lipid recovery was seen with DOX and VitK3 loading compared to empty liposomes.

3.1.2 *In vitro* drug release profiles

The purpose of drug delivery systems is to control the encapsulated drug's release, enhancing its bioavailability. Drug release studies evaluate the amount and pace of drugs released from the liposome's core, defining the liposomal system's pharmacological activity. The *in vitro* release profile provides an idea about the system's release behavior *in vivo*. When the active agents are encapsulated within liposomes, they are not therapeutically active. They are released from the liposomes expectantly at the target site and become active. The cumulative release profiles were done by membrane diffusion technique using dialysis tubing in the release medium; the drug has to diffuse from the liposome and then diffuse through the membrane

into the release medium, where it will be quantified (Solomon et al., 2017). Figure 12 shows the release profile of doxorubicin, free DOX release from dialysis tubing was used as a control.

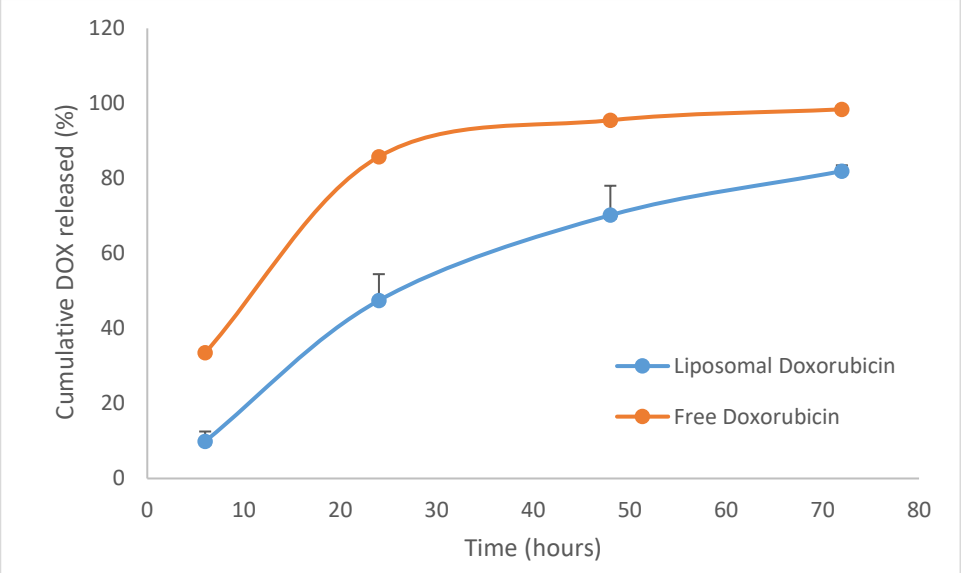

Figure 17: Liposomal and free doxorubicin release profiles in PBS, pH:7.4, at 37°C (n=3).

Table 3: Summary of release profiles

Sample	Drug % released after 6h	Drug % released after 72h	Time it took 50% of total drug to be released (t50)
Liposomal VitK3	20	24	-
Liposomal DOX	10	82	25 hours
Free DOX	33	98	9 hours

Figure 17 and Table 3 show that after 6 hours, 33.5% of free doxorubicin was released compared to 10% of the liposomal doxorubicin; this initial burst indicates that the diffusion of drugs out of the dialysis membrane can be neglected (Yamamoto et al., 2007). In less than 30 hours, around 90% of the initial amount of free

doxorubicin was released; meanwhile, even after 72 hours, 82% of doxorubicin was released from liposomes. Our results are like those of (Sabeti et al., 2014); in their study, about 80% of doxorubicin was released in 70 hours of incubation. This shows that encapsulating doxorubicin in liposomes controlled the release of the drug and allowed the slow release, which is expected to increase its bioavailability in blood circulation. PEGylation of liposomes sustains and stabilizes the release of drugs from liposomal formulations due increase rigidity of the phospholipid bilayer.

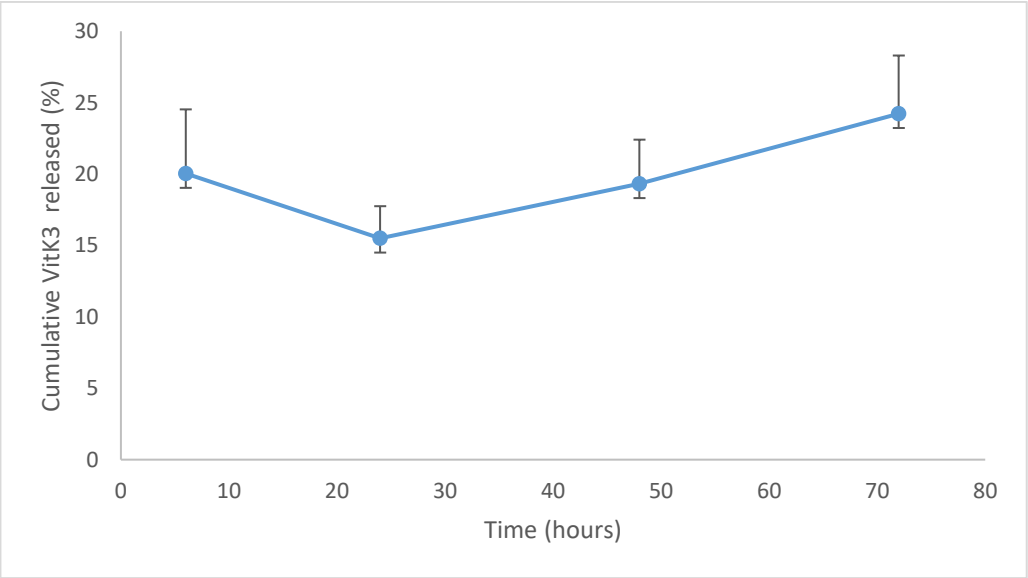

Figure 18: Liposomal Vitamin K3 release profile in PBS, pH:7.4 at 37°C (n=2)

As Figure 18 shows, the release profile of vitamin K3 liposomes, vitamin K3 was released in a burst manner after 6h of incubation, 20% of the encapsulated drug was released. After 24h of incubation, it was apparent that there was a decrease in the cumulative released amount according to the OD values being less than the previous (6h reading) from the same release media (only a small fraction was replaced with fresh media at 6th hour reading). This decrease was not expected to occur since vitamin K3 was protected from light at each step of liposome preparation and release. Besides, that VitK3 solution stability upon temperature exposure and incubations at room temperature was tested during calibration curve settling experiments.

However, after refreshing the total release media at 24 h, the release of VitK3 continued and gave about 3-5 % of the VitK3 into media each following day.

3.1.3 Liposomal particle size, polydispersity, and zeta potential measurement

To determine liposome particle size distribution and polydispersity and the effect of the encapsulated drugs and storage conditions on size distribution, particle size analysis was performed by dynamic light scattering on the liposomal formulations. Table 4 shows the structural analysis of the liposomal formulations produced.

Table 4: Size, PdI and zeta potential determination of liposomal formulations (PdI: Polydispersity index)

Liposome formulation	Z-Average (nm)	Peak Diameter (nm)	Width (nm)	PdI	Zeta Potential (mV)
Empty liposomes	148.1	142.0	36.32	0.038	-0.497
DOX-loaded liposomes	128.7	120.9	32.65	0.066	-0.389
VitK3-loaded liposomes	150.9	142.9	44.76	0.066	-3.610

During the preparation of liposomes by lipid film hydration, samples were extruded through 400, 200, and 100 nm porous polycarbonate membranes at 65°C, which is higher than the transition temperature and is enough to resize liposomes to obtain a 100 nm diameter. Particle size is an essential parameter in nanodrug delivery systems, as it affects molecule clearance, stability, biodistribution, liposome half-life in blood, and drug release. For enhanced penetration into the tumor blood vessel, nanoparticles should be able to fit within the range between 40-200 nm (Zein et al.,

2020). Nanocarriers smaller than 150 nm can penetrate fenestrated capillaries, like those in the surrounding tumor microenvironment. Larger than 100-150 nm liposomes can be recognized and taken up by the reticuloendothelial system and eliminated by the liver's phagocytes (Kupffer cells) and the spleen (Danaei et al., 2018). Therefore, for the chemotherapeutic drug (DOX) enhancement for anticancer effect, liposomes sized less than 150 nm can exploit the EPR effect and accumulate at the tumor site, releasing the drug locally. Thus, the average sizes of the liposomes produced mainly were within the required diameter (128-150 nm) for the administration of drugs in cancer therapy. The polydispersity index (PDI) refers to the population's size; if the sizes of liposomes are close to each other, the population is homogenous; if there is a broad difference between the sizes of the particles, the population is non-uniform, therefore heterogenous; having high PDI. The formulated products should be homogenous for a safe and stable administration of nanocarriers (Danaei et al., 2018). For liposomes, a population is considered monodisperse (homogenous) when PDI is less than 0.3 and is accepted for administration (Dragicevic-Curic et al., 2008). The liposomes produced in this study were within the required range regarding particle size and PDI.

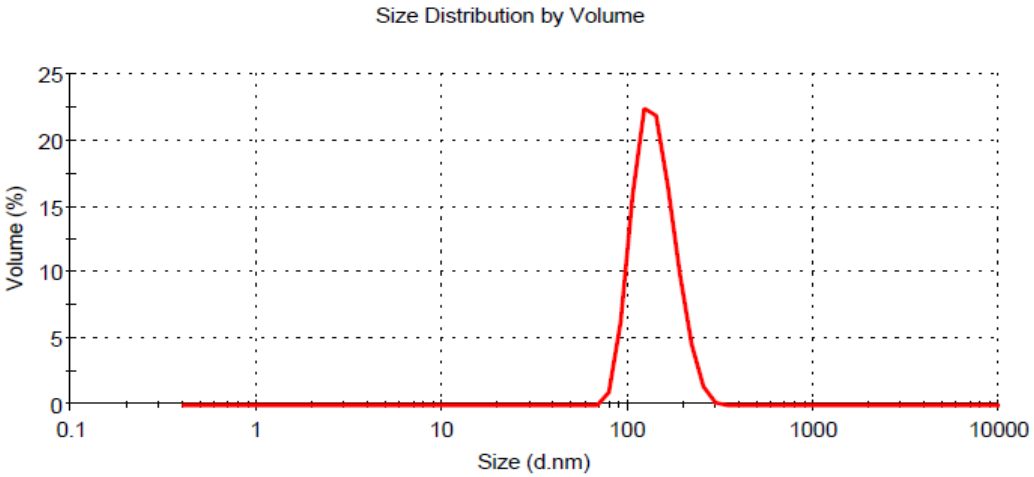

Figure 19: Size distribution by volume of empty liposomes

Figures (19-21) show the size distribution of the liposomal formulations; since all were functionalized by adding PEG, a hydrophilic molecule, a higher hydrodynamic

diameter greater than 100 nm was expected. It was also recognized that doxorubicin liposomes had the smallest size (average and peak size), and vitamin K3-loaded liposomes were slightly larger than empty liposomes.

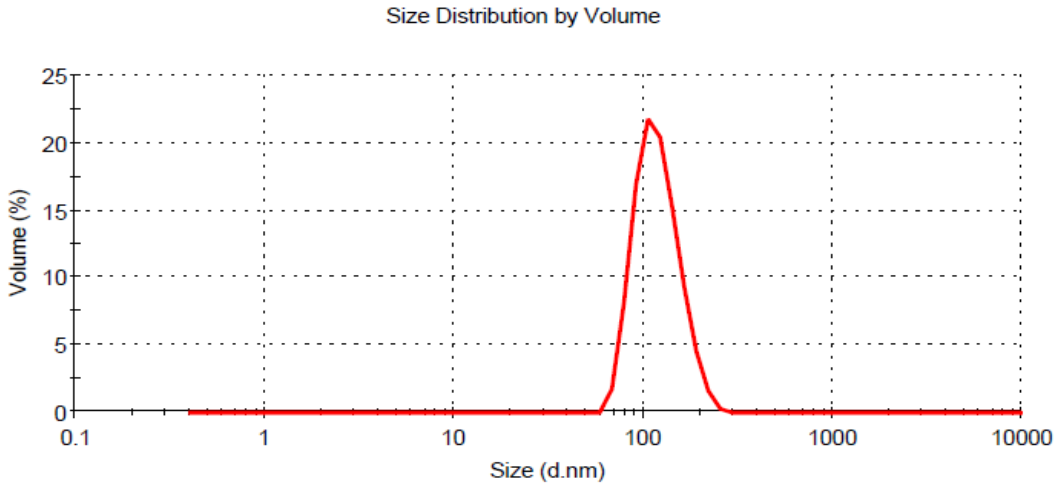

Figure 20: Size distribution by volume of doxorubicin loaded liposomes.

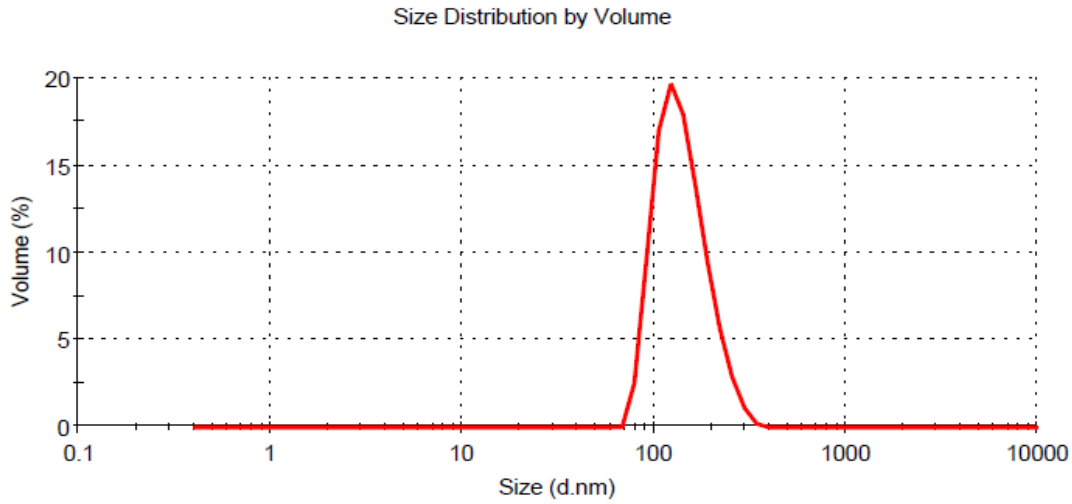

Figure 21: Size distribution by volume of vitamin K3-loaded liposomes

Zeta potential is another important physical parameter in evaluating liposomes for usage. Zeta potential describes the surface charge of liposomes; it is used to predict the stability of the suspended colloidal system, in this case, liposomes, in the medium (Hunter et al., 2001). Liposomes can be positive, neutral, or negative, depending on

the phospholipid used. In this thesis research, phosphatidylcholine was used, which is a neutral phospholipid (Hamilton et al., 1983). Neutral liposomes have very low interaction with cells and tend to aggregate over time, reducing their stability. Negative liposomes have higher lipid-cell interaction, increasing liposome uptake by target cells (Dicko et al., 2010). The addition of PEG has been shown to lower the negative charge of zeta potential (Garbuzenko et al., 2005). For liposomes preparation DSPE-PEG2000 was used, DSPE is a negatively charged molecule that anchors PEG onto the liposome bilayer, and long PEG chains (2000 molecular weight) neutralize the charge of the molecule (Holsæter et al., 2022). To indicate the stability of liposomes, zeta potential ranges more than 30mV and lower than -30mV, liposomes in the range between +30mV and -30mV are considered unstable and will aggregate over time (Manaia et al., 2017).

Table 4 shows the results of the zeta potential evaluation analysis; empty and doxorubicin liposomes had close zeta potential, but vitamin K3 liposomes had a lower higher zeta potential. Due to the negative charge of these liposomes, low aggregation is expected.

Table 5: Physical characterization of liposomal formulations after storage for 45 days, at 4°C

Liposomal formulation	Z-Average (nm)	PdI	Zeta Potential (mV)
Empty liposomes	139.9	0.035	-3.04
DOX-loaded liposomes	125.8	0.080	-1.86
VitK3-loaded liposomes	151.6	0.114	-3.98

Table 5 shows the results of particle size measurement, zeta potential and PdI after storage of the liposomal formulations for 45 days at 4°C. Particle size of the

liposomal formulations was somehow stable with simple change in the size, for the storage period and temperature, but the PdI of the liposomal vitamin K3, has increased more than empty liposomes, but it was still less than 0.3 making it administrable (Dragicevic-Curic et al., 2008). The zeta potential of Vitamin K3-loaded liposomes did not change much as it initially had a higher zeta value, and due to the electric repulsion, little aggregation occurred, meanwhile liposomal DOX and empty liposomes have shown a decrease in the zeta potential as these formulations initially had a lower zeta potential (Guldiken et al., 2018).

3.2 Cell Cytotoxicity

Determination of effective doses and drug treatment regimens for DO and Vitamin K3 with *in vitro* cell culture studies

3.2.1 Free Doxorubicin and Vitamin K3

Two cell lines were used for *in vitro* cytotoxicity studies; MCF-7 breast cancer cells and L929 mouse fibroblasts. MCF-7 cells originate from human epithelial adenocarcinoma, breast cancer cells. These cells express both estrogen and progesterone receptors (Comşa et al., 2015). They are commonly used as model cells for investigating drugs or other agents on breast cancer treatment potency (Lee et al., 2015). L929 cells originate from murine fibroblast cell lines, the oldest continuous cell line (Theerakittayakorn & Bunprasert, 2011); L929 cells originate from noncancerous, normal subcutaneous areolar and adipose tissue (Ma et al., 2012). L929 cells were used as a standard control to evaluate the specificity of anticancer agents to cancerous cells (Esghaei et al., 2018; Ghagane et al., 2017).

3.2.1.1 Doxorubicin cytotoxicity

To find the concentration of doxorubicin that inhibits 50 % of cell growth in MCF-7 cell line, cell viability was assessed after treatment with various concentrations (0.4 – 3 μg/mL) for 24 and 48 hours (Figure 22). IC50 was found to be around 1.5 μg/mL for 24 h and 0.6 μg/mL for 48 h treatments. In another study studying the effect of doxorubicin on MCF-7 cells, the IC50 was 0.68 μg/mL after 48 h of treatment; thus, our IC50 agrees with the literature (Fang et al., 2014). The concentration that caused 50% growth inhibition after 48 h was used as IC50 value for DOX for further cell culture studies: dual studies with DOX-VitK3 sequential treatment after pretreatment by vitamin K3, and liposomal formulations evaluations, as well as cell viability studies on L929 cell line. The viability percentage was calculated with respect to untreated MCF-7 cells as a control. As shown in the graph, the viability of MCF-7 was inversely proportional to the time of treatment and DOX concentration; therefore, the cytotoxicity was dose and time-dependent, but after certain concentrations, 1.5 μg/mL after 24 h and 0.8 μg/mL after 48 h, cells growth reaches a plateau and is not responsive to any increase in concentration. This dose effect stabilization might be due to the cell's resistance to DOX with the increase in passage number, the efflux pumps of cells, or cell saturation by doxorubicin.

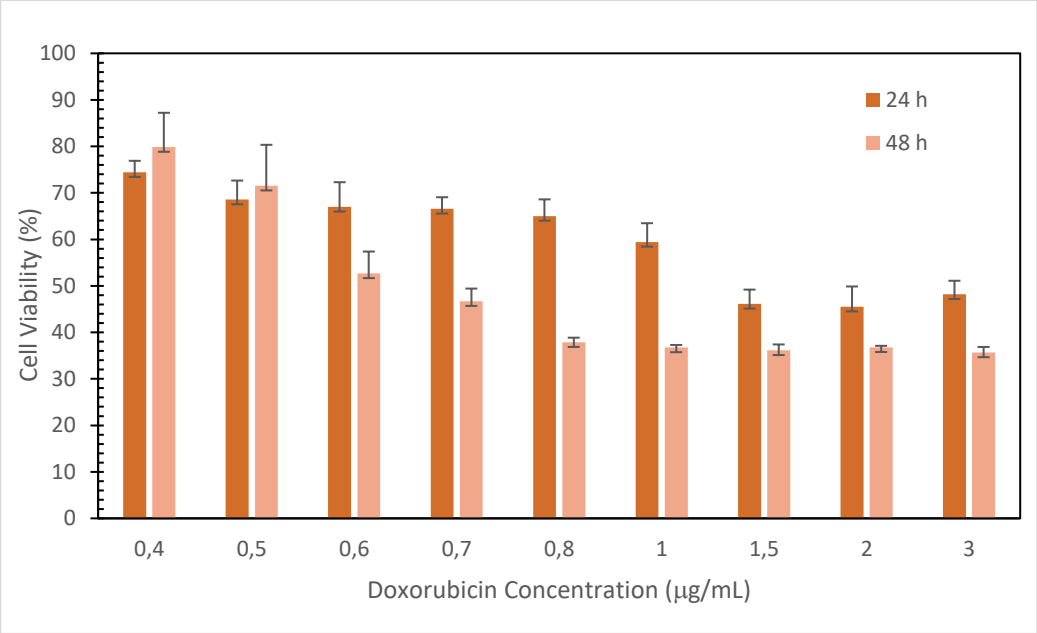

Figure 22: MCF-7 cell viability (%) in comparison to untreated cells, after treatment with various doxorubicin concentrations for 24 and 48 hours.(n=3)

To determine whether the cytotoxicity of doxorubicin was selective towards cancerous cells or if it will have similar cytotoxicity with used doses on nonmalignant cells, L929 cells were subjected to the same concentrations (0.4 – 3 µg/mL) of doxorubicin for 24 and 48 h. Figure 23 shows that L929 cells were not sensitive to doxorubicin between the concentrations (0.4-3 µg/mL) after 48h of treatment. After 72 h of exposure to doxorubicin, 50% inhibition occurred at 3 µg/mL. In a study by (Pyataev et al., 2019), 50% inhibition occurred at 2.85 µg/mL after 24 h of treatment, as their concentration is close to ours; just difference in treatment time might be due to the difference of seeded cells (2000 cells/well in their study, 4000cells/well in ours). DOX at a concentration of 3 µg/mL caused death in 24% of the L929 cells after 48 h, in contrast to 64% death in MCF-7 cancerous cells. At MCF-7's IC50 (0.6 µg/mL), only 17% of L929 cells were inhibited, showing that MCF-7 cells are more sensitive to DOX compared to L929 cells. The comparison of cytotoxicity on MCF-7 and L929 cell lines suggests that DOX has potent inhibitory activity on cancer cells.

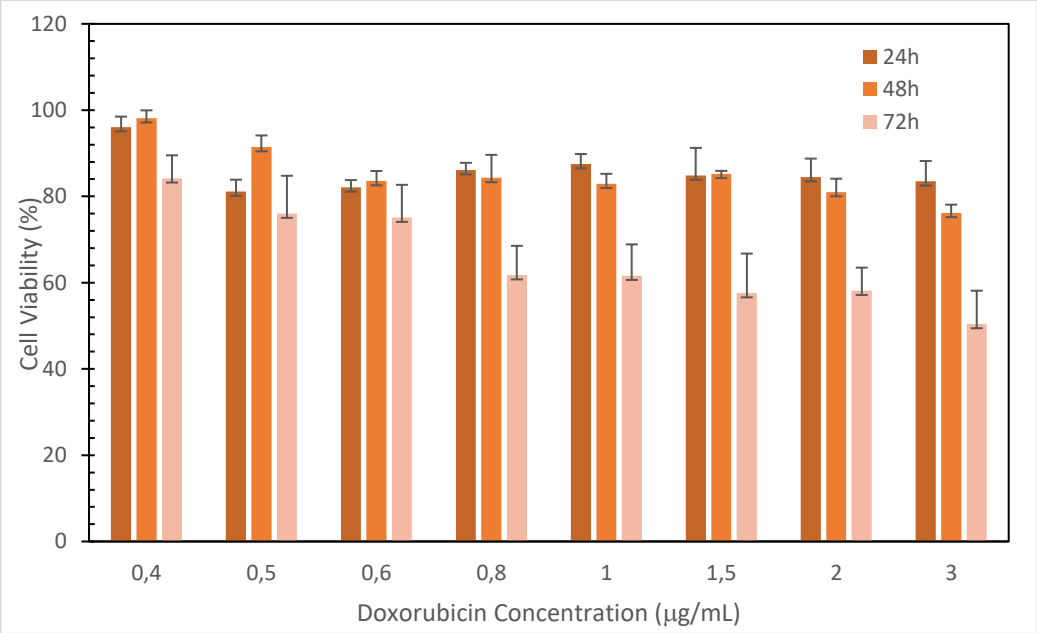

Figure 23: L929 cells viability (%) in comparison to untreated cells, after treatment with various doxorubicin concentrations after 24, 48 and 72 hours (n=3)

3.2.1.2 Vitamin K3 (menadione) cytotoxicity

MCF-7 and L929 cells were treated with different concentrations of vitamin K3 (MSB) (2.5,5, 6, 10 µg/mL) to evaluate the drug's cytotoxicity on cells. The cells were exposed to Vitamin K3 for 24, 48, and 72 hours, with the refreshment of the drug and media every 24 hours. Figures 24 and 25 show that both cell lines MCF-7 and L929 (respectively) are sensitive to Vitamin K3 cell growth inhibitory effects in a dose-dependent manner. Figure 24 shows that MCF-7 cells cytotoxicity is also time-dependent between concentration 2.5 µg/mL and 10 µg/mL, at concentrations higher than 2.5 µg/mL all dosage demonstrated a significant decrease in cell viability as early as 24 hours after treatment. Figure 25 shows 2.5 µg/mL did not show any significance change between 24 and 72 h in terms of cell viability meanwhile, 5 µg/mL demonstrated significant decrease in cytotoxicity as early as 24 hrs at the end 72 hrs Vitamin K3showed the lowest the cell viability at 5 µg/mL. After 5 µg/ml all of Vitamin K3 concentrations showed statistically significant lower cell viability

compared to 2.5 µg/mL. At 2.5 µg/mL L929 cell viability decreased from 74.24 % after 24 hours to 67.71% after 72 hours of treatment, showing at 2.5 µg/mL, MCF-7 were more sensitive to Vitamin K3 with time. Figure 24 shows that after 24 h of VitK3 treatment, 50% of cell inhibition in MCF-7 cells occurred around 6.5 µg/mL, assessing this concentration as IC50. In a study by (Yamada et al., 2015), they used menadione, the hydrophobic form of vitamin K3, the IC50 on MCF-7 was around 4.3 µg/mL, which is equivalent to 6.7 µg/mL of menadione sodium bisulfite (water-soluble form in this thesis research). At a concentration of 5 µg/mL, L929 cytotoxicity changed on day one from 46.77% to 30% on the second day to 6.11% on the third day of treatment, showing that at higher concentrations of VitK3 L929 cells are affected by the treatment time. In Figures 24 and 25, it is observed that L929 cells are more sensitive to Vitamin K3 inhibitory effect on the first two days at higher concentrations (6 µg/mL and 10 µg/mL) in comparison to MCF-7 cells with 53%, 48% after 24 and 48 hours respectively at 6 µg/mL compared to L929 cells viability of 13% and 11% after 24 and 48 hours respectively.

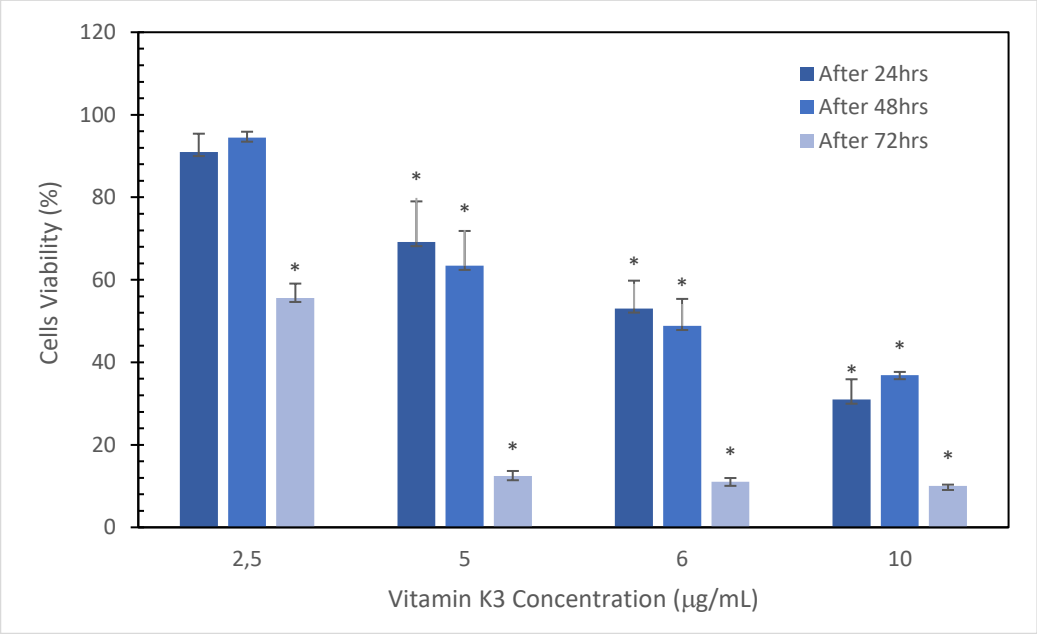

Figure 24: MCF-7 cell viability (%) in comparison to untreated cells, after treatment with different Vitamin K3 (menadione) concentrations for 24, 48 and 72hours (n=3), asterisks denote statistical significance at $p<0.005$.

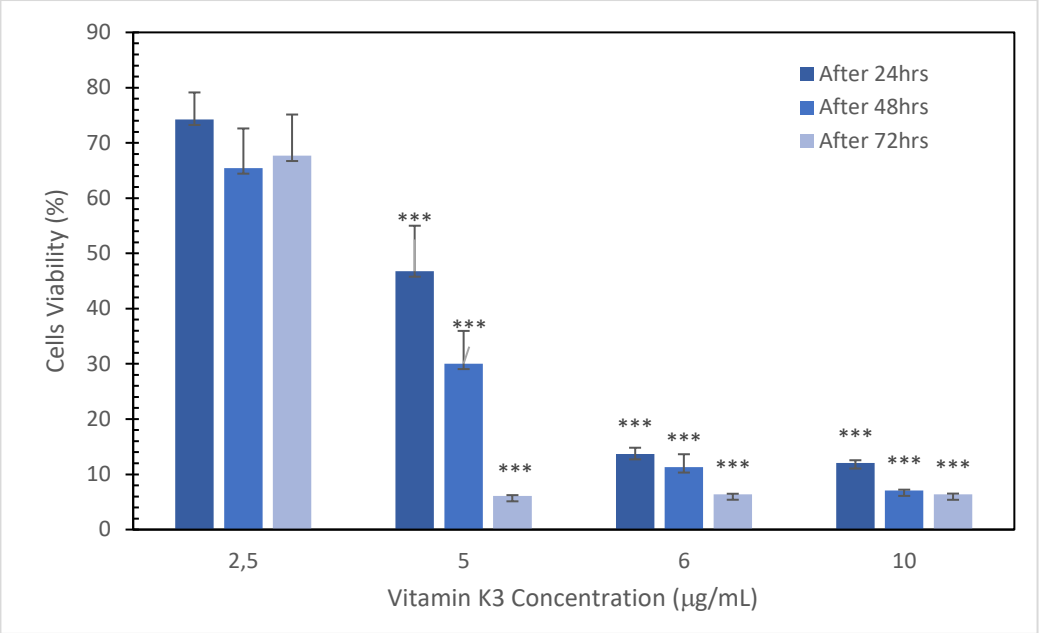

Figure 25: L929 cell viability (%) in comparison to untreated cells, after treatment with different vitamin K3 (menadione) concentrations for 24, 48 and 72hours, (n=3), (p***<0.001)

3.2.1.3 Doxorubicin and Vitamin K3 dual cytotoxicity

To study the combined effect of menadione and doxorubicin on cell viability, MCF-7 and L929 cells were treated with 0.65 µg/mL of doxorubicin and different concentrations of menadione (3, 4, 5, 6 µg/mL) together. In the MCF-7 cell line, 0.6 µg/mL was considered the IC50 after 48hrs, as it caused cytotoxicity to 50% of the cells, and in L929 cell line, the same concentration caused the same cytotoxicity for 24% of cells. MCF-7 and L929 cells were therefore treated with these doses, and results were demonstrated in Figures 26 and 27. The results were highly unexpected, so the entire experiment was repeated with triplicate sets, and the same result was verified. Accordingly, the two drugs inhibited each other's mechanism of action in terms of cytotoxicity. Thus, the cytotoxicity effect of doxorubicin at IC50 dose (observed for 2nd day with only DOX) was decreased, and it did not cause a 50% inhibitory effect even after 72 h of treatment for the cancer cell line, and the decrease in cell viability was statistically significant only after 72 hours of treatment, but there

was no significant decrease in cell viability with the increase of concentrations of the drugs on MCF-7 cells.

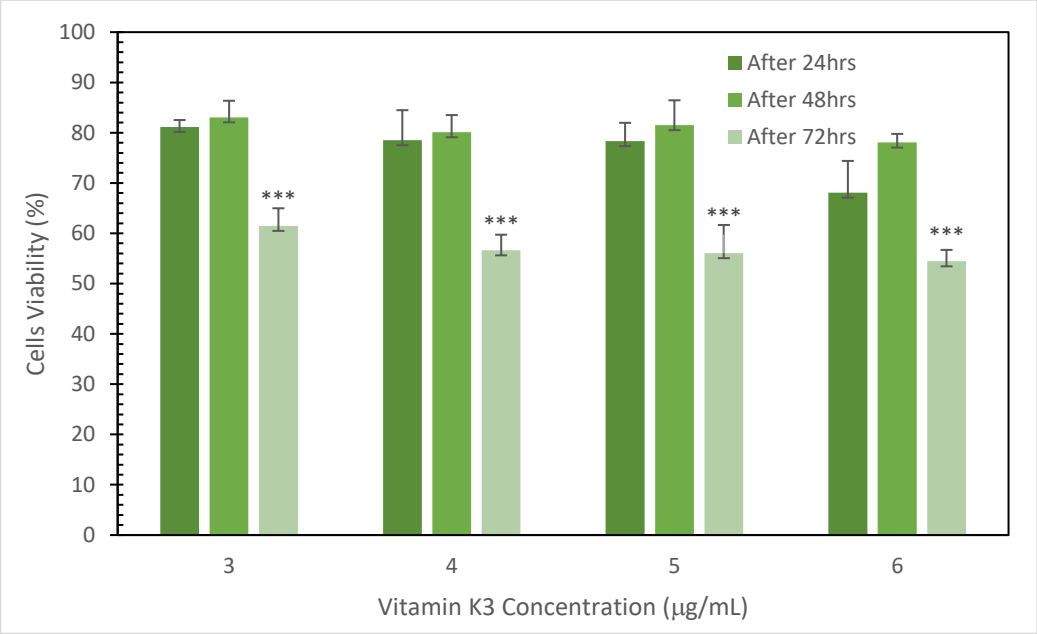

Figure 26: Dual treatment of MCF-7 (%) in comparison to untreated cells, cells, with different vitamin K3 concentrations and doxorubicin at 0.6 µg/mL (DOX IC50 to MCF-7) (n=3), p*** shows statistically significant at $p<0.001$

When L929 cells were treated with DOX at a dose of 0.6 µg/mL and menadione (3, 4, 5, 6 µg/mL) together, this dual treatment with menadione and DOX decreased the cytotoxicity effect of single VitK3 but increased it compared to single Dox. With only Dox (at 0.6 µg/mL), these cells had relatively high viability (>70%) for the 3-time points. However, it decreased slightly at 4 µg/mLVitK3 during dual use and more with higher doses (Figure 27). Dual treatment of L929 cells showed that both time and concentrations increase had a significant effect on cell viability of L929 cells.

In other words, after 48 hrs of treatment with 5 µg/mL of vitamin K3 alone, 70% cytotoxicity was achieved (with 30% viability), meanwhile when 5µg/mL was added with 0.6 µg/mL of DOX, cytotoxicity decreased to 46% (with app. 54% viability), thus showing that DOX has an inhibitory effect on vitamin K3 for these cells.

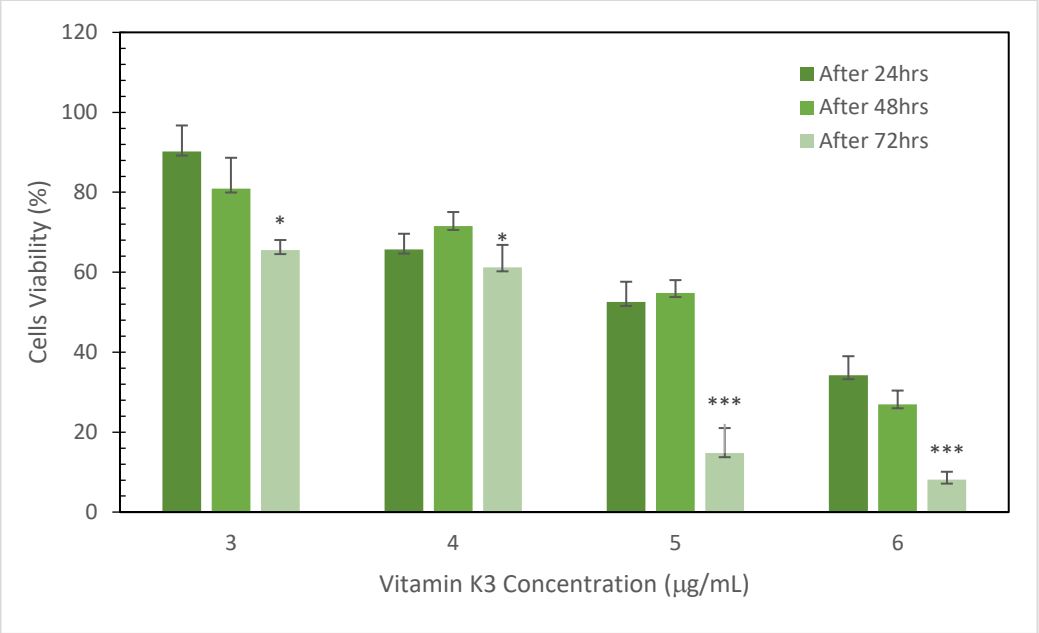

Figure 27: Dual treatment of L929 cells, with different vitamin K3 concentrations and doxorubicin at 0.6 µg/mL (DOX IC50 to MCF-7) (n=3), Asterisks show statistically significant at p*<0.05 and p**<0.001

3.2.1.4 Pretreatment of cells with Vitamin K3 before Doxorubicin

The experiments in this thesis research aimed to evaluate the synergy/additive effect of VitK3 on doxorubicin. However, inhibitory effects on cancer cells and enhancement effects on normal cell line was observed. Another study showed that pretreatment Ehrlich ascites carcinoma cells resistant to doxorubicin with 10 µg/mL of vitK3 lowered the IC50 from 22.3 µg/mL to 5.3 µg/mL (Xu, Zhang, Wang, & Zhang, 1998).

Considering these previous approaches for dual treatment, MCF-7 and L929 cell lines were pretreated for 72hrs with 3 µg/mL and 6 µg/mL of vitamin K3 group 1 and 2, respectively, while refreshing the drug and media daily and then treated with only DOX at 0.6 µg/mL at the 3rd day for only 24 hours. As a control, cells were treated with 0.6 µg/mLof doxorubicin alone. Figure 28 shows that all the groups are statistically significantly different than each other. Group 3 has the highest value,

and group 2 has the smallest value (p***<0.001). Figure 28 shows that pretreatment of cells with Vitamin K3 before the administration of Doxorubicin, showed no increase in the cytotoxicity. Comparing free administration of Vitamin K3 (Figure24) showed 11% cell viability on MCF-7 cells after treatment with 6 µg/mL VitK3, in the pretreatment experiment the cell viability of MCF-7 cells was around 18% after treatment with 6 µg/mL of Vitamin K3 for 72 hours followed by DOX IC50 for 24 hours. Showing that the pretreatment had no increase in the efficiency of vitamin K3 and DOX treatment

In Figure 29 the pretreated groups (1-2) showed significant decrease in cell viability when compared to DOX IC50. In the L929 cell line, 6 µg/mL VitK3 pretreatment before DOX IC50, had 74% cell viability and when compared to free administration of VitK3 (Figure 25) 6 µg/mL had only 7% cell viability after 72 hours. Figure 29 also shows that the treatment with DOX IC50 after pretreatment with vitamin K3 inhibited the effect of vitamin K3 on cells.

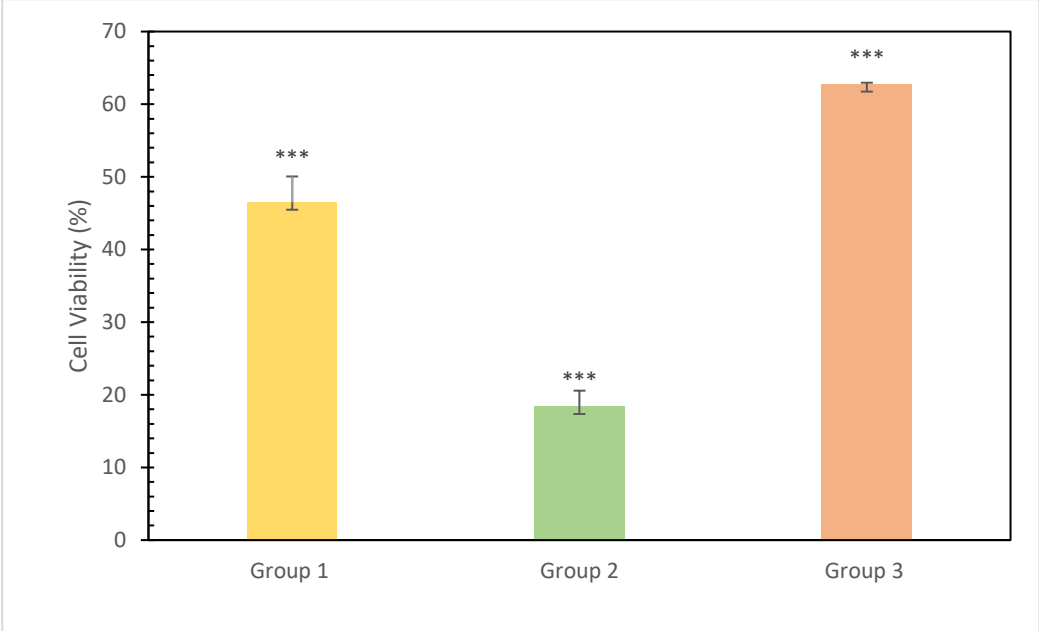

Figure 28: MCF-7 cell viability (%) in comparison to untreated cells, cells treated with different groups. Group 1: 3 µg/mL VitK3 pretreatment then addition of doxorubicin 0.6 µg/mL. Group 2: 6 µg/mL VitK3 pretreatment then addition of

Doxorubicin 0.6 μg/mL. Group 3: Doxorubicin 0.6 μg/mL alone on 3rd day (n=3), asterisks show statistical significance at p***<0.001

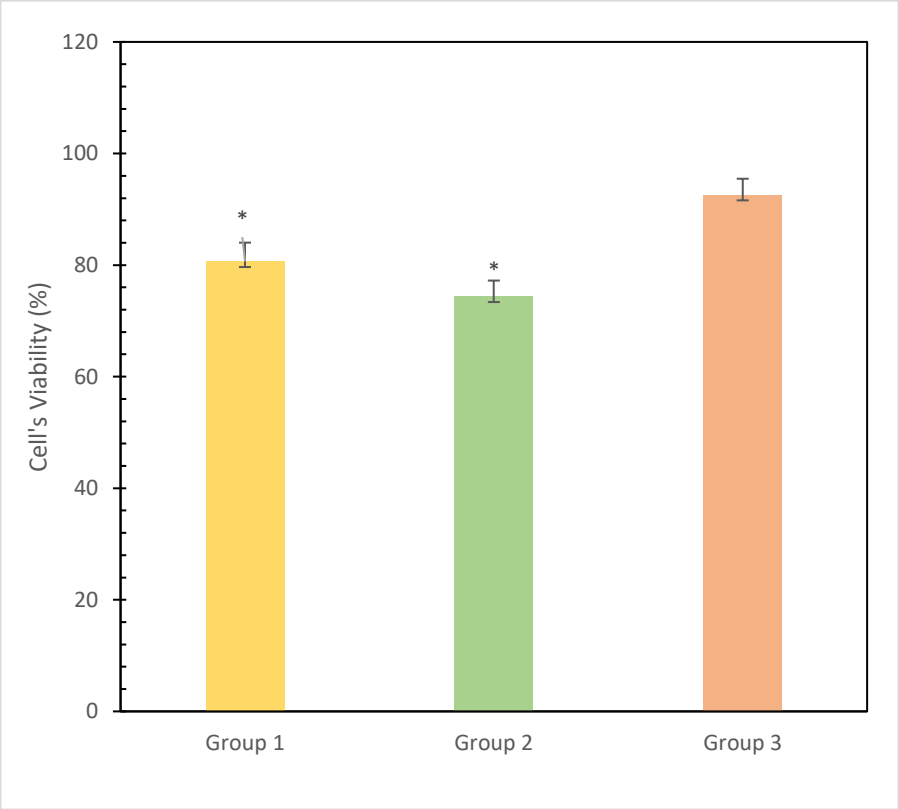

Figure 29: L929 cell viability (%) in comparison to untreated cells, cells treated with different groups. Group 1: 3 μg/mLVitK3 pretreatment then addition of doxorubicin 0.6 μg/mL. Group 2: 6 μg/mLVitK3 pretreatment then addition of Doxorubicin 0.6 μg/mL. Group 3: Doxorubicin 0.6 μg/mL alone on 3rd day (n=3), asterisks show statistical significance at p*<0.05.

3.2.2 Liposomal formulations cytotoxicity

Vitamin K3 and doxorubicin are both potent drugs against cancer, and their main drawback is the adverse side effects exerted on the patients. To enhance their therapeutic index, liposomal formulations of both drugs were produced. Doxorubicin HCl is hydrophilic, and menadione sodium bisulfite is the water-soluble form of vitamin K3, so they are entrapped in the aqueous core of liposomes. The cytotoxicity

of liposomal formulations of each drug was studied, as well as the pretreatment of liposomal doxorubicin with liposomal vitamin K3.

3.2.2.1 Empty liposomes cytotoxicity

MCF-7 cells were treated with different concentrations of empty liposomes to assess the cytotoxicity of HSPC lipids on cells. Cell viability was evaluated after 24, 48, and 72 hours of treatment with 100 – 200 – 300 – 400 – 500 nmol/mL of empty liposomes. Figure 30 shows that the cytotoxicity of empty liposomes ranged between 8.3% and 19%; the trend of cytotoxicity was the highest on the first day for all liposome concentrations, and then maintaining the cytotoxicity was approximately constant.

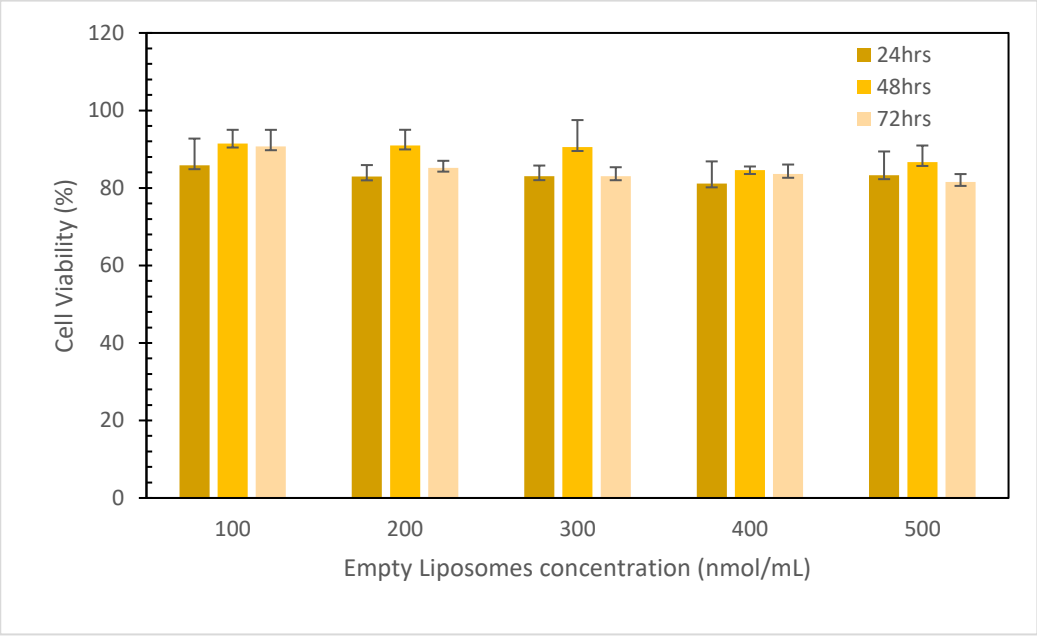

Figure 30: MCF-7 cell viability (%) in comparison to untreated cells, after treatment with empty liposomes for 24, 48 and 72 release hours (n=3)

3.2.2.2 Liposomal doxorubicin cytotoxicity

To evaluate the cytotoxicity of liposomal formulation of doxorubicin, MCF-7 and L929 cells were treated with different concentrations of DOX liposomes. Cells were treated with 50, 100, 200 nmol/mL of HSPC lipid containing 0.6, 1.2, 2.4 μg/mL of doxorubicin respectively. These concentrations were chosen as IC50 (0.6 μg/mL) concentration, 2xIC50 and 4xIC50 concentrations, respectively. Figures 31 and 32 show the treatment results for 24, 48, and 72 hours.

In Figure 31, it is shown that after 24h of incubation of doxorubicin loaded liposomes, it exhibited a very low level of cytotoxicity. However, with increased time and concentration, the cytotoxicity also increased; thereby, the cytotoxicity of doxorubicin loaded liposomes occurred in a dose and time-related manner. In another study evaluating the cytotoxicity of liposomal formulation of doxorubicin, it also showed that after 24 hours the cytotoxicity of doxorubicin loaded liposomes was less than that of free doxorubicin, and with the increase of time the cytotoxicity increased (Haghiralsadat et al., 2017). The release study of doxorubicin shows that doxorubicin is released slowly from liposomes. This explains the cytotoxicity increase with time for liposomes. Considering that after 72 hrs, when 80% of the drug is released to the cell culture media, approximately 0.5 μg/mL is released from 50 nmol/mL of liposomes, therefore, nearly IC50 (0.6 μg/mL) was reached. In treatment with free doxorubicin, MCF-7 cells showed resistance after the concentration of 1.5 μg/mL, but when these cells were treated with liposomal doxorubicin, the cells were responsive to liposomal DOX formulations with 4 times IC50 Concentrations (app. 2.4 μg/mL) and the decrease in cell viability was statistically significantly different in terms of treatment time and concentrations.

In Figure 32, it is shown that L929 cytotoxicity was also time-dependent, as it showed 50% cell growth inhibition after 72hrs of treatment, but it was not dose-dependent, in the range of 0.6 – 2.4 μg/mL of liposomal DOX formulations. After 24 and 48 hours of treatment, cell viability ranged between 82% and 84% for the 3

concentrations applied. IC50 of DOX on L929 cells decreased from 3 μg/mL to around 0.5 μg/mL of liposomal doxorubicin, considering that 80% of the drug is released after 72hrs. The decrease in cell viability was only significantly after 72 hours of treatment with liposomal doxorubicin.

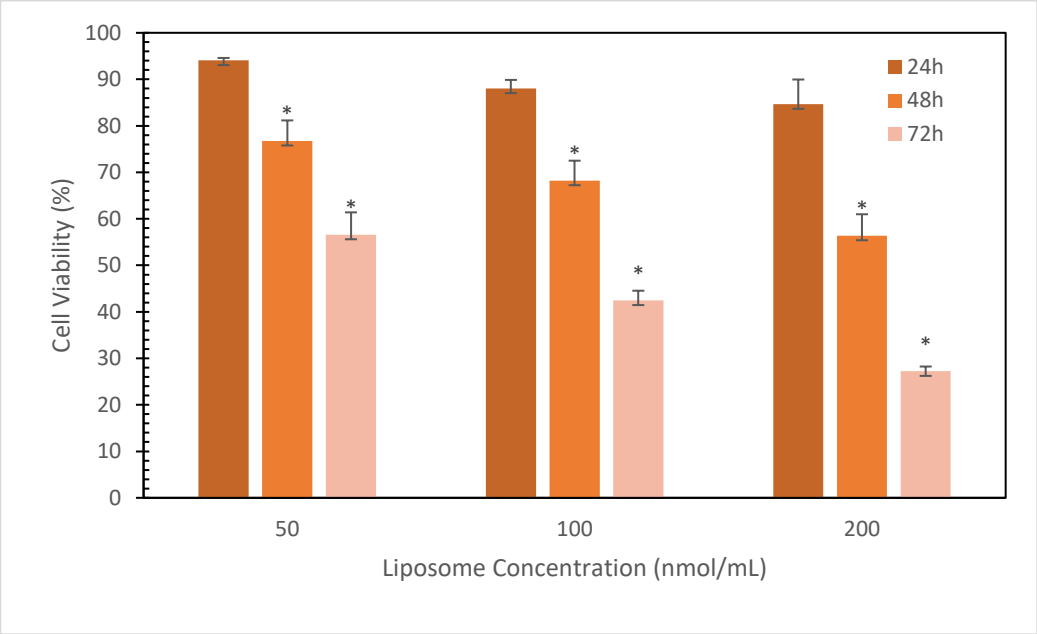

Figure 31: MCF-7 cell (%) in comparison to untreated cells, after treatment with doxorubicin loaded liposomes in different concentrations for 24, 48 and 72 hours 50 nmol/mL of liposomal DOX contains IC50 of DOX, 100 nmol/mL contains 2xIC50 of DOX, and 200 nmol/mL contains 4xIC50 of DOX (n=3), asterisks demonstrate statistical significance at p*<0.05.

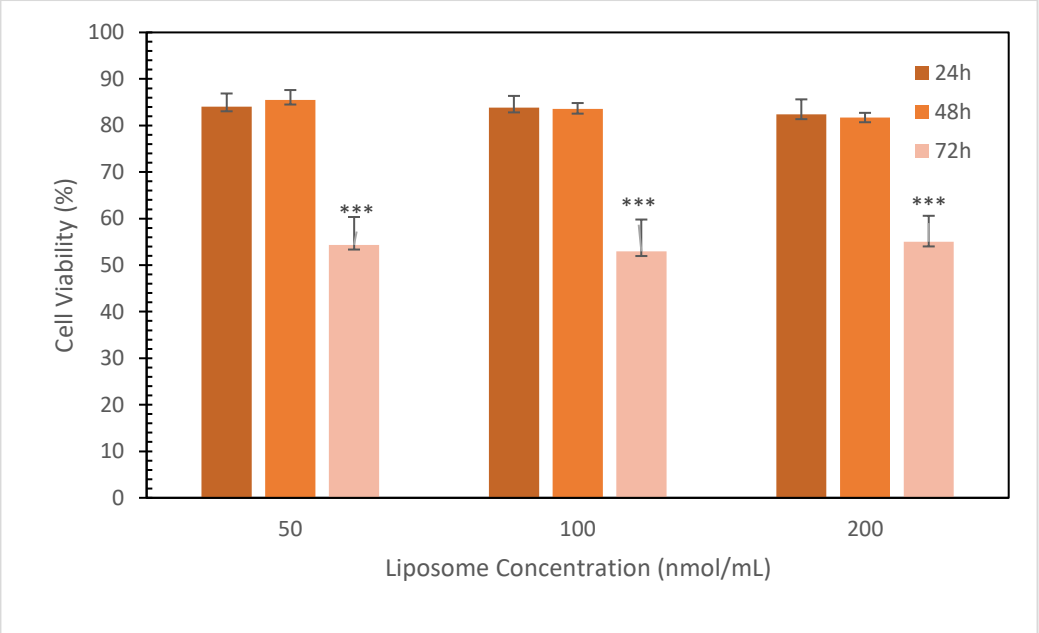

Figure 32: L929 cell viability (%) in comparison to untreated cells, after treatment with doxorubicin loaded liposomes in different concentrations for 24, 48 and 72 hours 50 nmol/mL of liposomal DOX contains IC50 of DOX, 100 nmol/mL contains 2xIC50 of DOX, and 200 nmol/mL contains 4xIC50 of DOX (n=3), asterisks demonstrate statistical significance at p***<0.001

3.2.2.3 Liposomal Vitamin K3 cytotoxicity

To assess the cytotoxicity of liposomal Vitamin K3, MCF-7 and L929 cells were treated with different concentrations of Vitamin K3 loaded liposomes, ranging from liposomal concentrations of 60, 120, and 240 nmol/mL, containing 6, 12, and 24 μg/mL of vitamin K3, respectively. These concentrations correspond to IC50, 2xIC50, and 4xIC50 doses of VitK3 obtained by previous results, IC50 being around 6 μg/mL.

Figure 33 shows that MCF-7 cells were not responsive to liposomal Vitamin K3 in a dose-dependent manner, instead only in a time-dependent way, inhibiting growth the most after 72 hours of incubation. During the first 2 days of incubation with liposomal Vitamin K3, the cell viability ranged from 86% to 89% for the 3 dosages administered, but on the third day of incubation, the cell viability ranged from 67%

to 73%. In Vitamin K3 liposomal formulations, only 24% of vitamin K3 was released in 72hrs, with the burst being after 6 hours, with 20% release; taking this into consideration, 1.2, 2.4, 4.8 µg/mL concentrations of Vitamin K3 were estimated to be released from the liposomal formulations respectively.

Figure 34 shows the cytotoxicity of liposomal Vitamin K3 on L929 cells; when cells were treated with 60 nmol/mL of liposomal Vitamin K3, containing IC50 dose, after 24hrs of treatment, cytotoxicity was around 9%, and this cytotoxicity did not increase with the increase of treatment time. On the other hand, when treated with higher concentrations of vitamin K3 loaded liposomes, cells were more responsive to the treatment time. The highest cytotoxicity with liposomal vitamin K3 exerted on L929 cells was 29%, at 240 nmol/mL liposomal vitamin K3 containing app. 4xIC50 dose.

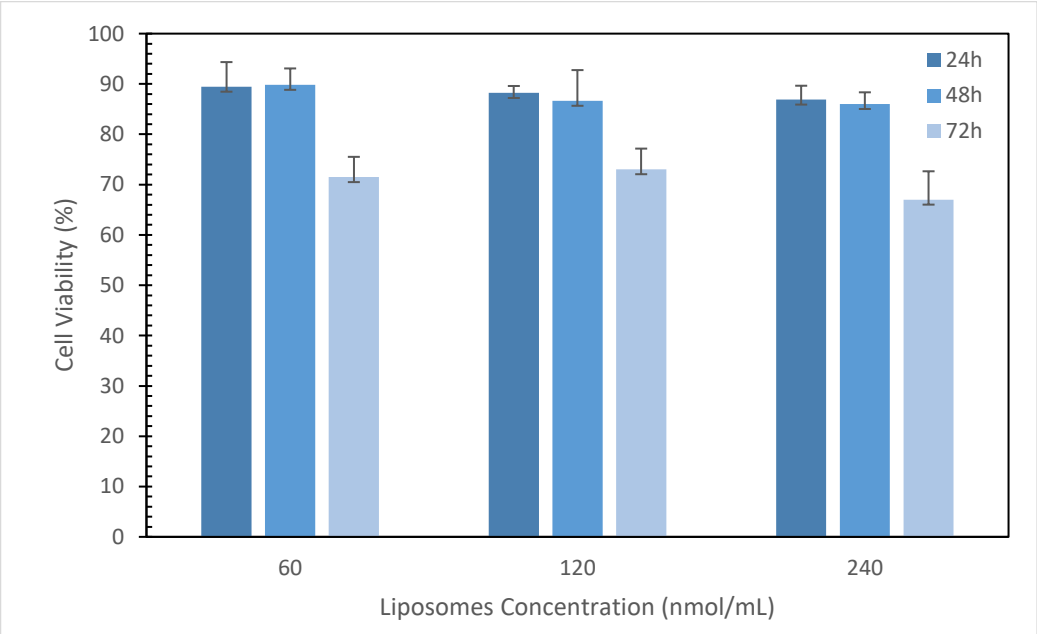

Figure 33: MCF-7 cell viability (%) in comparison to untreated cells, after treatment with different concentrations of VitaminK3 loaded liposomes for 24, 48 and 72 hours 60 nmol/mL of liposomal VitK3 contains IC50 of VitK3, 120 nmol/mL contains 2xIC50 of VitK3, and 240 nmol/mL contains 4xIC50 of VitK3 (n=3)

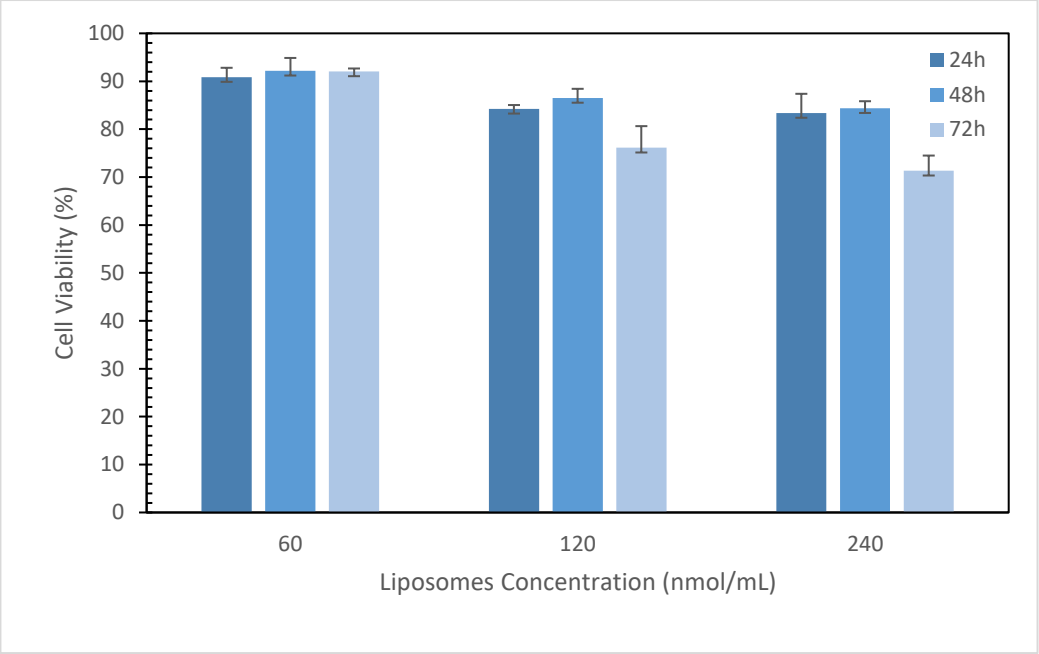

Figure 34: L929 cell viability (%) in comparison to untreated cells, after treatment with different concentrations of VitaminK3 loaded liposomes for 24, 48 and 72 hours 60 nmol/mL of liposomal VitK3 contains IC50 of VitK3, 120 nmol/mL contains 2xIC50 of VitK3, and 240 nmol/mL contains 4xIC50 of VitK3 (n=3)

In both cell lines, inhibition of 50% of cell growth was not reached; this might be due to the slow release of the drug from the liposomes. This might be advantageous as liposomal formulations will release most of the drugs when they reach the tumor site. A study showed that within the first 5 minutes, liposomes have accumulated at the tumor site and slightly start decreasing after 60 minutes (Viglianti et al., 2004). Due to the addition of PEG to liposomes, they are enabled to circulate for a more extended period (3-4 days) in the bloodstream, which means more extended time for accumulation at the tumor site due to the stealth property of PEG liposomes, avoiding RES (Olusanya et al., 2018).

3.2.2.4 Treatment with liposomal VitK3 before liposomal DOX Cytotoxicity

MCF-7 cells were pretreated with different liposomal groups; all groups were treated with liposomal vitamin K3 for 3 days, then replaced with liposomal doxorubicin for 3 days. Group 1 was treated with IC50 of VitK3 for 3 days, followed by treatment

with liposomal DOX for 3 days; group 2 was firstly treated with 2xIC50 VitK3, then 2xIC50 DOX, and group 3 cells were treated with 4xIC50 of VitK3 followed by 4xIC50 of DOX. Firgure 35, shows that MCF-7 cytotoxicity increased with the increase of the administered doses, demonstrating that cytotoxicity was dose-dependent as the decrease in cell viability was statistically significant at p***<0.001. The results of this regime were similar to the results of liposomal doxorubicin cytotoxicity on the third day showing no enhancement of its anticancer effects on MCF-7 cells. Results did not show high cytotoxicity compared with the pretreatment regime of free vitamin K3 and doxorubicin. Group 1 of this study and group 2 of the pretreatment of cells with free drugs both contained VitK3 IC50 and DOX IC50, which caused 82% cytotoxicity, and liposomal pretreatment only caused 25% cytotoxicity. The difference in the experimental setting is that in free drug pretreatment, vitamin K3 was refreshed every 24h; therefore, cells were exposed to IC50 of VitK3 on 3 occasions. As seen in the release studies, liposomal formulations gradually release drugs; after 72h, only 24% of vitamin K3 was released from the liposomal form, and 82% of DOX from its liposomal form. This slow release, specifically from VitK3 liposomes, might be the reason for the lower cytotoxicity.

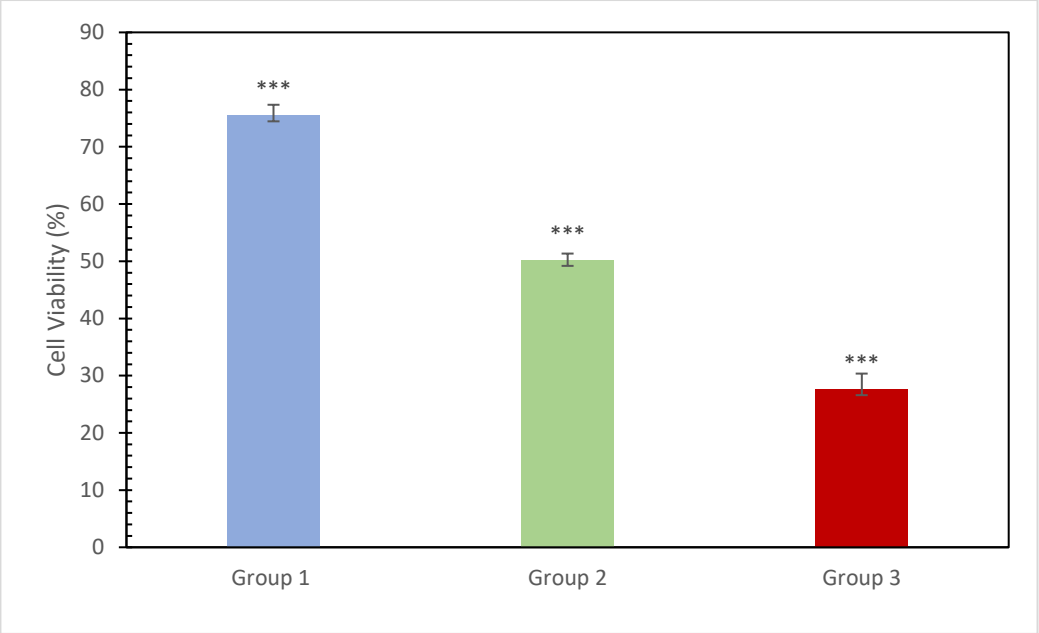

Figure 35: MCF-7 cell viability (%) in comparison to untreated cells, cells were pretreated with liposomal VitaminK3, before treatment with liposomal Doxorubicin: Group 1: IC50 VitK3 + IC50 DOX, Group 2: 2xIC50 VitK31+ 2xIC50 DOX, Group 3: 4xIC50 VitK3 + 4xIC50 DOX (n=3), asterisks demonstrate statistical significance at p***<0.001

CHAPTER 4

CONCLUSION

Liposomes are extensively used in the clinical setting as chemptherapeutic nanocarries. This study aimed to enhance the development of coadministration of doxorubicin and vitamin K3, , using liposomal formulations. Vitamin K3-loaded liposomes and DOX-loaded liposomes were prepared and characterized for encapsulation efficiencies, loading efficiency, and lipid recovery. The liposomal formulations were studied for their particle size, zeta potential, and release studies. After preliminary cell culture studies of dual drug treatment Vitamin K3 seemed to inhibit doxorubicin cytotoxicity; therefore, dual-loaded liposomes were not produced. Cytotoxicity studies have revealed that pretreatment of MCF-7 cells with 3 days of vitamin K3, followed by 24h of doxorubicin treatment, had no effect on increasing the cytotoxicity of the VitK3 but did increase the cytotoxicity of DOX.. Hence, pretreatment of cells with the liposomal formulations of drugs was conducted, with 3 days of treatment with liposomal vitamin K3 followed by 3 days with doxorubicin. The results have shown that this liposomal pretreatment has lower cytotoxicity than the free drug pretreatment; this might be due to the slow release of drugs from liposomes. Another reason might be that in free drug treatment, cells were exposed to Vitamin K3 daily for 3 days, followed by doxorubicin treatment for 24h. This regime is inapplicable in clinical settings due to the cytotoxicity of vitamin K3; this way, the patient would be exposed to a high dosage of Vitamin K3, which would be unfavourable due to side effects. Thus, even with lower cytotoxicity outcomes compared to free form, better in vivo achievements might be expected with VitK3 loaded liposomes alone or together with DOX loaded liposomes considering passive targeting related enhanced drug delivery with less amount of drug exposure to overall system and hence less side effects. These results pave the way for future works by trying dual liposomal administration.

REFERENCES

Abraham, A., Kattoor, A. J., Saldeen, T., & Mehta, J. L. (2019). Vitamin E and its anticancer effects. Critical Reviews in Food Science and Nutrition, 59(17), 2831–2838. https://doi.org/10.1080/10408398.2018.1474169

Abraham, S. A., Waterhouse, D. N., Mayer, L. D., Cullis, P. R., Madden, T. D., & Bally, M. B. (2005). The liposomal formulation of doxorubicin. Methods in Enzymology, 391(SPEC. ISS.), 71–97. https://doi.org/10.1016/S0076-6879(05)91004-5

Acharya, B. R., Choudhury, D., Das, A., & Chakrabarti, G. (2009). Vitamin K3 disrupts the microtubule networks by binding to tubulin: A novel mechanism of its antiproliferative activity. Biochemistry, 48(29), 6963–6974. https://doi.org/10.1021/bi900152k

Aghebati-Maleki, A., Dolati, S., Ahmadi, M., Baghbanzhadeh, A., Asadi, M., Fotouhi, A., Yousefi, M., & Aghebati-Maleki, L. (2019). Nanoparticles and cancer therapy: Perspectives for application of nanoparticles in the treatment of cancers. Journal of Cellular Physiology, 235(3), 1962–1972. https://doi.org/10.1002/jcp.29126

Akiyoshi, T., Matzno, S., Sakai, M., Okamura, N., & Matsuyama, K. (2009). The potential of vitamin K3 as an anticancer agent against breast cancer that acts via the mitochondria-related apoptotic pathway. Cancer Chemotherapy and Pharmacology, 65(1). https://doi.org/10.1007/s00280-009-1016-7

Allahou, L. W., Madani, S. Y., & Seifalian, A. (2021). Investigating the Application of Liposomes as Drug Delivery Systems for the Diagnosis and Treatment of Cancer. International Journal of Biomaterials, 2021. https://doi.org/10.1155/2021/3041969

Allen, T. M., & Cullis, P. R. (2004). Drug Delivery Systems : Entering the Mainstream. American Association for the Advancement of Science, 303(5665), 1818–1822. http://www.jstor.org/stable/3836507

Allen, T. M., K. Cheng, W. W., Hare, J. I., & Laginha, K. M. (2008). Pharmacokinetics and Pharmacodynamics of Lipidic Nano-Particles in Cancer. Anti-Cancer Agents in Medicinal Chemistry, 6(6), 513–523. https://doi.org/10.2174/187152006778699121

Ang, A., Pullar, J. M., Currie, M. J., & Vissers, M. C. M. (2018). Vitamin C and immune cell function in inflammation and cancer. Biochemical Society Transactions, 46(5), 1147–1159. https://doi.org/10.1042/BST20180169

Ansari, L., Shiehzadeh, F., Taherzadeh, Z., Nikoofal-Sahlabadi, S., Momtazi-Borojeni, A. A., Sahebkar, A., & Eslami, S. (2017). The most prevalent side effects of pegylated liposomal doxorubicin monotherapy in women with metastatic breast cancer: A systematic review of clinical trials. Cancer Gene Therapy, 24(5), 189–193. https://doi.org/10.1038/cgt.2017.9

Bangham, A. D. (1963). Physical Structure and Behavior of Lipids and Lipid Enzymes. In Advances in lipid research (Vol. 64). ACADEMIC PRESS INC. https://doi.org/10.1016/b978-1-4831-9937-5.50008-9

Bazak, R., Houri, M., El Achy, S., Kamel, S., & Refaat, T. (2015). Cancer active targeting by nanoparticles: a comprehensive review of literature. Journal of Cancer Research and Clinical Oncology, 141(5), 769–784. https://doi.org/10.1007/s00432-014-1767-3

Berkner, K. L., & Runge, K. W. (2004). The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. Journal of Thrombosis and Haemostasis, 2(12), 2118–2132. https://doi.org/10.1111/j.1538-7836.2004.00968.x

Bisker, G., Yeheskely-Hayon, D., Minai, L., & Yelin, D. (2012). Controlled release of Rituximab from gold nanoparticles for phototherapy of malignant cells. Journal

of Controlled Release, 162(2), 303–309. https://doi.org/10.1016/j.jconrel.2012.06.030

Bloemen, M. (2015). Immunomagnetic separation of bacteria by iron oxide nanoparticles. Ku Leuven, May, 1–157.

Booth, S. L. (2012). Vitamin K: Food composition and dietary intakes. Food and Nutrition Research, 56, 1–5. https://doi.org/10.3402/fnr.v56i0.5505

Bos, G. M. J. (2019). The Effect of Vitamin C (Ascorbic Acid) in the Treatment of Patients with Cancer : A Systematic Review. Nutrients, 11(977), 1–15.

Bozzuto, G., & Molinari, A. (2015). Liposomes as nanomedical devices. International Journal of Nanomedicine, 10, 975–999. https://doi.org/10.2147/IJN.S68861

Britt, K. L., Cuzick, J., & Phillips, K. A. (2020). Key steps for effective breast cancer prevention. Nature Reviews Cancer, 20(8), 417–436. https://doi.org/10.1038/s41568-020-0266-x

Briuglia, M. L., Rotella, C., McFarlane, A., & Lamprou, D. A. (2015). Influence of cholesterol on liposome stability and on *in vitro* drug release. Drug Delivery and Translational Research, 5(3), 231–242. https://doi.org/10.1007/s13346-015-0220-8

Buchholz, T. A., Stivers, D. N., Stec, J., Ayers, M., Clark, E., Bolt, A., Sahin, A. A., Symmans, W. F., Hess, K. R., Kuerer, H. M., Valero, V., Hortobagyi, G. N., & Pusztai, L. (2002). Global gene expression changes during neoadjuvant chemotherapy for human breast cancer. Cancer Journal, 8(6), 461–468. https://doi.org/10.1097/00130404-200211000-00010

Buzdar, A. U. (2006). Topoisomerase IIα gene amplification and response to anthracycline-containing adjuvant chemotherapy in breast cancer. Journal of Clinical Oncology, 24(16), 2409–2411. https://doi.org/10.1200/JCO.2006.05.9113

Cao, Y., Dong, X., & Chen, X. (2022). Polymer-Modified Liposomes for Drug Delivery : From Fundamentals to Applications. Pharmaceutics, 14(4), 1–27. https://doi.org/10.3390/pharmaceutics14040778

Caliskan, Y., Dalgic, A. D., Gerekci, S., Gulec, E. A., Tezcaner, A., Ozen, C., & Keskin, D. (2019). A new therapeutic combination for osteosarcoma: Gemcitabine and Clofazimine co-loaded liposomal formulation. International Journal of Pharmaceutics, 557(May 2018), 97–104. https://doi.org/10.1016/j.ijpharm.2018.12.041

Carvalho, C., Santos, R., Cardoso, S., Correia, S., Oliveira, P., Santos, M., & Moreira, P. (2009). Doxorubicin: The Good, the Bad and the Ugly Effect. Current Medicinal Chemistry, 16(25), 3267–3285. https://doi.org/10.2174/092986709788803312

Chatterjee, K., Zhang, J., Honbo, N., & Karliner, J. S. (2010). Doxorubicin cardiomyopathy. Cardiology, 115(2), 155–162. https://doi.org/10.1159/000265166

Chen, S., Li, L., Zhao, C., & Zheng, J. (2010). Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials. Polymer, 51(23), 5283–5293. https://doi.org/10.1016/j.polymer.2010.08.022

Chlebowski, R. T., Akman, S. A., & Block, J. B. (1985). Vitamin K in the treatment of cancer. Cancer Treatment Reviews, 12(1), 49–63. https://doi.org/10.1016/0305-7372(85)90012-X

Choi, H. S., Liu, W., Misra, P., Tanaka, E., Zimmer, J. P., Itty Ipe, B., Bawendi, M. G., & Frangioni, J. V. (2009). Renal Clearance of Nanoparticles. Nature Biotechnology, 25(10), 1165–1170. https://doi.org/10.1038/nbt1340.

Chun, R. F., Liu, P. T., Modlin, R. L., Adams, J. S., & Hewison, M. (2014). Impact of vitamin D on immune function: Lessons learned from genome-wide analysis. Frontiers in Physiology, 5(151), 1–15. https://doi.org/10.3389/fphys.2014.00151

Comşa, Ş., Cîmpean, A. M., & Raica, M. (2015). The story of MCF-7 breast cancer cell line: 40 Years of experience in research. Anticancer Research, 35(6), 3147–3154.

Condorelli, R., & Vaz-Luis, I. (2018). Managing side effects in adjuvant endocrine therapy for breast cancer. Expert Review of Anticancer Therapy, 18(11), 1101–1112. https://doi.org/10.1080/14737140.2018.1520096

Contini, C., Schneemilch, M., Gaisford, S., & Quirke, N. (2018). Nanoparticle–membrane interactions. Journal of Experimental Nanoscience, 13(1), 62–81. https://doi.org/10.1080/17458080.2017.1413253

Cortés-Funes, H., & Coronado, C. (2007). Role of anthracyclines in the era of targeted therapy. Cardiovascular Toxicology, 7(2), 56–60. https://doi.org/10.1007/s12012-007-0015-3

Cranenburg, E. C. M., Schurgers, L. J., & Vermeer, C. (2007). Vitamin K: The coagulation vitamin that became omnipotent. Thrombosis and Haemostasis, 98(1), 120–125. https://doi.org/10.1160/TH07-04-0266

Dalgic, A. D., Tezcaner, A., Elci, P., Sarper, M., Arpaci, F., Avcu, F., & Keskin, D. (2016). Improvement of a liposomal formulation with a native molecule: Calcitriol. RSC Advances, 6(83), 80158–80167. https://doi.org/10.1039/c6ra19187h

DAM, H. (1935). Cliv. the Antihaemorrhagic Vitamin of the Chick. Biochemical Journal, 29(6), 1273–1285. https://doi.org/10.1111/j.1753-4887.1973.tb05050.x

Danaei, M., Dehghankhold, M., Ataei, S., Hasanzadeh Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S., & Mozafari, M. R. (2018). Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics, 10(2), 1–17. https://doi.org/10.3390/pharmaceutics10020057

Dasari, S., Ali, S. M., Zheng, G., Chen, A., Dontaraju, V. S., Bosland, M. C., Kajdacsy-Balla, A., & Munirathinam, G. (2017). Vitamin K and its analogs:

Potential avenues for prostate cancer management. Oncotarget, 8(34), 57782–57799. https://doi.org/10.18632/oncotarget.17997

Decuzzi, P., Pasqualini, R., Arap, W., & Ferrari, M. (2009). Intravascular delivery of particulate systems: Does geometry really matter? Pharmaceutical Research, 26(1), 235–243. https://doi.org/10.1007/s11095-008-9697-x

DeLuca, H. F. (2004). Overview of general physiologic features and functions of vitamin D. The American Journal of Clinical Nutrition, 80(6 Suppl), 1689–1696. https://doi.org/10.1093/ajcn/80.6.1689s

Dicko, A., Kwak, S., Frazier, A. A., Mayer, L. D., & Liboiron, B. D. (2010). Biophysical characterization of a liposomal formulation of cytarabine and daunorubicin. International Journal of Pharmaceutics, 391(1–2), 248–259. https://doi.org/10.1016/j.ijpharm.2010.02.014

Doroshow, J. H. (1986). Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. Proceedings of the National Academy of Sciences of the United States of America, 83(12), 4514–4518. https://doi.org/10.1073/pnas.83.12.4514

Dragicevic-Curic, N., Scheglmann, D., Albrecht, V., & Fahr, A. (2008). Temoporfin-loaded invasomes: Development, characterization and *in vitro* skin penetration studies. Journal of Controlled Release, 127(1), 59–69. https://doi.org/10.1016/j.jconrel.2007.12.013

Esghaei, M., Ghaffari, H., Esboei, B. R., Tapeh, Z. E., Salim, F. B., & Motevalian, M. (2018). Evaluation of anticancer activity of Camellia sinensis in the Caco-2 colorectal cancer cell line. Asian Pacific Journal of Cancer Prevention, 19(6), 1697–1701. https://doi.org/10.22034/APJCP.2018.19.6.1697

Fang, X. J., Jiang, H., Zhu, Y. Q., Zhang, L. Y., Fan, Q. H., & Tian, Y. (2014). Doxorubicin induces drug resistance and expression of the novel CD44st via NF-κB in human breast cancer MCF-7 cells. Oncology Reports, 31(6), 2735–2742. https://doi.org/10.3892/or.2014.3131

Feghhi, M., Sharif Makhmalzadeh, B., Farrahi, F., Akmali, M., & Hasanvand, N. (2020). Anti-microbial Effect and *in Vivo* Ocular Delivery of Ciprofloxacin-loaded Liposome through Rabbit's Eye. Current Eye Research, 45(10), 1245–1251. https://doi.org/10.1080/02713683.2020.1728777

Fritze, A., Hens, F., Kimpfler, A., Schubert, R., & Peschka-Süss, R. (2006). Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient. Biochimica et Biophysica Acta - Biomembranes, 1758(10), 1633–1640. https://doi.org/10.1016/j.bbamem.2006.05.028

Furie, B., & Furie, B. C. (1990). Molecular Basis of Vitamin K-Dependent y-Carboxylation. The Journal of The American Society of Hematology, 75(9), 1753–1762. https://doi.org/10.1182/blood.v75.9.1753.1753

Gabizon, A., Shmeeda, H., & Barenholz, Y. (2003). Pharmacokinetics of pegylated liposomal doxorubicin: Review of animal and human studies. Clinical Pharmacokinetics, 42(5), 419–436. https://doi.org/10.2165/00003088-200342050-00002

Garbuzenko, O., Zalipsky, S., Qazen, M., & Barenholz, Y. (2005). Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers. Langmuir, 21(6), 2560–2568. https://doi.org/10.1021/la0479105

Gewirtz, D. A. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochemical Pharmacology, 57(7), 727–741. https://doi.org/10.1016/S0006-2952(98)00307-4

Ghagane, S. C., Puranik, S. I., Kumbar, V. M., Nerli, R. B., Jalalpure, S. S., Hiremath, M. B., Neelagund, S., & Aladakatti, R. (2017). *In vitro* antioxidant and anticancer activity of Leea indica leaf extracts on human prostate cancer cell lines. Integrative Medicine Research, 6(1), 79–87. https://doi.org/10.1016/j.imr.2017.01.004

Gil, Á., Plaza-Diaz, J., & Mesa, M. D. (2018). Vitamin D: Classic and Novel Actions. Annals of Nutrition and Metabolism, 72(2), 87–95. https://doi.org/10.1159/000486536

Gill, P. S., Wernz, J., Scadden, D. T., Cohen, P., Mukwaya, G. M., von Roenn, J. H., Jacobs, M., Kempin, S., Silverberg, I., Gonzales, G., Rarick, M. U., Myers, A. M., Shepherd, F., Sawka, C., Pike, M. C., & Ross, M. E. (1996). Randomized phase III trial of liposomal daunorubicin versus doxorubicin, bleomycin, and vincristine in AIDS-related Kaposi's sarcoma. Journal of Clinical Oncology, 14(8), 2353–2364. https://doi.org/10.1200/JCO.1996.14.8.2353

Gong, X., Gutala, R., & Jaiswal, A. K. (2008). Quinone Oxidoreductases and Vitamin K Metabolism. Vitamins and Hormones, 78(07), 85–101. https://doi.org/10.1016/S0083-6729(07)00005-2

Gonzalez Gomez, A., Syed, S., Marshall, K., & Hosseinidoust, Z. (2019). Liposomal Nanovesicles for Efficient Encapsulation of Staphylococcal Antibiotics. ACS Omega, 4(6), 10866–10876. https://doi.org/10.1021/acsomega.9b00825

Guimarães, D., Cavaco-Paulo, A., & Nogueira, E. (2021). Design of liposomes as drug delivery system for therapeutic applications. International Journal of Pharmaceutics, 601(April). https://doi.org/10.1016/j.ijpharm.2021.120571

Halwani, A. A. (2022). Development of Pharmaceutical Nanomedicines: From the Bench to the Market. Pharmaceutics, 14(1), 1–21. https://doi.org/10.3390/pharmaceutics14010106

Hamilton, J. A., Miller, K. W., & Small, D. M. (1983). Solubilization of triolein and cholesteryl oleate in egg phosphatidylcholine vesicles. Journal of Biological Chemistry, 258(21), 12821–12826. https://doi.org/10.1016/s0021-9258(17)44044-0

Haran, G., Cohen, R., Bar, L. K., & Barenholz, Y. (1993). Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochimica et Biophysica Acta, 1151(2), 201–215. https://doi.org/10.1016/0005-2736(93)90105-9

Hassan, G. S. (2013). Menadione. In Profiles of Drug Substances, Excipients and Related Methodology (Vol. 38). https://doi.org/10.1016/B978-0-12-407691-4.00006-X

Holsæter, A. M., Wizgird, K., Karlsen, I., Hemmingsen, J. F., Brandl, M., & Škalko-Basnet, N. (2022). How docetaxel entrapment, vesicle size, zeta potential and stability change with liposome composition–A formulation screening study. European Journal of Pharmaceutical Sciences, 177(April), 106267. https://doi.org/10.1016/J.EJPS.2022.106267

Husain, K., Francois, R. A., Yamauchi, T., Perez, M., Sebti, S. M., & Malafa, M. P. (2011). Vitamin E δ-tocotrienol augments the antitumor activity of gemcitabine and suppresses constitutive NF-κB activation in pancreatic cancer. Molecular Cancer Therapeutics, 10(12), 2363–2372. https://doi.org/10.1158/1535-7163.MCT-11-0424

Hunter, R. J., Midmore, B. R., & Zhang, H. (2001). Zeta potential of highly charged thin double-layer systems. Journal of Colloid and Interface Science, 237(1), 147–149. https://doi.org/10.1006/jcis.2001.7423

Huynh, C. T., Lee, D. S., & Macromolecules, T. (2020). Encyclopedia of Polymeric Nanomaterials. Encyclopedia of Polymeric Nanomaterials, 1–12. https://doi.org/10.1007/978-3-642-36199-9

Iyer, A. K., Khaled, G., Fang, J., & Maeda, H. (2006). Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discovery Today, 11(17–18), 812–818. https://doi.org/10.1016/j.drudis.2006.07.005

Jain, V., Kumar, H., Anod, H. V., Chand, P., Gupta, N. V., Dey, S., & Kesharwani, S. S. (2020). A review of nanotechnology-based approaches for breast cancer and triple-negative breast cancer. Journal of Controlled Release, 326(April), 628–647. https://doi.org/10.1016/j.jconrel.2020.07.003

Kamaly, N., Yameen, B., Wu, J., & Farokhzad, O. C. (2016). Degradable Controlled-Rlease Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug

Release. Chem Rev., 116(4), 2602–2663. https://doi.org/10.1021/acs.chemrev.5b00346

Kanwal, U., Irfan Bukhari, N., Ovais, M., Abass, N., Hussain, K., & Raza, A. (2018). Advances in nano-delivery systems for doxorubicin: an updated insight. Journal of Drug Targeting, 26(4), 296–310. https://doi.org/10.1080/1061186X.2017.1380655

Kapoor, M. S., D'Souza, A., Aibani, N., Nair, S. S., Sandbhor, P., kumari, D., & Banerjee, R. (2018). Stable Liposome in Cosmetic Platforms for Transdermal Folic acid delivery for fortification and treatment of micronutrient deficiencies. Scientific Reports, 8(1), 1–12. https://doi.org/10.1038/s41598-018-34205-0

Kurosu, M. (2017). Vitamin K2 Biosynthesis: Drug Targets for New Antibacterials. In Vitamin K2 - Vital for Health and Wellbeing (pp. 281–295). https://doi.org/10.5772/65487

Lamson, D. W., & Plaza, S. M. (2003). The anticancer effects of vitamin K. In Alternative Medicine Review (Vol. 8, Issue 3).

Lee, A. V., Oesterreich, S., & Davidson, N. E. (2015). MCF-7 Cells - Changing the Course of Breast Cancer Research and Care for 45 Years. Journal of the National Cancer Institute, 107(7), 1–4. https://doi.org/10.1093/jnci/djv073

Liao, W. C., Wu, F. Y., & Wu, C. W. (2000). Binary/ternary combined effects of vitamin K3 with other antitumor agents in nasopharyngeal carcinoma CG1 cells. International Journal of Oncology, 17(2), 323–328. https://doi.org/10.3892/ijo.17.2.323

Licata, S., Saponiero, A., Mordente, A., & Minotti, G. (2000). Doxorubicin metabolism and toxicity in human myocardium: Role of cytoplasmic deglycosidation and carbonyl reduction. Chemical Research in Toxicology, 13(5), 414–420. https://doi.org/10.1021/tx000013q

Liu, P., Chen, G., & Zhang, J. (2022). A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. Molecules, 27(4). https://doi.org/10.3390/molecules27041372

Liu, W., Hou, Y., Jin, Y., Wang, Y., Xu, X., & Han, J. (2020). Research progress on liposomes: Application in food, digestion behavior and absorption mechanism. Trends in Food Science and Technology, 104(August), 177–189. https://doi.org/10.1016/j.tifs.2020.08.012

Lyseng-Williamson, K. A., Duggan, S. T., & Keating, G. M. (2013). Pegylated liposomal doxorubicin: A guide to its use in various malignancies. BioDrugs, 27(5), 533–540. https://doi.org/10.1007/s40259-013-0070-1

Ma, Z., Hu, Y., Jiang, G., Hou, J., Liu, R., Lu, Y., & Liu, C. (2012). Spontaneous generation of germline characteristics in mouse fibrosarcoma cells. Scientific Reports, 2, 1–10. https://doi.org/10.1038/srep00743

Mainardes, R. M., & Silva, L. (2004). Drug Delivery Systems: Past, Present, and Future. Current Drug Targets, 5(5), 449–455. https://doi.org/10.2174/1389450043345407

Manaia, E. B., Abuçafy, M. P., Chiari-Andréo, B. G., Silva, B. L., Oshiro Junior, J. A., & Chiavacci, L. A. (2017). Physicochemical characterization of drug nanocarriers. International Journal of Nanomedicine, 12, 4991–5011. https://doi.org/10.2147/IJN.S133832

Martins-Teixeira, M. B., & Carvalho, I. (2020). Antitumour Anthracyclines: Progress and Perspectives. ChemMedChem, 15(11), 933–948. https://doi.org/10.1002/cmdc.202000131

Maruyama, K. (2011). Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. Advanced Drug Delivery Reviews, 63(3), 161–169. https://doi.org/10.1016/j.addr.2010.09.003

Master, A. M., & Sen Gupta, A. (2012). EGF receptor-targeted nanocarriers for enhanced cancer treatment. Nanomedicine, 7(12), 1895–1906. https://doi.org/10.2217/nnm.12.160

Matsubara, K., Kayashima, T., Masaharu, M., Yoshida, H., & Mizushina, Y. (2008). Inhibitory effects of vitamin K3 on DNA polymerase and angiogenesis. International Journal of Molecular Medicine, 22(4), 381–387. https://doi.org/10.3892/ijmm_00000034

McKee, R. W., Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., & Doisy, E. A. (1939). The Isolation of Vitamins K1 and K2. Journal of the American Chemical Society, 61(5), 1295–1295. https://doi.org/10.1021/ja01874a507

Milton Harris, J., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. Nature Reviews Drug Discovery, 2(3), 214–221. https://doi.org/10.1038/nrd1033

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., & Gianni, L. (2004). Anthracyclines: Molecular advances and pharmacologie developments in antitumor activity and cardiotoxicity. Pharmacological Reviews, 56(2), 185–229. https://doi.org/10.1124/pr.56.2.6

Mohammadabadi, M. R., & Mozafari, M. R. (2018). Enhanced efficacy and bioavailability of thymoquinone using nanoliposomal dosage form. Journal of Drug Delivery Science and Technology, 47(July), 445–453. https://doi.org/10.1016/j.jddst.2018.08.019

Mohd Zaffarin, A. S., Ng, S. F., Ng, M. H., Hassan, H., & Alias, E. (2020). Pharmacology and pharmacokinetics of Vitamin E: Nanoformulations to enhance bioavailability. International Journal of Nanomedicine, 15, 9961–9974. https://doi.org/10.2147/IJN.S276355

Mordente, A., Meucci, E., Silvestrini, A., Martorana, G. E., & Giardina, B. (2009). New Developments in Anthracycline-Induced Cardiotoxicity. Current Medicinal Chemistry, 16(13), 1656–1672.

Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of Lmmunological Methods, 65(83), 55–63. https://doi.org/10.1039/c6ra17788c

Nakhaei, P., Margiana, R., Bokov, D. O., Abdelbasset, W. K., Jadidi Kouhbanani, M. A., Varma, R. S., Marofi, F., Jarahian, M., & Beheshtkhoo, N. (2021). Liposomes: Structure, Biomedical Applications, and Stability Parameters With Emphasis on Cholesterol. Frontiers in Bioengineering and Biotechnology, 9(September), 1–23. https://doi.org/10.3389/fbioe.2021.705886

Nagaraja, P., Vasantha, R. A., & Yathirajan, H. S. (2002). Spectrophotometric methods for the rapid determination of menadione and menadione sodium bisulphite and their application in pharmaceutical preparations. Journal of Pharmaceutical and Biomedical Analysis, 28(1), 161–168. https://doi.org/10.1016/S0731-7085(01)00599-4

Ngo, E. O., Sun, T. P., Chang, J. Y., Wang, C. C., Chi, K. H., Cheng, A. L., & Nutter, L. M. (1991). Menadione-induced DNA damage in a human tumor cell line. Biochemical Pharmacology, 42(10), 1961–1968. https://doi.org/10.1016/0006-2952(91)90596-W

Niu, G., Cogburn, B., & Hughes, J. (2010). Preparation and characterization of doxorubicin liposomes. Methods in Molecular Biology (Clifton, N.J.), 624, 211–219. https://doi.org/10.1007/978-1-60761-609-2_14

Northfelt, D. W., Martin, F. J., Working, P., Voiberding, P. A., Martin, J., & Kaplan, D. (1996). Doxorubicin Encapsulated in Liposomes Containing Polyethylene Glycol : Tumor and Safety in Patients with Kaposi ' s Sarcoma. Journal of Clinical Pharmacology, 36(1), 55–63. https://doi.org/10.1002/j.1552-4604.1996.tb04152.x.

Nussbaumer, S., Bonnabry, P., Veuthey, J. L., & Fleury-Souverain, S. (2011). Analysis of anticancer drugs: A review. Talanta, 85(5), 2265–2289. https://doi.org/10.1016/j.talanta.2011.08.034

Nutter, L. M., Ngo, E. O., Fisher, G. R., & Gutierrez, P. L. (1992). DNA strand scission and free radical production in menadione-treated cells. Correlation with cytotoxicity and role of NADPH quinone acceptor oxidoreductase. Journal of Biological Chemistry, 267(4), 2474–2479. https://doi.org/10.1016/s0021-9258(18)45903-0

Ogawa, M., Nakai, S., Deguchi, A., Nonomura, T., Masaki, T., Uchida, N., Yoshiji, H., & Kuriyama, S. (2007). Vitamins K2, K3 and K5 exert antitumor effects on established colorectal cancer in mice by inducing apoptotic death of tumor cells. International Journal of Oncology, 31(2), 323–331. https://doi.org/10.3892/ijo.31.2.323

Okano, T., Shimomura, Y., Yamane, M., Suhara, Y., Kamao, M., Sugiura, M., & Nakagawa, K. (2008). Conversion of phylloquinone (vitamin K1) into menaquinone-4 (vitamin K2) in mice: Two possible routes for menaquinone-4 accumulation in cerebra of mice. Journal of Biological Chemistry, 283(17), 11270–11279. https://doi.org/10.1074/jbc.M702971200

Olusanya, T. O. B., Ahmad, R. R. H., Ibegbu, D. M., Smith, J. R., & Elkordy, A. A. (2018). Liposomal drug delivery systems and anticancer drugs. Molecules, 23(4), 1–17. https://doi.org/10.3390/molecules23040907

Pati, R., Shevtsov, M., & Sonawane, A. (2018). Nanoparticle vaccines against infectious diseases. In Frontiers in Immunology (Vol. 9, Issue OCT). https://doi.org/10.3389/fimmu.2018.02224

Pattni, B. S., Chupin, V. V., & Torchilin, V. P. (2015). New Developments in Liposomal Drug Delivery. Chemical Reviews, 115(19), 10938–10966. https://doi.org/10.1021/acs.chemrev.5b00046

Pawlowska, E., Szczepanska, J., & Blasiak, J. (2019). Pro- And antioxidant effects of Vitamin C in cancer in correspondence to its dietary and pharmacological concentrations. Oxidative Medicine and Cellular Longevity, 2019. https://doi.org/10.1155/2019/7286737

Peer, D., Karp, J. M., Hong, S., Farokhzad, O. C., Margalit, R., & Langer, R. (2007). Nanocarriers as an emerging platform for cancer therapy. Nature Nanotechnology, 2(12), 751–760. https://doi.org/10.1038/nnano.2007.387

Perrie, Y. (2008). Gregory Gregoriadis: Introducing liposomes to drug delivery. Journal of Drug Targeting, 16(7–8), 518–519. https://doi.org/10.1080/10611860802228376

Prasad, K. N., Edwards-Prasad, J., & Sakamoto, A. (1981). Vitamin K3 (Menadione) inhibits the growth of mammalian tumor cellls in culture. Life Sciences, 29(13), 1387–1392. https://doi.org/10.1016/0024-3205(81)90683-4

Pyataev, N. A., Petrov, P. S., Minaeva, O. V., Zharkov, M. N., Kulikov, O. A., Kokorev, A. V., Brodovskaya, E. P., Yurlov, I. A., Syusin, I. V., Zaborovskiy, A. V., & Balykova, L. A. (2019). Amylase-sensitive polymeric nanoparticles based on dextran sulfate and doxorubicin with anticoagulant activity. Polymers, 11(5). https://doi.org/10.3390/polym11050921

Rabbani, A., Finn, R. M., & Ausió, J. (2005). The anthracycline antibiotics: Antitumor drugs that alter chromatin structure. BioEssays, 27(1), 50–56. https://doi.org/10.1002/bies.20160

Ramalho, M. J., Coelho, M. A. N., & Pereira, M. C. (2017). Nanoparticles for Delivery of Vitamin D: Challenges and Opportunities. A Critical Evaluation of Vitamin D - Clinical Overview. https://doi.org/10.5772/64516

Rannels, S. R., Gallaher, K. J., Wallin, R., & Rannels, D. E. (1987). Vitamin K-dependent carboxylation of pulmonary surfactant-associated proteins. Proceedings of the National Academy of Sciences of the United States of America, 84(16), 5952–5956. https://doi.org/10.1073/pnas.84.16.5952

Rivankar, S. (2014). An overview of doxorubicin formulations in cancer therapy. Journal of Cancer Research and Therapeutics, 10(4), 853–858. https://doi.org/10.4103/0973-1482.139267

Ross, D., Thor, H., Orrenius, S., & Moldeus, P. (1985). Interaction of menadione (2-methyl-1,4-naphthoquinone) with glutathione. Chemico-Biological Interactions, 55(C), 177–184. https://doi.org/10.1016/S0009-2797(85)80126-5

Sabeti, B., Noordin, M. I., Mohd, S., Hashim, R., Dahlan, A., & Akbari Javar, H. (2014). Development and characterization of liposomal doxorubicin hydrochloride with palm oil. BioMed Research International, 2014. https://doi.org/10.1155/2014/765426

Sata, N., Stumpe-Klonowski, H., Han, B., Haüssinger, D., & Niederau, C. (1997). Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR4-2J cells. Free Radical Biology and Medicine, 23(6), 844–850. https://doi.org/10.1016/S0891-5849(97)00064-6

Semkova, S., Zhelev, Z., Miller, T., Sugaya, K., Aoki, I., Higashi, T., & Bakalova, R. (2020). Menadione/ascorbate induces overproduction of mitochondrial superoxide and impairs mitochondrial function in cancer: Comparative study on cancer and normal cells of the same origin. Anticancer Research, 40(4), 1963–1972. https://doi.org/10.21873/anticanres.14151

Shi, J., Kantoff, P. W., Wooster, R., & Farokhzad, O. C. (2017). Cancer nanomedicine: Progress, challenges and opportunities. Nature Reviews Cancer, 17(1), 20–37. https://doi.org/10.1038/nrc.2016.108

Shinde, V. R., Revi, N., Murugappan, S., Singh, S. P., & Rengan, A. K. (2022). Enhanced Permeability and Retention Effect: A key facilitator for solid tumor targeting by nanoparticles. Photodiagnosis and Photodynamic Therapy, 39(June 2021), 102915. https://doi.org/10.1016/j.pdpdt.2022.102915

Shu, Y., Pi, F., Sharma, A., Rajabi, M., Haque, F., Shu, D., Leggas, M., Evers, B. M., & Guo, P. (2014). Stable RNA nanoparticles as potential new generation drugs for cancer therapy. Advanced Drug Delivery Reviews, 66, 74–89. https://doi.org/10.1016/j.addr.2013.11.006

Singh, Z., & Kaur, H. (2019). Toxicological aspects of antineoplastic drugs Doxorubicin and Epirubicin. Journal of Clinical and Molecular Medicine, 2(2), 1–2. https://doi.org/10.15761/jcmm.1000130

Sivadasan, D., Sultan, M. H., Madkhali, O. A., Alessa, A. A., & Alsabei, S. H. (2022). Stealth Liposomes (PEGylated) Containing an Anticancer Drug Camptothecin: *In Vitro* Characterization and *In Vivo* Pharmacokinetic and Tissue Distribution Study. Molecules, 27(3). https://doi.org/10.3390/molecules27031086

Smith, M. T. (1985). Quinones as mutagens, carcinogens, and anticancer agents: Introduction and overview. Journal of Toxicology and Environmental Health, 16(5), 665–672. https://doi.org/10.1080/15287398509530776

Solomon, D., Gupta, N., Mulla, N. S., Shukla, S., Guerrero, Y. A., & Gupta, V. (2017). Role of *In Vitro* Release Methods in Liposomal Formulation Development: Challenges and Regulatory Perspective. AAPS Journal, 19(6), 1669–1681. https://doi.org/10.1208/s12248-017-0142-0

Stewart, J. C. M. (1980). Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Analytical Biochemistry, 104(1), 10–14. https://doi.org/10.1016/0003-2697(80)90269-9

Sweatt, A., Sane, D. C., Hutson, S. M., & Wallin, R. (2003). Matrix Gla protein (MGP) and bone morphogenetic protein-2 in aortic calcified lesions of aging rats. Journal of Thrombosis and Haemostasis, 1(1), 178–185. https://doi.org/10.1046/j.1538-7836.2003.00023.x

Tacar, O., Sriamornsak, P., & Dass, C. R. (2013). Doxorubicin: An update on anticancer molecular action, toxicity and novel drug delivery systems. Journal of Pharmacy and Pharmacology, 65(2), 157–170. https://doi.org/10.1111/j.2042-7158.2012.01567.x

Tareen, B., Summers, J. L., Jamison, J. M., Neal, D. R., McGuire, K., Gerson, L., & Diokno, A. (2008). A 12 week, open label, phase I/IIa study using Apatone® for the

treatment of prostate cancer patients who have failed standard therapy. International Journal of Medical Sciences, 5(2), 62–67. https://doi.org/10.7150/ijms.5.62

Taurin, S., Nehoff, H., & Greish, K. (2012). Anticancer nanomedicine and tumor vascular permeability; Where is the missing link? Journal of Controlled Release, 164(3), 265–275. https://doi.org/10.1016/j.jconrel.2012.07.013

Tetef, M., Margolin, K., Ahn, C., Akman, S., Chow, W., Leong, L., Morgan, R. J., Raschko, J., Somlo, G., & Doroshow, J. H. (1995). Mitomycin C and menadione for the treatment of lung cancer: a phase II trial. Investigational New Drugs, 13(2), 157–162. https://doi.org/10.1007/BF00872865

Theerakittayakorn, K., & Bunprasert, T. (2011). Differentiation capacity of mouse L929 fibroblastic cell line compare with human dermal fibroblast. World Academy of Science, Engineering and Technology, 50(February 2011), 373–376.

Torchilin, V. P. (2000). Drug Targeting. European Journal of Pharmaceutical Sciences, 11, S81–S91. https://doi.org/10.1016/s0928-0987(00)00166-4

Torchilin, V. P. (2008). Multifunctional Pharmaceutical Nanocarriers. In Springer (pp. 1–33). Springer US. https://doi.org/10.1007/978-0-387-76554-9

Ventola, C. L. (2012). The nanomedicine revolution: Part 2: Current and future clinical applications. P and T, 37(10), 582–591.

Viglianti, B. L., Abraham, S. A., Michelich, C. R., Yarmolenko, P. S., MacFall, J. R., Bally, M. B., & Dewhirst, M. W. (2004). *In vivo* monitoring of tissue pharmacokinetics of liposome/drug using MRI: Illustration of targeted delivery. Magnetic Resonance in Medicine, 51(6), 1153–1162. https://doi.org/10.1002/mrm.20074

Waks, A. G., & Winer, E. P. (2019). Breast Cancer Treatment: A Review. JAMA - Journal of the American Medical Association, 321(3), 288–300. https://doi.org/10.1001/jama.2018.19323

Wang, T., Ph, D., Hartner, W. C., Ph, D., Gillespie, J. W., Praveen, K. P., Ph, D., Yang, S., Ph, D., Mei, L. A., Petrenko, V. A., Ph, D., Torchilin, V. P., & Ph, D. (2014). Enhanced Tumor Delivery and Antitumor Activity *in Vivo* of Liposomal Doxorubicin Modified with MCF-7-Specific Phage Fusion Protein. Nanomedicine : Nanotechnology, Biology, and Medicine, 10(2), 421–430. https://doi.org/10.1016/j.nano.2013.08.009.Enhanced

Wartlick, H., Michaelis, K., Balthasar, S., Strebhardt, K., Kreuter, J., & Langer, K. (2004). Highly specific HER2-mediated cellular uptake of antibody-modified nanoparticles in tumour cells. Journal of Drug Targeting, 12(7), 461–471. https://doi.org/10.1080/10611860400010697

Waterhouse, D. N., Tardi, P. G., Mayer, L. D., & Bally, M. B. (2001). A comparison of liposomal formulations of doxorubicin with Drug Administered in free form: Changing toxicity profiles. Drug Safety, 24(12), 903–920. https://doi.org/10.2165/00002018-200124120-00004

Wood, M. J., Irwin, W. J., & Scott, D. K. (1990). Photodegradation of doxorubicin, daunorubicin and epirubicin measured by high-performance liquid chromatography. Journal of Clinical Pharmacy and Therapeutics, 15(4), 291–300. https://doi.org/10.1111/j.1365-2710.1990.tb00387.x

Wu, D. C., & Ofner, C. M. (2013). Adsorption and degradation of doxorubicin from aqueous solution in polypropylene containers. AAPS PharmSciTech, 14(1), 74–77. https://doi.org/10.1208/s12249-012-9885-1

Wu, F. Y. H., & Sun, T. P. (1999). Vitamin K3 induces cell cycle arrest and cell death by inhibiting Cdc25 phosphatase. European Journal of Cancer, 35(9), 1388–1393. https://doi.org/10.1016/S0959-8049(99)00156-2

Wu, Xingyong, Liu, H., Liu, J., Haley, K. N., Treadwat, J. A., Larson, J. P., Ge, N., Peale, F., & Bruchez, M. P. (2003). Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. Nature Biotechnology, 21, 41–46. https://doi.org/10.1038/nbt764

Wu, Xu, Hu, W., Lu, L., Zhao, Y., Zhou, Y., Xiao, Z., Zhang, L., Zhang, H., Li, X., Li, W., Wang, S., Cho, C. H., Shen, J., & Li, M. (2019). Repurposing vitamin D for treatment of human malignancies via targeting tumor microenvironment. Acta Pharmaceutica Sinica B, 9(2), 203–219. https://doi.org/10.1016/j.apsb.2018.09.002

Yadav, D., Sandeep, K., Pandey, D., & Dutta, R. K. (2017). Liposomes for Drug Delivery. Journal of Biotechnology & Biomaterials, 07(04). https://doi.org/10.4172/2155-952x.1000276

Yamada, A., Osada, S., Tanahashi, T., Matsui, S., Sasaki, Y., Tanaka, Y., Okumura, N., Matsuhashi, N., Takahashi, T., Yamaguchi, K., & Yoshida, K. (2015). Novel therapy for locally advanced triple-negative breast cancer. International Journal of Oncology, 47(4), 1266–1272. https://doi.org/10.3892/ijo.2015.3113

Yamamoto, T., Yokoyama, M., Opanasopit, P., Hayama, A., Kawano, K., & Maitani, Y. (2007). What are determining factors for stable drug incorporation into polymeric micelle carriers? Consideration on physical and chemical characters of the micelle inner core. Journal of Controlled Release, 123(1), 11–18. https://doi.org/10.1016/j.jconrel.2007.07.008

Yiang, G. T., Chen, T. Y., Chen, C., Hung, Y. T., Hsueh, K. C., Wu, T. K., Pan, Y. R., Chien, Y. C., Chen, C. H., Yu, Y., & Wei, C. W. (2021). Antioxidant vitamins promote anticancer effects on low-concentration methotrexate-treated glioblastoma cells via enhancing the caspase-3 death pathway. Food Science and Nutrition, 9(6), 3308–3316. https://doi.org/10.1002/fsn3.2298

Zein, R., Sharrouf, W., & Selting, K. (2020). Physical Properties of Nanoparticles That Result in Improved Cancer Targeting. Journal of Oncology, 2020. https://doi.org/10.1155/2020/5194780

Zylberberg, C., & Matosevic, S. (2016). Pharmaceutical liposomal drug delivery: a review of new delivery systems and a look at the regulatory landscape. Drug Delivery, 23(9), 3319–3329. https://doi.org/10.1080/10717544.2016.1177136

APPENDICES

A. Calibration curves

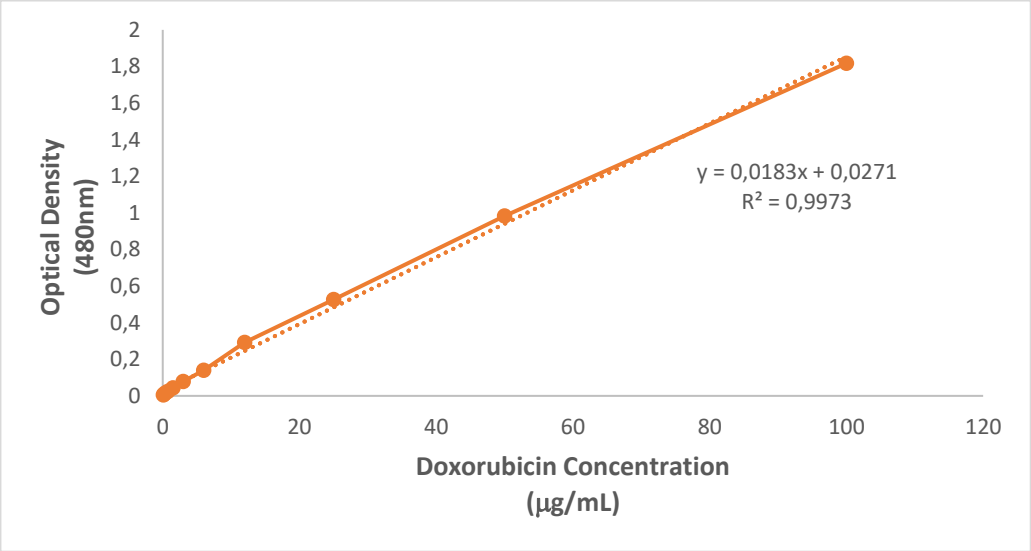

Figure 36: Doxorubicin calibration curve in PBS (0.01M)) (n=3)

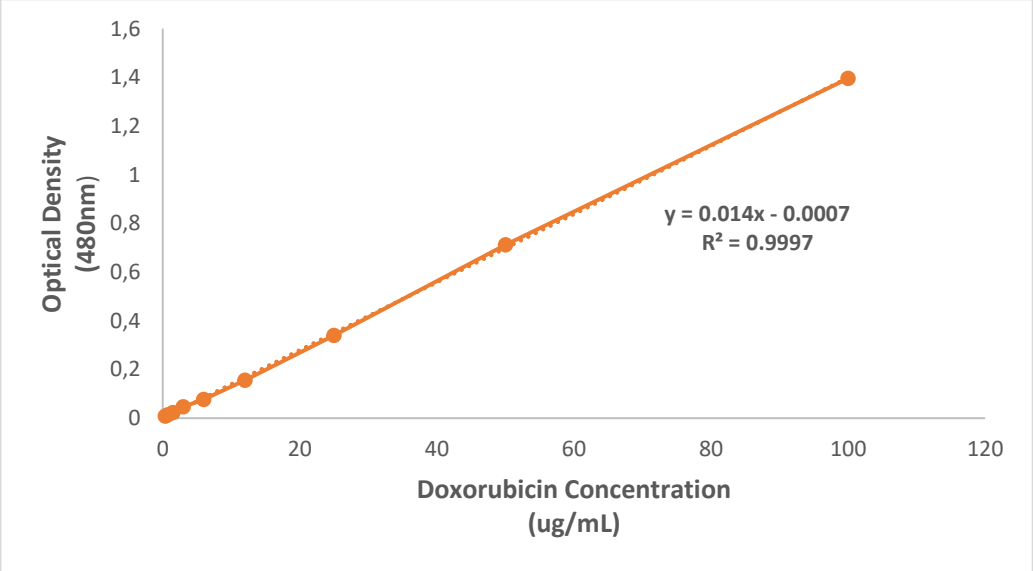

Figure 37: Doxorubicin calibration curve in methanol (n=3)

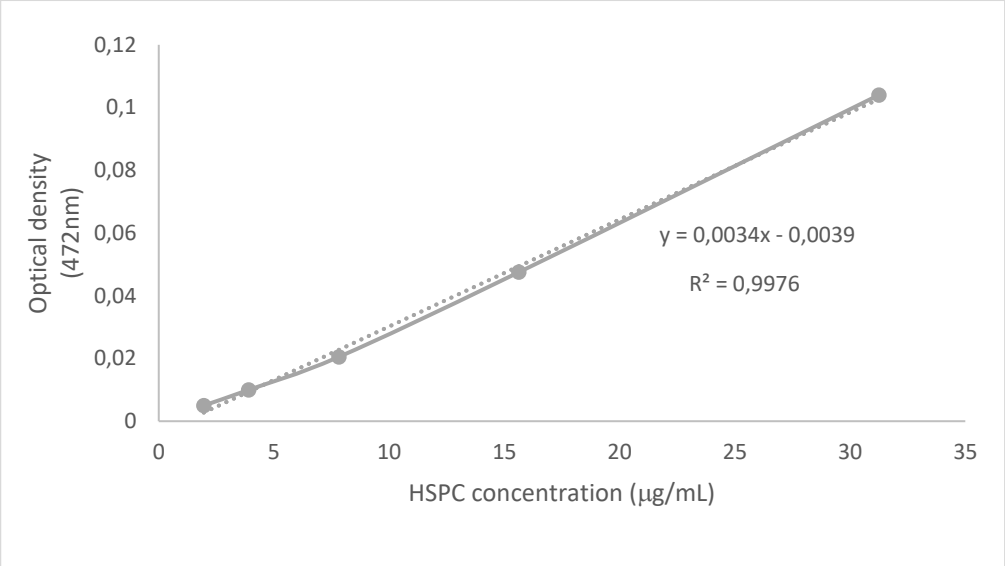

Figure 38: HSPC calibration curve (n=3)

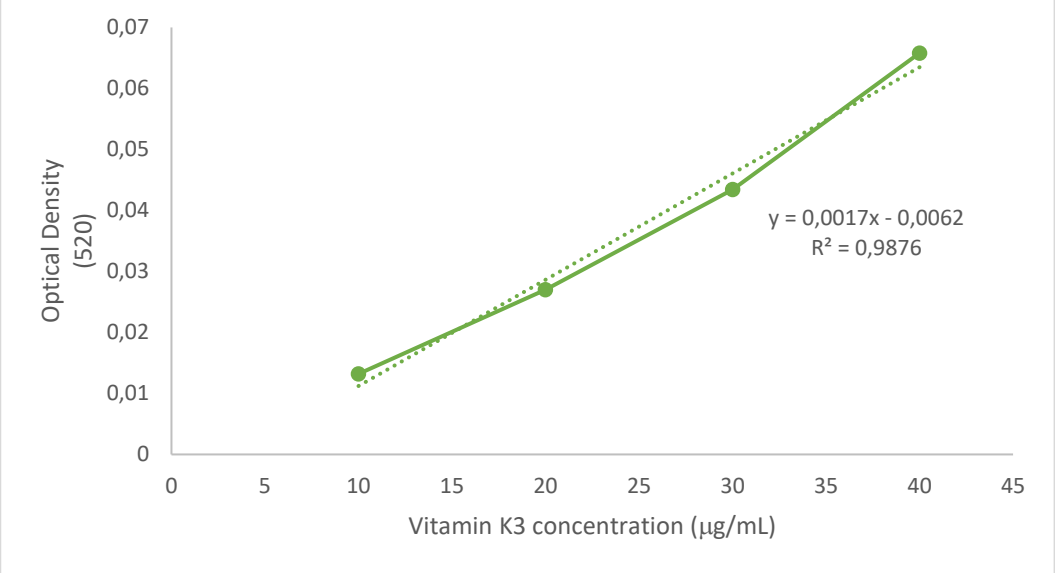

Figure 39: Vitamin K3, menadione (MSB) calibration curve