DEVELOPMENT OF A NEW TARGETED LIPOSOMAL DELIVERY SYSTEM OF DOXORUBICIN MODIFIED WITH LYMPHOMA CELL SPECIFIC ANTIBODY

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DEVELOPMENT OF A NEW TARGETED LIPOSOMAL DELIVERY SYSTEM OF DOXORUBICIN MODIFIED WITH LYMPHOMA CELL SPECIFIC ANTIBODY

submitted by **ALİ DENİZ DALGIÇ** in partial fulfillment of the requirements for the degree of **Master of Science in Engineering Sciences Department, Middle East Technical University by,**

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

Prof. Dr. Murat Dicleli
Head of Department, **Engineering Sciences** _____

Assoc. Prof. Dr. Dilek Keskin
Supervisor, **Engineering Sciences Dept., METU** _____

Assoc. Prof. Dr. Ayşen Tezcaner
Co-Supervisor, **Engineering Sciences Dept., METU** _____

Examining Committee Members:

Assoc. Prof. Dr. Ayşen Tezcaner
Engineering Sciences Dept., METU _____

Assoc. Prof. Dr. Dilek Keskin
Engineering Sciences Dept., METU _____

Prof. Dr. Fikret Arpacı
Medical Onchology Dept., GATA _____

Assist. Prof. Dr. İrem Erel
Chemistry Dept., METU _____

Assoc. Prof. Dr. Sreeparna Banerjee
Biology Dept., METU _____

Date: _____

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

Name, Last name: Ali Deniz Dalgıç

Signature:

ABSTRACT

DEVELOPMENT OF A NEW TARGETED LIPOSOMAL DELIVERY SYSTEM OF DOXORUBICIN MODIFIED WITH LYMPHOMA CELL SPECIFIC ANTIBODY

Ali Deniz Dalgıç

M.S., Department of Engineering Sciences

Supervisor: Assoc. Prof. Dr. Dilek Keskin

Co-Supervisor: Assoc. Prof. Dr. Ayşen Tezcaner

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Three doxorubicin loaded drug carrier systems were prepared against Lymphoma diseases. The first approach was to target DXR loaded liposomal system by attaching cell specific antibodies. Non-internalizing anti-CD20 antibody was used to target Namalwa cells and internalizing anti-CD30 antibody was used to target B lymphoma cells. Targeted DXR loaded liposomes were prepared and their cytotoxicity against Lymphoma cells were compared with untargeted DXR loaded liposomes. The targeted liposome formulations were optimized to have around "92" anti-CD20 and "31" anti-CD30 antibodies per single 100 nm sized liposome (HSPC:CHOL:DSPE-PEG; 2:1:0.2). After 48 hours of incubation with Namalwa cells, anti-CD20 targeted DXR loaded liposomes caused over 87% toxicity while untargeted liposomes resulted with 19% cell death. However, anti-CD30 targeted liposomes toxicity improvement compared to untargeted liposomes was not clear due to less number of targeting moieties/cell in the study. So, this system will be further optimized. A second study was performed to investigate possible doxorubicin cytotoxicity enhancement by calcitriol pretreatment on Namalwa cells. The combinational effect of both agents was investigated firstly by treating Namalwa cells with their free form. We showed that Namalwa cells are more susceptible to DXR after 72 hours of calcitriol pretreatment. Then both agents co-loaded to liposomes and cytotoxicity compared with only doxorubicin loaded liposomes. Calcitriol and DXR co-loading to

liposome, enhances cytotoxicity with in a shorter time with lower concentrations. Both modified liposomal systems prepared successfully and caused improved toxicity relative to DXR loaded liposomes.

Keywords: B lymphocytes, anti-CD20, anti-CD30, doxorubicin, calcitriol.

ÖZ

LENFOMA TEDAVİSİNE YÖNELİK LENFOSİT HÜCRELERİNE ÖZGÜN ANTİKOR İLE HEDEFLİ, DOKSORUBİSİN İÇEREN ÇİFT ETKİLİ YENİ LİPOZOMAL SALIM SİSTEMİ GELİŞTİRİLMESİ

Ali Deniz Dalgıç
Yüksek Lisans, Mühendislik Bilimleri
Tez Yöneticisi: Doç. Dr. Dilek Keskin
Ortak Tez Yöneticisi: Doç. Dr. Ayşen Tezcaner
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Lenfoma hücrelerine karşı, doksorubisin içeren üç farklı sistem oluşturulmuştur. İlk yaklaşım, doksorubisin içeren lipozomları antikor bağlayarak hedeflemekti. Hücre içine alınma özelliği bulunmayan CD20 antikorları, Namalwa hücrelerini hedeflemek için kullanıldı ve hücre içine alınma özelliği olan CD30 antikoru ile lipozomlar B lenfosit hücrelerine hedeflendi. Hedefli, doksorubisin yüklü, iki ayrı lipozom sistemi geliştirildi ve lenfoma hücrelerine karşı sitotoksiteleri hedefli olmayan lipozom formülü ile karşılaştırıldı. Hedefli lipozom sistemleri, 100nm'lik bir lipozom (HSPC:Kolesterol:DSPE-PEG; 2:1:0.2) yüzeyine "92" adet anti-CD20 veya "31" adet anti-CD30 antikoru bağlanabilicek şekilde optimize edildi. Namalwa hücreleriyle 48 saat etkileşimden sonra, anti-CD20 antikoru ile hedefli lipozomlar %87 sitotoksisiteye neden olurken, hedefli olmayan lipozomlar sadece %19 sitotoksisite yaratabilmiştir. Ancak CD30 antikoru ile hedefli lipozomların sebep olduğu hücre ölüm yüzdesindeki artış, kullanılan anti-CD30 antikor sayısının yetmemesinden dolayı yeterince açık gözlenemedi. Bu yüzden sistemin optimizasyonu devam etmektedir. İkinci çalışmamızda, doksorubisin'in Namalwa hücreleri üzerindeki toksik etkisinin, hücreler önceden calcitriol'e maruz bırakıldığında artabilme olasılığı araştırılmıştır. İlaçların birlikte oluşturduğu etki ilk olarak Namalwa hücreleri üzerinde ilaçları serbest olarak etkileştirerek gözlendi. Bu

çalışmada Namalwa hücrelerinin calcitriol ile 72 saatlik ön maruzundan sonra doksorubisine çok daha duyarlı olduklarını gösterdik. Daha sonra iki ilaç birlikte lipozom içine hapsedildi ve oluşturdukları toksisite sadece doksorubisin yüklü lipozomların oluşturduğu ile karşılaştırıldı. Doksorubisin ve calcitriol aynı lipozoma yüklenmesinin daha hızlı ve daha az konsantrasyonlarda, daha yüksek toksisiteye neden olduğu görüldü. Geliştirilmiş lipozom sistemleri başarıyla üzretildi ve sadece doksorubisin yüklü lipozomlar ile karşılaştırıldığında daha üstün bir toksisite elde edildi.

Keywords: B lenfosit hücreleri, anti-CD20, anti-CD30, doksorubisin, calcitriol.

to the people who are seeking for the true meaning of life and are not be afraid of being lost…

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ANOVA: Analysis of Variance

CHOL: Cholesterol

DLS: Dynamic Light Scattering

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl Sulphoxide

DNA: Deoxyribonucleic acid

DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DSPE-mPEG(2000): 1,2 - distearoyl-sn-glycero - 3 - phosphoethanolamine - N - methoxy (polyethylene glycol) - 2000

DXR: Doxorubicin

EE: Encapsulation efficiency

ELISA: Enzyme-Linked immunosorbent assay

EPR: Enhanced permeability and retention

FBS: Fetal bovine serum

FISH: Fluorescence in situ hybridization

HDL: High density lipoproteins

HIV: Human immunodeficiency virus

HPLC: High-performance liquid chromatography

HSPC: Hydrogenated (Soy) L-α-phosphatidylcholine

LUVs: Large unilamellar vesicles

MLVs: Multilamellar vesicles

mPEG: Methoxy polyethylene glycol

MPS: mononuclear phagocyte system

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

MW: Molecular weight

MWCO: Molecular weight cut-off

PB: Phosphate buffer

PDI: Polydispersity index

PE: Phosphoethanolamine

PEG: Poly ethylene glycol

pNP: Para-nitro phenyl carbonyl

RES: Reticuloendothelial system

PP: Polypropylene

RS: Reed Sternberg

RT: Room temperature

SUVs: Small unilamellar vesicles

TEM: Transmission electron microscopy

TBS: Tris buffered saline

UV: Ultraviolet

VDR: Calcitriol receptor

Z-average diameter: Average hydrodynamic diameter

CHAPTER 1

INTRODUCTION

1.1. Liposomes

The liposomes were first described by British hematologist Alec D. Bangham and he first published his work on liposomes in 1964 [1]. Liposomes are artificially made, lipid based vesicles that form in an aqueous environment. The bilayer structure consists of two sheets of lipid molecules in which molecules align side by side. Phospholipid molecules consist of a hydrophilic head group and a hydrophobic tail region and they are amphiphilic in nature. In bilayer structure hydrophobic tail regions of the inner and outer membrane face towards each other and hydrophilic head groups face aqueous environment as shown in Figure 1.1 forming bilayered closed vesicles called liposomes [3].

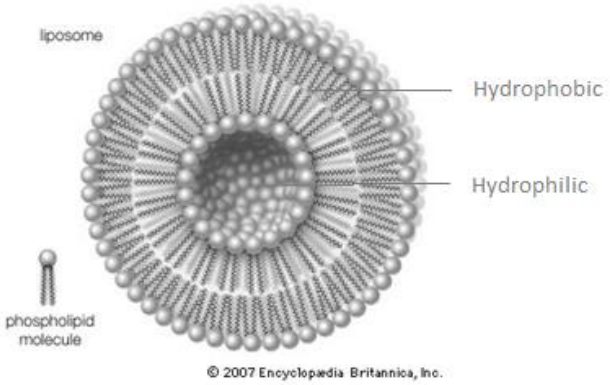


Figure 1.1. Liposome bilayer structure [53]

Liposomes can be as small as 20 nm and can be as large as 10 μm in diameter. Smaller liposome size is preferred for biological applications and larger liposome size is preferred in the study of physical and chemical properties of the bilayer structure [4]. Size, bilayer structure and lipid composition of liposomes is determined according to purpose of application. Unilamellar liposomes can be grouped in three; small (20-40 nm), medium (40-80 nm) and large (100-1000 nm) unilamellar vesicles. Multilamellar liposomes have several bilayers separated by aqueous environment. Liposomes can also be grouped according to their composition and application [5]. Conventional unilamellar liposome is the simplest unilamellar formulation which mainly consists of neutral and negative lipids. The encapsulated substance diffuse through bilayer in time and the rate of release depends on the lipid composition and drug related parameters. For example, an increased amount of cholesterol will provide a slower release rate because cholesterol enhances rigidity and stability of the liposomal bilayer [6, 7]. Cationic liposomes are formed by introducing positively charged lipids and are favored in gene therapy applications due to their interaction between negatively charged DNA structure and cell membrane [8]. Conventional liposomes are passively targeted to the tumor tissue, determined mainly by the size of the liposomes. The tumor tissue forms new vessels to get nutrition as it grows but new veins cannot be properly structured. The veins of a tumor have some deformations that cause leakage of the blood content at the tumor site. The nano sized conventional liposomes are able to leak through these deformations which is defined as enhanced permeability and retention (EPR) effect [9].

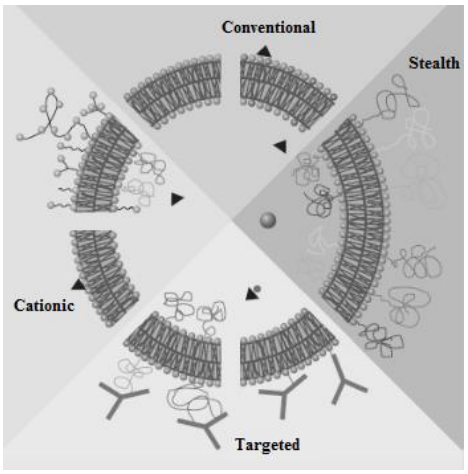


Figure 1.2. Liposome types [10]

The conventional liposome forms have proved themselves by their ability to encapsulate substances with different chemical characteristics. However, an obstacle arises when the intravenous administration is to be considered. They are quickly eliminated from the circulation. One of the reasons for elimination is mononuclear phagocyte system (MPS). When liposomes are administered intravenously their interaction with the blood components is inevitable. Blood proteins called opsonins are responsible for binding to foreign substances thereby making them available to macrophages [11]. The opsonin interaction with liposome surface depends on several characteristics; charge, size, fluidity and hydrophobicity [12, 13]. Negative and positive charged liposomes have more affinity to opsonins than neutral liposomes. Also elimination rate increases with increasing size and hydrophobicity. Altering these characteristics can partially prevent the MPS uptake of liposomes. To overcome MPS recognition, different polymer coatings are applied to provide a hydrophilic liposomal surface.

One of the best results was obtained by using poly ethylene glycol (PEG) chain. PEG chain has biocompatibility, solubility in aqueous and organic media and very low immunogenicity [7]. PEG chain can be bonded terminally to a lipid molecule which can be incorporated to the liposome bilayer [14]. By this mean, PEG residues face

outwards and create a hydrophilic area around the liposome surface. PEG chains prevent opsonins from binding to liposomal surface so eliminate the MPS effect. After PEGylation usually a larger liposome size is obtained which reduces kidney filtration of liposomes. Blood solubility of the liposome also increases by incorporated hydrophilic PEG chains [15]. PEG incorporated liposomes are called ‘‘stealth liposomes’’. Life time of a stealth liposome is much longer than a conventional type liposome. Long circulation life-time of the stealth liposomes gives them necessary time to reach and leak to the tumor site. Such long circulation time also allows releasing of a drug for a long period of time while circulating. Sustained release rate of the stealth liposome can be obtained to achieve to make the circulating drug dose at effective therapeutic dose all the time [7].

Stealth liposomes have many advantages like long circulating time and stability. However, PEGylation is not enough to overcome side effects. Therefore, homing devices are now being added on liposomes to eliminate the side effects of drugs at other tissues. Many targeting molecules can be directly linked to PEG chains which are on the liposomal surface. The targeting molecule can be an antibody or a recognition sequence that recognize a specific antigen on the surface of a cancer cell [5].

The bilayer structure creates a hydrophobic region between the bilayer membranes and an aqueous core inside the liposome which are the key components of a liposome as a carrier system. This dual nature of the liposome enables the loading of both hydrophobic and hydrophilic molecules. Today liposomal carriers are used in many areas including medicine, immunology, diagnostics, cosmetics, ecology, cleansing and the food industry [16].

1.2. Liposomal Drug Delivery Systems

One of the major aplication areas of liposomes are used is drug delivery. Liposomes can be simply formed and modified by using several methods at industrial scales.

Easy management of the liposomes enables their modification and use in many different fields like in biotechnology, drug delivery, diseases diagnosis and treatment. Once the drug is encapsulated into liposome, pharmacokinetics of the drug is changed. Possible biodistribution of the drug decreases when administered in liposomal form and this is expected to decrease the toxic effects of the drug in other tissues [17]. Liposomal drug is concentrated and actively preserved in the formulation until it reaches to target site. Hence bioavailability is higher when the drug is encapsulated into liposome [18]. There are many application areas of liposomes as drug carriers, they are either passively targeted or actively targeted by a homing device. Liposomes are successfully used in clinics for cancer treatment, metabolic disorders, fungal disease, respiratory illness, eye disorder and in many other fields [19].

The encapsulation method is based on the chemical characteristics of the drug molecule. Hydrophobic drugs are encapsulated within the lipid bilayer but hydrophilic drugs are encapsulated into the aqueous core. Lipid film is formed by dissolving lipids in an organic solution which is then evaporated. The simplest liposome encapsulation of drug can be performed during lipid film hydration procedure (Fig. 1.3). Hydrophobic drugs can be incorporated into the lipid mixture, in this step. After hydration, large lipid vesicles are formed which are extruded to obtain nano sized liposomes. This is the first drug encapsulation method presented by Bangham et al [2].

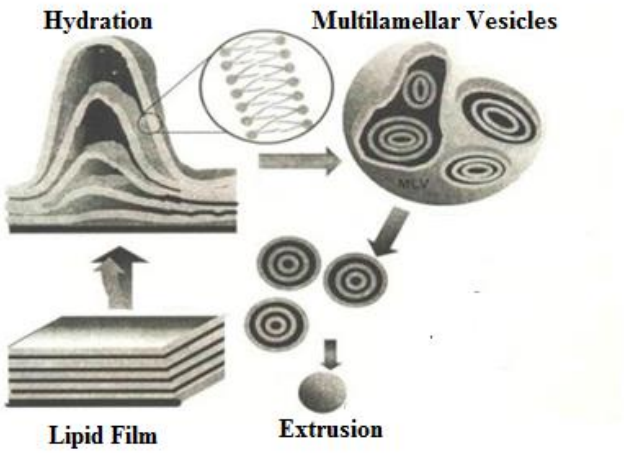


Figure 1.3. Lipid film hydration method [3]

Lipid film hydration is the simplest method but usually results in low drug to lipid ratio. Methods that use charge gradients for encapsulation provide higher drug to lipid ratio. Many antibiotics and anthracyclins are successfully entrapped by using transmembrane pH gradient [20, 22]. An interior solution with low pH and an exterior buffer solution with higher pH are the two key components of the method. Liposomes are altered to carry many drugs to their specific target for years now and continuously evolving for new treatment approaches.

1.3. Lymphoma Disease

Lymphoma is a cancer type that occurs when immune system cells become cancerous. The cancerous lymphocytes start to proliferate in excess amounts and may spread through lymphatic system. The activated lymphocytes can be found in tissues, circulation system and lymph canals [23]. The lymphoma cell types are B cells and T cells which become abnormal in lymphoma disease. In a healthy body, B cells recognize foreign bodies and produce specific antibodies against them and T cells are responsible for cell-mediated immunity. Once lymphoma occurs, number of

B or T cells starts to increase uncontrollably and the cancerous cells cannot accomplish their tasks. Lymphoma types can be investigated under two; Hodgkin's lymphoma and non-Hodgkin's lymphoma. Hodgkin's lymphoma has five subcategories 4 of them being classical Hodgkin's lymphoma and the last one is nodular Hodgkin's lymphoma [23]. The main property of Hodgkin's lymphoma is the formation of Reed Sternberg (RS) cells that have more than one nucleus [24]. There is no certain reason described for having the illness but weakened immune system is the leading cause. Infection of Epstein-Bar Virus is shown as one of the reasons, which is detected in 40% of the patients. Also HIV is mentioned as a cause but HIV only supports the disease by weakening the immune system. Prolonged exposure to exotoxins and excess human growth hormone use are some other reasons [23].

1.3.1. Treatment of the Disease

Symptoms of the lymphoma can be divided as systemic and local. Local symptoms can be seen as swelling of lymph nodes, painless swelled areas, fatigue, itching. The systemic symptoms are sweating, fever and weight loss [25]. First diagnosis can be made by physical examination which includes checking for any abnormalities around lymph nodes. In the case of lymphoma abnormal values for blood count and chemistry are expected. After lymphoma diagnosis, several tests should be performed to characterize the disease. The characterization is important because treatment method changes with the lymphoma type. After several cell type detection tests (immunophenotyping, FISH testing and etc.) results can be covered by asking questions like whether or not it is a Hodgkin lymphoma, whether replicating cells are T cell or B cell, where do the cells arise from [26]. The treatment mainly covers radiotherapy, biological therapy and chemotherapy. The dose of treatment depends on how spread is the disease, sex, age and several physical conditions. Today surviving rates of Hodgkin's lymphoma patients are high with the improvements in treatment methods. Non-Hodgkin's lymphoma has the same aspect but different approaches are used for different types and treatment depends upon histology [23].

1.4. Anticancer Agents

Nearly hundred different types of cancer are available today which is why there is not a single cure for the cancer. Every cancer has a specific mutagenized cell type for its own. Mutagenic cells, also called activated cells, have a key similarity; being immortal and uncontrollably proliferative [23]. On the other hand, every cancer cell has unique properties some of which are different from their healthy phenotype (i.e., cell surface antigens). Anti-cancer agents, therefore, may present affects which can be toxic on more than one cancer cell type. However, a resistance can occur against the drug [27]. That is why chemotherapy includes more than one anti-cancer agent. There are three common ways that anticancer agents work. The first and direct way is damaging the target DNA of cells. The anti-cancer agent can directly break the unity of the DNA directly or indirectly. The second option is to inhibit any new DNA synthesis which will stop tumor growth. The third way is to destabilize mitosis. Low molecular weight drugs are preferred for chemotherapy as they are able to kill or suppress cancer cells. However, drugs also show the same effect on healthy cells. The anticancer agents are classified by their mechanism of action; DNA-interactive agents, antimetabolites, antitubulin agents, molecular targeting agents, hormones, monoclonal antibodies and other biological agents [28]. Antimetabolites are the agents that interrupt the metabolic pathways which are necessary for the cells to live, like protein synthesis. DNA interactive agents have many different pathways which interact with DNA itself and there are different groups of these agents. The group that cause alkylation of DNA bases is called alkylating agents (Dacarbazine and temozolomine). Crosslinking agents form bridges with strong bonds in-between the DNA structure which disables metabolic act of DNA (cisplatin, carboplatin and oxaliplatin). Intercalating agents bind between base pairs and interrupt the DNA sequence. Drugs in this group mainly inhibit the actions of DNA metabolism enzymes [29]. The last group of DNA interactive agents is DNA-cleaving agents that disrupt the DNA strands.

Doxorubicin (Adriamycin) is an anthracycline antibiotic that is commonly used in cancer treatments. In 1950 the first form of the drug was synthesized from soil bacteria called ‘Streptomyces peucetius’. The cytotoxicity occurs when the drug is intercalated into DNA. By interacting with DNA and topoisomerase II enzyme, doxorubicin prevents DNA replication and causes apoptosis [30]. However, doxorubicin has an important side effect which causes cardio-toxicity [32].

1.5. Drug Delivery Mechanism and Kinetic of Liposomal Delivery Systems

Liposomal drug delivery kinetics is one of the most important factors that determines the in vivo success of liposomal system. Ideal drug delivery kinetics changes with treatment purpose of the liposomal system which may be a constant rate of release in circulation or direct target delivery of whole liposomal content. In every case, ideal liposomal formulation should sustain an optimum stability which improves drug delivery by decreasing the required dose of drug. Every lipid molecule has a unique transition temperature which affects the final transition temperature of lipid mixture. When lipids with higher transition temperature are used, the stability of liposome in blood increases. Lipid choice also affects size and drug encapsulation efficiency of the liposome. Optimum formulation can be found by altering lipid ratios in order to obtain preferred transition temperature and stability [6, 45]. In vitro studies are important prior to any in vivo study which includes stability, encapsulation and leakage of encapsulated compound. Basically, release kinetics is determined in physiological like serum containing media in which liposomes are placed in a dialysis bag. Several other techniques present such as gel filtration, ultracentrifugation, density gradient centrifugation and mini-column centrifugation [46]. Drug release kinetics of the liposomes is basically studied by dialysis respect to some mathematical models. In mathematical methods liposome is accepted as a circular vesicle that has a constant size. Higuchi equation is widely used to show the linear correlation between square-root of time and total amount of released material [52].

$$\frac{M_t}{A} = \sqrt{2C_{ini}DC_s t}$$

Where;

t is considered release time

M_t is cumulative amount of drug release

A is total surface area of the dialysis membrane exposed to dialysis

C_{ini} is initial drug concentration

C_s is drug solubility

Among the liposomal formulations liposomal doxorubicin formulation reached the market and is used in clinics. The two forms of doxorubicin are available Doxil (PEGylated-Stealth Liposome) and Myocet (not Pegylated) liposomal formulations. At first the doxorubicin was directly administered intravenously. Dose accumulation of the doxorubicin caused serious damage in the cardiac tissue [32]. The first liposomal formulation was "Myocet" which was not a stealth form. The efficiency of the formulation was low due to rapid clearance from the blood circulation. Then the PEGylated stealth liposomal doxorubicin "Doxil" was developed. There is evidence that the Doxil and Myocet formulations minimized the cardio-toxicity experienced by patients. However, it was observed that Doxil causes Hand-Foot Syndrome which is a skin reaction that appears on the palms of the hands and the soles of the feet. Because the drug accrues at hands and feet, reaction occurs on skin [31, 33]. These experiences point out that a homing device should be used to improve the therapeutic efficiency of the drug.

1.6. Antibodies as Targeting Agents

The antibodies and antibody fragments received the most attention for years as targeting moieties. When a healthy cell mutates into a cancer cell, new antigens arise on the surface. Each cancer cell type has specific receptors and antigens, which are perfect for cancer targeting, especially if over-expressed. After the specific target has

been determined a targeting molecule has to be produced. Monoclonal antibody technology is one of the best methods to produce a certain homing device for a specific target. Monoclonal antibodies are typically made by fusing myeloma cells with the spleen cells from a mouse or B-cells of a rabbit that has been immunized with the desired antigen. Polyethylene glycol (PEG) is used to fuse adjacent plasma membranes. When they show an immune reaction they produce the specific antibody and the antibodies secreted by the different clones are then assayed for their ability to bind to the antigen. The purity and efficiency of the antibody may then be tested with ELISA or Antigen Microarray Assays.

The ability to produce desired antibodies enabled targeting of the liposomes to desired locations in the body [34]. The antibody molecule was preferred in many studies as an agent for targeting and antibodies were increasing the specificity of the drug delivery system. The immunoliposome needs an antibody which is conjugated to the surface of the liposome. The antibody can be conjugated either directly to the surface or onto the PEG chain on the surface. Coupling the antibodies to the distal ends of PEG units is a more efficient way to target the liposome rather than binding antibodies to liposome surface. If antibodies are coupled directly to the liposomal membrane the PEG units will surround them and block some of their activity [35]. The antibody coupling is performed through different reactive groups on the poly ethylene glycol chain, a reactive methoxy end or a p-nitrophenylcarbonyl derivative are two of the main groups used for coupling.

The coupling of antibodies to liposomes can be achieved by several methods; the most common method is to use Mal-PEG-DSPE for coupling of the thiolated antibody molecule. Torchilin et al (2001) presented a simplified and applicable coupling method for single-step binding of a large variety of a primary amino group-containing substances, including proteins and small molecules [36]. Briefly, they used p-nitrophenylcarbonyl group as a reactive group and antibody was coupled to this end. The coupling occurs when the pH is in the basic range. The phosphoethanolamine (PE) residue, which was firstly incorporated into the liposome,

makes the structure able to incorporate into liposomes bilayer and the antibody is able to bind the water exposed pNP groups. This binding forms a stable, non-toxic urethane (carbamate) bond. The study of Lukyanov et al (2004) reported that the method permits the binding of several dozen protein molecules per single 200 nm liposome. They also concluded that liposome and all bound proteins completely preserved their specific activity [37].

1.7. Calcitriol as an Anticancer Agent

Vitamin D can be synthesized by the interaction of sunlight on skin and can be obtained by diet. After being metabolized, vitamin D acts as steroid hormone that regulates calcium homeostasis and bone metabolism. Vitamin D metabolism starts in the skin with conversion of 7-dehydrocholesterol to vitamin D_3 which is hydroxylated in liver to turn into $25(OH)D_3$. This molecule is converted into Calcitriol ($1,25(OH)_2D_3$) in the kidney [47]. Calcitriol interacts with a cell by vitamin D receptor (VDR) which induces several metabolic pathways and gene transcription. The interaction of calcitriol with VDR on intestine cells induces calcium homeostasis. However, it is reported that VDR is also present in some other tissues that do not contribute to calcium homeostasis but reveals other possible physiologic acts. Other biological actions of calcitriol include induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems [48]. Healthy tissues like intestine, kidney, bone and heart naturally have VDR. In addition to that some activated cell types like activated B and T cells express VDR. Calcitriol has antineoplastic activity in a broad range of tumor types. Synergistic effects of calcitriol with cytotoxic chemotherapy, radiation, and other cancer drugs have been reported. The mechanism of the additive effect of calcitriol differs with different chemotherapy agents and tumor models [49]. Mechanism of anti-cancer activity of calcitriol has not yet been certainly described but there are studies concluding that its anticancer activity is based on a genomic pathway, which is mediated through the VDR. Synergistic effect of calcitriol with DXR has been reported in the study of Ravid et al [50]. The recognition of calcitriol by VDR, which

is present on the surface of breast cancer cells, inhibits the production of cytoplasmic anti-oxidant enzyme (Cu/Zn superoxide dismutase). By this aspect it was shown that the oxidative damage of doxorubicin can be enhanced by pretreatment with calcitriol [50].

1.8. Aim of the Study

Commercial form of liposomal doxorubicin, Doxil, has partially overcome the cardiotoxicity problem of DXR free use. However, Doxil still causes some side effects like hand-foot syndrome [32]. To overcome these side effects, several targeted liposomal doxorubicin formulations which targets lymphoma and other cancer types are present in literature. For lymphoma disease over expressed cell antigens are distinguished as targets for liposomal therapy such as retinoic acid receptor, Bcr-Abl protein, CD20 and CD30 antigens [40, 41, 51]. Several studies showed the therapeutic effect of anti-CD30 and anti-CD20 antibody among lymphoma without using any drugs [40, 41, 51]. The superiority of CD30 antigen among others is its only presence on cancerous cells and its ability to internalize.

The aim of this study is to develop modified doxorubicin loaded liposomal systems that have higher cytotoxicity against Lymphoma cell lines. Two of the prepared systems were doxorubicin loaded liposomes targeted with anti-CD20 and anti-CD30. Non-internalizing anti-CD20 modified liposomes were targeted to Namalwa cells and it was expected to see dual therapeutic effect by doxorubicin and the target antibody (anti-CD20) which is also a clinically used drug itself (Rutiximab). An internalizing anti-CD30 antibody was used to produce a second targeted liposomal system that targets B lymphocytes which also has a dual therapeutic activity through anti-CD30 and doxorubicin. In vitro cytotoxicity studies with B lymphoma cells and anti-CD30 are made with the hypothesis that internalizing system will have higher toxicity than non-internalizing ones. An initial fast therapeutic act was expected from both targeted systems, firstly by target molecule and secondly by doxorubicin release. Schematic presentation of the targeted liposomal formulation is given in Fig. 1.4.

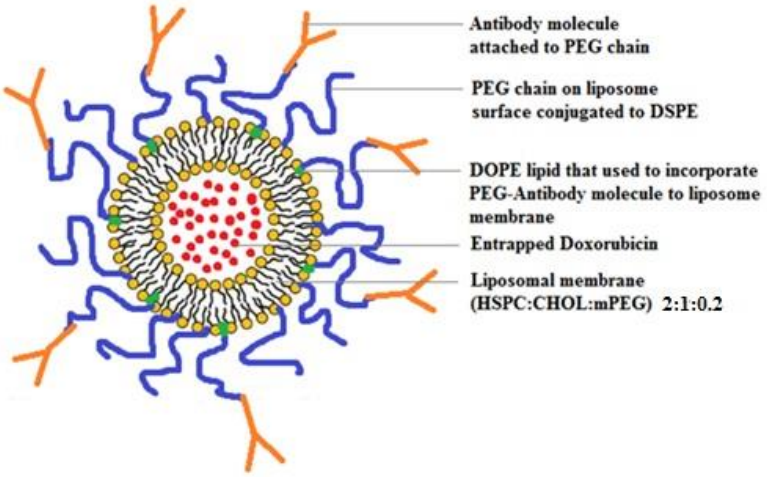


Figure 1.4. Schematic illustration of Doxorubicin loaded liposomes targeted to CD20 or CD30 positive lymphoma cells. HSPC: CHOL: DSPE-PEG (2: 1: 0.2)

A third liposomal system was developed to improve cytotoxicity against Namalwa cells. A second bioactive agent (Calcitriol) was added to liposomal formulation, to enhance the toxic effect of DXR. Calcitriol is a fat soluble molecule and it is possible to incorporate calcitriol into the lipid bilayer. Prior to liposome incorporation experiments, the combined effect of free calcitriol and DXR was tested over Namalwa cells. Calcitriol pretreatment was applied for 24, 48 and 72 hours which were followed by 24 hours of Doxorubicin incubation. After the cytotoxicity enhancement was shown with free form of agents, liposome encapsulation of dual agents were performed.

Liposomal characteristics like drug release profile, size distribution, drug encapsulation efficiency, loading, lipid recovery and antibody binding efficiency were experimentally optimized for both systems to achieve better targeting, treatment efficiency and intravenous application purpose. In order to observe the specific therapeutic action of the liposomal system in vitro cytotoxicity experiments were performed with Namalwa and B lymphoma cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene Glycol)2000 (18:00 mPEG(2000)-DSPE), L-α-phosphatidylethanolamine-N-(lissamine Rhodamine B sulfonyl) (Ammonium Salt) (Egg-transphosphatidylated, Chicken), Mini-extruder set, filter supports, Nucleopore Track-Etch Membranes (100, 400, 800 nm) were purchased from Avanti Polar Lipids, Inc. (USA). Doxorubicin Hydrochloride, cholesterol, methoxypolyethylene glycol p-nitrophenyl carbonate, fluorescamine, dialysis sacks, benzoylated dialysis tubing, chloroform (HPLC grade), methanol (HPLC grade), ethanol (HPLC grade) were the products of Sigma Aldrich Chem. Co. (USA). Sephadex G-75, PD-10 Disposable Columns were purchased from GE Healthcare (UK). Calcitriol was obtained from Cayman Chemical Company (USA). Oregon green was purchased from Life Technologies (USA). Anti-CD20 (Rituximab), anti-CD30 antibodies were purchased from Abcam (USA). Namalwa cell line and B Lymphoma cell lines were kindly provided by Gulhane Medical Faculty, Cancer and Stem Cell Research Center. These cell lines were originally obtained from ATCC (USA). RPMI 1640 medium without phenol red obtained from GIBCO-BRL Biochrom AG (Germany). 10% FBS was purchased from Biological Industries, (Israel), and 2 mM L-glutamine and 1× penicillin-streptomycin was obtained from Invitrogen. MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was obtained from Invitrogen (USA). Cell culture plastic-wares were the products of Orange Scientific (Germany).

2.2. Methods

2.2.1. Preparation of Liposome

HSPC:DSPE-PEG:CHOL at a molar ratio of 2:0.2:1 was used as the liposome composition. Lipid film hydration method was used to form MLVs in aqueous salt solution. Powder lipid stocks were incubated at room temperature for 15 minutes to equilibrate the temperature of lipids. After weighing, lipids were transferred to round bottom eppendorf tubes and dissolved in 100 µl chloroform. After obtaining a homogenous mixture, N_2 gas was applied for 2 hours to obtain lipid film. Formed lipid film was incubated overnight in vacuum oven (Nüve EV 018, Turkey) in order to remove chloroform completely. Ammonium sulfate solution (120 mM) was prepared in 1 mL dH_2O as hydration solution. Hydration was performed by continuous two minute cycles of heating in water bath and vortex (CAT VM3, France) for about an hour. The water bath temperature was fixed to 65°C which is transition temperature of lipid mixture. To reduce the size of MLVs, extrusion was applied. Briefly, vesicles were forced through membranes with reducing sizes, sequentially through 800, 400 and 200 and/or 100 nm polycarbonate filters. Extrusion was performed by passing liposome suspension 11 times through each membrane at 65°C. Final size around 100 nm was achieved and LUVs were obtained.

2.2.1.1. Preparation of Anticancer Drug Loaded Liposome

Doxorubicin (DXR) was loaded into liposomes by ammonium sulfate gradient method [22]. LUVs were formed in 120 mM solution of ammonium sulfate that forms both intra and extra-liposomal aqueous phases. Dialysis method was used to change extra-liposomal environment with 0.9% NaCl solution for 20 hours. For this purpose liposome sample was dialyzed against 0.9% NaCl solution for 12h. DXR (500 µg) in NaCl solution (0.9%) was used per 25 mg lipid mixture. Liposome and drug solutions were heated up to transition temperature of the lipid mixture and solutions were mixed together. They were incubated at 65°C for 10 minutes and

quickly dipped into ice bath. Loading procedure and following steps were continued in dark due to drug's light sensitivity. Unencapsulated drug was removed with Sephadex-G75 column (GE Healthcare, USA). Column was prepared by dissolving Sephadex powder in 0.9 NaCl solution. Solution was incubated under vacuum for 5 hours in order to remove any air bubbles (Nüve EV 018, Turkey). Liposomes were introduced into column and fractions of 1 ml were collected. Turbidity reading of each tube was performed at 410 nm by using UV-Visible spectrophotometer (Hitachi U-2800A, Japan). According to turbidity reading, liposome fractions were pooled. Liposomes were freshly prepared before characterization and cell culture studies. They were stored at 4°C at most 1 day before analysis.

2.2.1.2. Preparation of Anticancer Agent and Calcitriol Loaded Liposome

Calcitriol was loaded into the lipid membrane of liposome due to its hydrophobic nature. Calcitriol (50 µg) was dissolved with the mixture of lipids (HSPC:DSPE-PEG:CHOL) in chloroform then thin film is obtained. Steps explained in Section 2.2.1.1, were used to prepare drug loaded liposomes that were co-loaded with calcitriol. Unencapsulated Doxorubicin and calcitriol were removed using Sephadex G-75 chromatography column (GE Healthcare, USA) and liposome samples were pooled.

2.2.2. Preparation of Targeted Liposome

The Antibody-PEG-PE unit was conjugated to readily formed drug loaded liposome to form immuno-liposome. Antibody coupling method was formed by combination of two methods from literature [36, 37]. The study of Torchilin et al [36], presented a coupling method in which empty liposomes were added with pNP-PEG-PE molecules at hydration step. However, this protocol would change many parameters in our drug loaded liposomes as they formed by ammonium sulfate gradient method. For example the hydration solution used in Torchilin's study was citrate and octyl glucoside (pH:5.1) which is improper for loading with ammonium sulfate gradient.

pNP-PEG-PE unit was synthesized according to study of Torchilin at all, [36]. Therefore, a second method, Lukyanov et al, [37], that couple antibodies to a drug loaded liposomal system by using pNP-PEG-PE units reacted with antibodies and conjugated to readily formed liposomes was combined to form antibody targeted liposomes. Antibody conjugated PEG-PE molecule was integrated into liposomes by co-incubation. Unattached antibodies were separated by centrifugation at 8000 rpm for 2 hours (4ºC) through Vivaspin 300.000 MW filter (Sartorius Stedim, DEU).

2.2.2.1. Synthesis of pNP-PEG-DOPE molecule

The first step was to combine 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) with (pNP_2)-PEG molecule (Fig. 2.1) Eight fold molar excess $(pNP)_2$-PEG over PE was weighed and both were dissolved in 1 mL chloroform. Trietylamine was added 2 fold molar excess over (pNP_2)-PEG. Solution was incubated overnight at RT under N_2 gas. Next day, chloroform was removed with N_2 gas and by incubating 4 hours in vacuum oven. A dried film was obtained at the bottom of the glassware. A solution of 0.01M HCl and 0.15M NaCl were prepared in dH_2O for hydration. Hydration solution was added onto film and sonication was applied to form micelles of (pNP_2)-PEG-PE. Column chromatography was used to separate unreacted molecules. Disposable column (PD-10, 10 cm length and 2 cm width) was packed with Sepharose CL-4B swollen in 0.15M NaCl solution containing 0.01M HCl. The solution was passed through column and collected in 1 mL tubes. The tubes containing pNP-PEG-PE micelles were determined with UV measurements at 300 nm. Obtained micelle solution was kept frozen at -80°C overnight. The frozen sample was freeze-dried and pNP-PEG-PE was extracted by dissolving in chloroform. The solution was kept at -80°C until use.

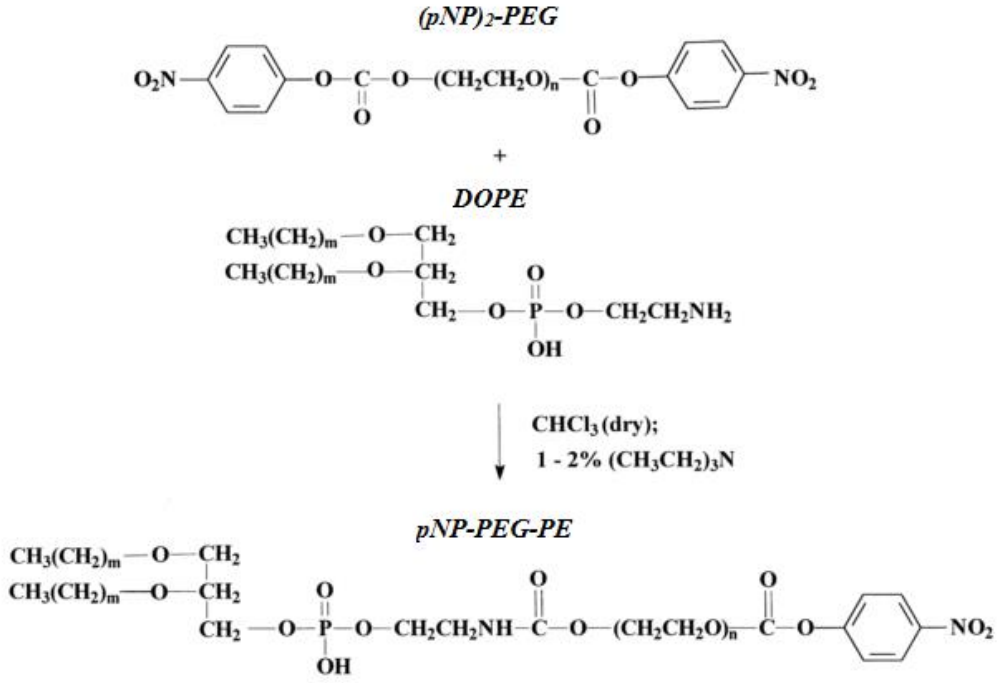


Figure 2.1. General reaction scheme of pNP-PEG-PE synthesis presented. Eight fold molar excess $(pNP)_2$-PEG over PE was reacted together [36].

2.2.2.2. Production of Antibody-PEG-DOPE Conjugate

Antibodies were attached to liposomes via PEG-DOPE molecules with activated bis(p-nitrophenylcarbonyl) distal end. A non-toxic urethane (carbamate) bond was formed between amino groups of antibody and PEG-PE molecules after the reaction [37].

(pNP_2)-PEG-PE solution (125 µl) which was 2% mol of total lipids in liposome, was dried under N_2 gas. $C_6H_5Na_3O_7$ (5 M) and NaCl (0.15 M) in 4 mL of dH_2O was used as the hydration solution. Octylglucoside solution (40 µl) was added. Anti-CD30 or anti-CD20 antibody solution at a concentration of 1 mg/mL was prepared in Tris Buffered Saline (TBS). Two solutions were mixed and incubated at $4^{\circ}C$ overnight. Antibody-PEG-PE molecule was formed after incubation.

2.2.2.2.1. Determination of Antibody Conjugation Efficiency

Fluorescamine, an amino group reactive dye, was used to determine the pNP-PEG-DOPE conjugation efficiency as described by Lukyanov et al [37]. Sample solution with antibody or antibody-PEG-PE was prepared in PBS (0.01 M, pH 7.4) at 0.1 mg/mL concentration. Fluorescamine solution was prepared in acetone (3 mg/mL) and added to antibody or antibody-PEG-PE solution at a 3:1 volume ratio. The solution was then incubated at dark for 30 minutes at RT. The optical density of fluorescamine was measured using UV-Visible spectrophotometer. The peak absorbance was detected at 359 nm after performing a wavelength scan. Absorbance readings of the solutions were compared for fluorescamine reacted with antibody alone and after reacted with antibody-PEG-PE. The amount of pNP-PEG-PE molecule conjugation to a single antibody molecule was calculated with the absorbance decrease ratio. All procedure was run in dark due to fluorescamine's light sensitivity.

2.2.2.3. Incorporation of Antibody-PEG-DOPE Molecules to Liposome

Antibody-PEG-PE molecules were incorporated into the preformed liposomes by post-insertion method. Antibody-PEG-PE molecules were produced in a salt solution that contains Octyl-glucoside detergent as explained in section 2.2.2.2. Antibody-PEG-PE solution was mixed with liposome solution and incubated at 4°C for 5 hours. Unattached antibody-PEG-PE molecules were separated through Vivaspin filtration (300.000 MW cutoff).

2.2.2.3.1. Determination of Incorporation Efficiency of Antibody to Liposome

Oregon green was used to estimate the number of antibodies incorporated on a single liposomal surface. Oregon green reacts with the unprotonated aliphatic amine groups on antibody molecule. The calibration of oregon green 488 coupled antibodies was performed using 496nm excitation wavelength with spectrophotometer. To determine

antibody attachment efficiency immunoliposomal formulation was prepared with Oregon green 488 labelled antibodies incorporated into liposome. As the first step, pure antibody solution was reacted with Oregon Green 488 dye and a calibration curve was plotted accordingly. Then antibody solution was reacted with Oregon Green 488 prior to pNP-PEG-PE conjugation and liposome incorporation procedure was run. Antibody amount on liposomes was calculated with absorbance value of Oregon Green 488 modified antibodies. All procedure was run in dark.

Antibody solution (5 mg/mL) was prepared in 0.1 M sodium bicarbonate (NaH_2CO_3) buffer (pH 8.3). Oregon Green stock solution was prepared in DMSO and mixed with antibody solution to obtain 15:1 final molar ratio. The mixture was incubated at 25°C for an hour. Microdialysis against 0.1 M sodium bicarbonate solution was performed for 12 hours in order to remove excess dye. The Oregon Green labelled antibodies were conjugated with 40 fold molar excess pNP-PEG-PE and incorporated into liposomes with post-insertion method. Unattached antibody-PEG-PE molecules were filtered out with Vivaspin (300 000 MW) (Sartorius Stedim, DEU). Oregon Green 488 optical densities were measured in UV-Visible spectrophotometer and antibody amounts were calculated from Oregon Greens and antibody reaction's calibration curve.

2.2.3. Quantification of Doxorubicin

DXR was quantified with UV-Visible spectrophotometer at 480 nm. DXR was dissolved in methanol and separated into HPLC vials such that each vial had 500 µg DXR. Vials were placed in vacuum oven (Nüve EV 018, Turkey) in order to evaporate methanol, which were then incubated at 4°C until used. Calibration curve was constructed with DXR in the concentration range 1-50 µg/mL (Appendix A). Liposomal DXR was determined after rupturing liposomes in methanol. Absorbance was measured at 480 nm and DXR amount was calculated from the calibration curve. Non-interference of lipids at the same wavelength was also checked.

2.2.4. Quantification of Calcitriol

High performance liquid chromatography was used in quantitation of calcitriol. C18 reverse-phase separation column (5 μm, 4.6x250 mm) was used and MetOH:dH_2O (98:2) was used as the mobile phase. Retention time of calcitriol was observed after 5 minutes. Absorbance readings were performed at 255 nm. Column was heated up to 35°C. Flow rate was 1mL/min and samples were injected in 20 μl volume.

2.2.5. Quantification of Phospholipids (HSPC)

Phospholipid amount was determined with the method by Stewart Assay in order to determine the lipid amount that contributed in liposome formation [54]. Mechanism of the assay is based on the formation of a complex between phospholipid and ferrothiocyanate molecules. Liposome samples were dried by incubating them overnight in vacuum oven (Nüve EV 018, Turkey). Dried samples were dissolved in 2 mL chloroform solution in a glass tube to which 2 mL ferrothiocyanate solution was added. Then, solutions were vortex mixed for 1 minute and centrifuged at 2000 rpm for 5 minutes (Hettich EBA 20, UK). After centrifugation the chloroform phase was removed and absorbance was measured at 488 nm. HSPC amount was calculated according to the calibration curve constructed with known amounts of HSPC.

2.2.6. Characterization of Liposomes

2.2.6.1. Particle Size Measurement

Dynamic light scattering method was used to measure the mean particle size and particle size distributions of the liposome samples. The liposome samples were diluted to 1:10 with NaCl solution (0.9%) prior to measurements. The average hydrodynamic diameter of liposomes was determined using particle sizer (Malvern Mastersizer 2000) at Middle East Technical University Central Laboratory.

2.2.6.2. Morphological Characterization

Transmission electron microscopy (TEM) was used to study morphological characteristics of liposome samples. A drop of liposomal sample was allowed to dry on copper grid at room temperature. Then, sample was dyed with a drop of 2% uranyl acetate (Sigma-Aldrich Co., USA) solution. They were examined under the transition electron microscope (Philips, JEM-100CX) at 80 kV in METU Central Laboratory.

2.2.6.3. Encapsulation Efficiency and Loading

Encapsulation efficiencies of DXR and calcitriol were calculated by the ratio of encapsulated drug to the initially added drug. Equation is as follows:

$$\text{EE\%} = \frac{\text{amount of encapsulated drug}}{\text{initial amount of drug added}} \times 100\%$$

Percent drug loading was calculated by the ratio of encapsulated drug to liposomal lipid amount as according to the equation below:

$$\text{Drug loading \%} = \frac{\text{amount of drug encapsulated (mg)}}{\text{amount of liposomal lipid (mg)}} \times 100\%$$

2.2.6.4. Doxorubicin and Calcitriol Release Profile

Release profiles of DXR from liposomes at all formulations were studied. Briefly, 1 mL of liposome sample was placed into dialysis bag (12.000 Da MWCO, Sigma-Aldrich, USA) and the dialysis bag was transferred into polypropylene (PP) tubes containing 10 mL PB (0.1 M, pH 7.4). Tubes were placed in a shaking water bath (NÜVE ST 402, TUR) set at 37°C. Samples (1 mL) were taken from the release

medium (PB) for absorbance measurements at predetermined times; 2, 6, 12, 24 and 48 hours and the total release media was changed with fresh PB at all time points. Cumulative release amounts vs time graphs were plotted for each formulation (n=3).

2.2.6.5. Stability of Liposome

Stability of the liposome formulations was evaluated with periodic particle size measurements. Two different temperatures were used for storage, $4^{\circ}C$ and $25^{\circ}C$. Particle size measurements were recorded for 3 months for separate samples. Dynamic Light Scattering (Malvern Nano ZS90, Middle East Technical University Central Laboratory) of liposome samples were obtained and compared with fresh liposome formulations.

2.2.7. Cell Culture

2.2.7.1. Cell Culture Conditions

Namalwa cell line was cultured in RPMI 1640 medium without phenol red, 10% FBS and 2 mM L-glutamine and 1× penicillin-streptomycin. Cells were incubated at 37ºC under humidified atmosphere of 5% CO^2 – 95% air in incubator (SHEL LAB, USA). The medium was refreshed every two or three days. Cultures were maintained by replacement of fresh medium. When the cells reached at least 70-80% confluency, they were passaged.

2.2.7.2. In Vitro Cytotoxicity Assay

Cytotoxicity experiments were performed with free and liposomal formulations of both DXR and calcitriol on Namalwa cells and B lymphocytes. Cells were seeded in 96 wells at an initial seeding density of 300 cells/well. MTT tests for DXR were performed with experimental groups of free DXR (1.33 μg/well), pegylated liposomal DXR and targeted liposomal DXR. Samples of 20 μl were added to each

well and each group had 6 replicates. Liposomal groups were prepared in order to have 100, 200, 300 and 400 nmol/mL lipid concentrations per well. Doxorubicin amount in every drug loaded liposomal group was around 10 μg/well. Control group was seeded with cells only. Cytotoxicity results of 24 and 48 hours of exposure were investigated.

Calcitriol based experiments were performed with 24, 48 and 72 hours free calcitriol pretreatment groups. After calcitriol pretreatment, 24 hours of DXR treatment at IC_{50} concentration was applied to each group to study the effect of combined treatment. After promising results of free calcitriol with DXR treatment, DXR and calcitriol were co-loaded into liposomes. Namalwa cells were incubated with liposomes and toxicity data was collected at 24, 48 and 72 hours.

After complete treatment, media were removed from wells and cells were washed with PBS (0.01 M, pH 7.4). MTT test solution (500 μl) was added to each well and plate was incubated at dark for 4 hours. The test solution was removed and wells were treated with DMSO (500 μl) and cells were lysed by continuous shaking for 15 minutes. The absorbance values of plates were measured in microplate spectrophotometer (GMI Biotech 3550, USA) at 570 nm.

2.2.8 Statistical Analysis

One-way Analysis of Variance (ANOVA) test was applied to compare groups for single parameter. Tukey's Multiple Comparison Test for the post-hoc pairwise comparisons (SPSS-22 Software Programme, SPPS Inc., USA) was used; $p < 0.05$ was considered as statistically significant results.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Characterization of Liposome

3.1.1. Drug Encapsulation Efficiency and Percent Lipid Recovery of Doxorubicin Loaded Liposome

Drug encapsulation efficiency and percent lipid recovery were determined for conventional, PEGylated and CD20 targeted liposomes. The DXR encapsulation and release optimization was performed only with anti-CD20 antibody since anti-CD30 antibody was expensive and low in amount. DXR was loaded into liposome by ammonium sulfate gradient loading method. Over 95% encapsulation of DXR was achieved with this method for both conventional and PEGylated liposomes. The high encapsulation efficiency is related to DXR encapsulation to readily formed SUVs by ion exchange which happens after the shape and size formation steps; hydration and extrusion. With this method, it was suggested that DXR was encapsulated more than its solubility even accumulating as aggregates inside the liposome due to high ion gradient force [55, 56].

Ammonium sulfate gradient loading is accepted as an efficient method used for doxorubicin (DXR) loading [55, 56, 60, 61, 62]. Briefly, Ammonium sulfate solution was encapsulated into liposomes and the solution which surrounds the liposomes was changed with a salt solution. Due to creation of ammonia concentration difference, ammonia ions rush outside and leave a protonated hydrogen ion inside. Protonated hydrogen ions create an acidic environment that will attract doxorubicin into liposome. When doxorubicin enters the liposome it conjugated with SO_4^- and

aggregates inside the liposome that enables encapsulation beyond doxorubicin solubility. However, there are some major differences between methods like molarity of hydration solution, solution that liposomes were dialyzed in and purification methods. Ammonium sulfate gradient loading method was optimized by choosing steps with efficient results obtained through several tryouts.

Ammonium sulfate solution with different molarities was used for lipid film hydration with different extra-liposomal solutions, in the literature. 10% sucrose, 5% glucose and 0.9% NaCl were among three of the solutions used to exchange extra-liposomal media. In several studies, when ammonium sulfate solution with higher molarity (250-300 mM) was used as hydration solution, more saturated extra-liposomal solutions (10% sucrose or 5% glucose) were preferred [56, 60]. The reason of this was explained as the balance between hydration and extra-liposomal solution molarity should be adjusted by considering possible osmotic forces that can affect liposomes. As an example, ammonium sulfate solution (360 mM) was reported as hypertonic to 0.9% NaCl, but isotonic to 2.7% NaCl. When hypertonic ammonium sulfate was used a failure in the liposome structure was accrued to match the tonicity which reduces loading efficiency through osmotic swelling [55].

The extra-liposomal solution was changed by dialyzing against 0.9% NaCl solution. NaCl solution with low salt concentration was preferred in order to prevent any high absorbance peaks of salt that can interfere with drug or lipid detection. Several DXR loading experiments were performed with ammonium sulfate (250 mM) and 0.9% NaCl solutions. The encapsulation efficiency was around 30% which was due to hypertonic ammonium sulfate solution that was used. When lower concentration of ammonium sulfate (120 mM) was used, over 95% encapsulation was achieved.

Lipid recovery ranged between 60% and 75% percent for conventional and PEGylated liposomes as summarized in Table 3.1. A gradual decrease in lipid recovery was observed with increasing liposome modification. The lowest lipid recovery was observed for targeted liposome, which was thought to be caused by

vivaspin filtration step. This centrifugation step might have caused rupture of some liposome and washed them out through filter. The encapsulated drug with the rupture of some liposome was also washed out and for this aspect percent loading was not changed more than 2.37%.

Table 3.1. Comparison of drug encapsulation efficiencies (drug EE%) and percent lipid recoveries of doxorubicin loaded liposome formulations; conventional liposome (HSPC:CHOL 2:1), PEGylated liposome (HSPC:CHOL:DSPE-PEG, 2:1:0.2) and anti-CD20 targeted liposome (HSPC:CHOL:DSPE-PEG, 2:1:0.2).

Liposome	Drug EE (%)	Drug Loading (%)	Lipid Recovery (%)
Conventional	97.15±1.35	8.47±0.84	71.99±3.47
Stealth	96.56±1.20	8.27±0.56	62.70±4.86
Targeted	71.86±4.56	5.79±0.81	48.73±3.36

Drug encapsulation efficiency was also investigated after antibody (Anti-CD20) conjugation. It was observed that nearly 70% of the encapsulated DXR remained and not more than 30% of DXR leaked from liposomes during antibody incorporation step. The main reason of drug leakage in this step could be the disruption of liposome membrane by the detergent octyl glucoside, during antibody incorporation, as reported in a similar study [37]. Lukyanov et al, used readily formed commercial liposome Doxil and modified liposomes with monoclonal antibody 2C5. The nucleosome specific antibody was modified with pNP-PEG-PE molecule and incorporated into liposomes by post-insertion method. They reported that the DXR loss after antibody incorporation was not more than 20% [37]. The DXR retained after antibody incorporation in the study was reported 80% which was around 10% more than what we achieved. Lukyanovs' study has two main differences which were the use of readily purchased commercial Doxil and the separation method of unconjugated antibody-PEG-PE molecules. Separation method used in Lukyanovs' study was dialysis with cellulose ester tube with 300,000 Da cut off size which was a

nonaggressive method. However, in our study liposomes with similar composition were prepared by combining several different ammonium sulfate gradient loading methods and optimized. Unconjugated antibody-PEG-PE molecules were separated by centrifugation through 300,000 Da pored membrane that imposed gravitational force on liposomes which could have caused further DXR leakage.

The calcitriol combination groups were also analyzed for a possible effect of calcitriol co-loading on DXR encapsulation efficiency. DXR encapsulation was again over 95% which showed that membrane incorporated calcitriol had no effect on exchange between DXR and ammonia ions.

Percent loading was calculated for DXR loaded conventional, PEGylated and CD20 targeted liposomes. Percent DXR loading was 8.27±0.56% for stealth liposomes and 5.79±0.81% for CD20 targeted liposomes. There was 2.37% decrease in DXR loading which was the result of 30% drug loss during antibody incorporation. The commercial Doxil liposome formulation contains 2 mg/mL doxorubicin which corresponds to DXR percent loading of 20% which is 2.5 times higher than we achieved. The initial drug amount for encapsulation in our study was chosen low in order to sustain DXR release from liposome that is just enough to sustain IC_{50} value when the liposome concentration was 400 nmol/mL lipid in cytotoxicity experiments.

3.1.2. In Vitro Release Profile

Cumulative percent doxorubicin release was compared for conventional (HSPC:CHOL 2:1), stealth (HSPC:CHOL:DSPE-PEG, 2:1:0.2) and CD20 targeted liposomal formulations. The release media were collected and amount of DXR released was determined spectrophotometrically at 2, 6, 12, 24 and 48 hours. Release profiles were obtained as shown in Figure 3.1. A burst release from liposome formulations was observed at 2 hours. Conventional and PEGylated liposomes showed similar burst release outcomes; 14% and 16% of the total drug encapsulated,

respectively. However, targeted liposomes showed lower amount of burst release compared to other two formulations which was only 9% of total drug encapsulated. Also the extra PEG chains which were added in antibody attachment step to liposome surface might have enhanced this smaller burst release. In the step of pNP-PEG-DOPE modification of antibodies, some of the -pNP- groups hydrolyzed before antibody attachment and formed PEG-DOPE molecules which were incorporated into liposomes as additional PEG chains. Fastest release was observed for conventional type liposome which released half of its drug content in 48 hours. PEGylated (Stealth) liposome showed a slower and sustained release profile. Stealth liposomes released 26% of encapsulated DXR at the end of 48 hours. In literature, PEGylation was mentioned as a method to stabilize liposome structure and thereby sustain release [59]. Targeted liposome formulation had the same lipid and PEG ratio with stealth liposome which supports the similar drug release profile observed with stealth liposomes. Release rate of targeted liposome was, however, slightly slower. This small difference can be explained by addition of extra PEG chains which were used for antibody conjugation. Slow release profiles of PEGylated and targeted liposome groups were a desired result for the purpose of liposomal system developed in this study. Here it is aimed to send the liposomal system directly to the target cells and release their content in or at the vicinity of these cells. That's why they should hold as much drug as they can until they reach their target cells. To this end, HSPC phospholipid was used in the composition for decreasing drug release before reaching to the target.

HSPC has saturated and long fatty acyl chains that forms more stable liposomal membrane which can lower drug release. When compared with other phospholipids, a slower release rate was observed when HSPC is used as the main phospholipid in liposome [58]. Also in this study, same lipid composition with the commercial liposomal doxorubicin (Doxil) was used. The slow release profile (1% in 2 hours) of Doxil liposome formulation was reported, that is slower than our liposome release profile at 2 hours [57]. This difference could be due to difference in release media of two studies. (10 mM HEPES in Fritze et al, [57] and 10 mM PBS in our study). After

bust release our stealth liposomal formulation did not release more than 1% of encapsulated drug for more than 12 hour which shows a similar release profile. The release rate of DXR from similar liposomal formulations are reported as slow and sustained for long periods [63]. Conventional liposomes released 56% of DXR after 48 hours.

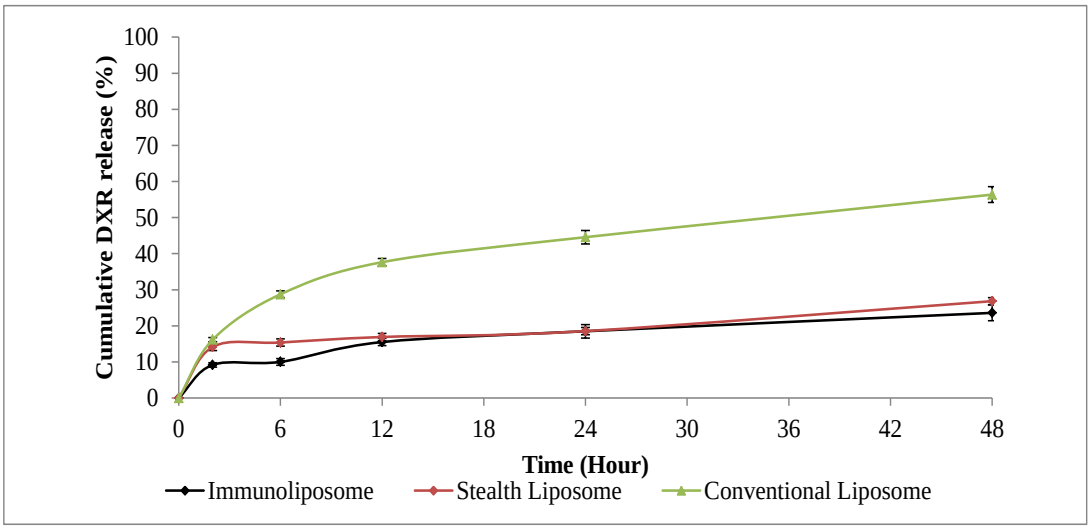


Figure 3.1. Comparison of cumulative DXR release profiles from conventional liposome (HSPC:CHOL 2:1), PEGylated liposome (HSPC:CHOL:DSPE-PEG 2:1:0.2) and anti-CD20 targeted liposome (HSPC:CHOL:DSPE-PEG 2:1:0.2) in PBS (0.1 M, pH 7.2), at 37°C through 48 hours.

3.1.3. Particle Size Distribution and Stability of Liposome Formulations

Size is one of the important parameters affecting the clearance rate of liposomes from the blood circulation. In literature, it is stated that larger sized liposomes have more tendency to be eliminated from circulation than smaller liposomes [7]. When administered intravenously, opsonization of these liposomes results with their recognition as foreign by the macrophages and accumulation in tissues of reticuloendothelial system (RES), a system responsible for main clearance in the body. In liver there are fenestrated endothelium pores with a mean diameter of 100-

150 nm. So, from these pores, liposomes below this size can escape into blood circulation [64]. Blood contains high density lipoproteins (HLD) that are responsible of transferring liposomal lipids to serum lipoproteins. This results with disruption of liposomal membrane and release of encapsulated content. It was reported that smaller liposomes are more susceptible to HDL effect in circulation [65, 66]. Besides that, particles below 100 nm are more sensitive to hepatocyte interaction that causes removal from circulation via hepatic uptake [69]. The optimum size range for stealth liposomes was reported as 120-200 nm considering size of fenestrations in the hepatic sinusoidal endothelium [70]. For our study, optimum size range for liposomal formulations was, therefore, chosen as between 100 nm and 200 nm as also commonly used in literature.

Conventional and PEGylated liposomes were prepared by a final extrusion through 100 nm pore sized polycarbonate membranes. The extrusion temperature was high enough to resize liposomes and size around 100 nm was easily obtained after eleven passages through the extruder.

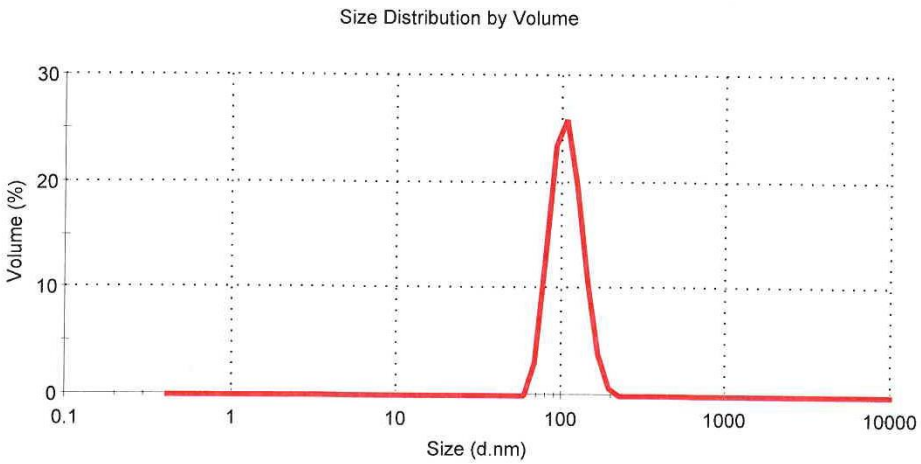
Dynamic Light Scattering (DLS) method was used to determine particle size of the liposomes and Mie Theory was used to calculate hydrodynamic diameter of the liposomes in aqueous form. The diameter obtained by this technique refers to a sphere having the same translational diffusion coefficient as the liposome being measured.

Table 3.2 shows the size distribution comparison of doxorubicin loaded conventional liposomes and doxorubicin loaded targeted liposomes. Size difference observed can be explained by the modification steps; PEGylation and addition of antibodies to liposome surface. Although the average size increased after these modifications, average size of targeted liposomes was still in the optimum liposome size interval (100-150 nm). The mean hydrodynamic diameters of the liposomes also had monodisperse size distribution (PdI $\leq$ 0.1).

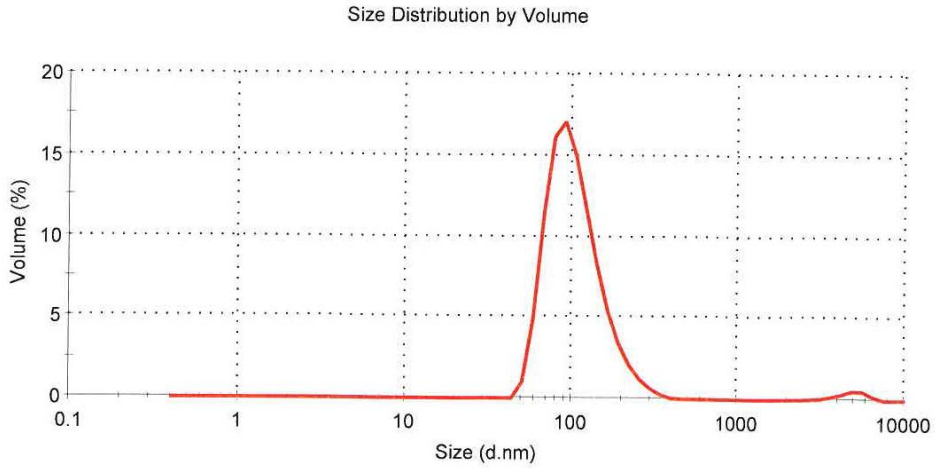
Table 3.2. Size distribution results of doxorubicin loaded PEGylated and targeted liposomes

Liposome Type	90th Percentile	Z-average (nm)	Peak Diameter (nm)	Width (nm)	PdI
PEGylated (HSPC:CHOL:DSPE-PEG)	141.8	114.7	107.8	23.62	0.04
CD20 targeted (HSPC:CHOL:DSPE-PEG)	164.2	131.6	142.0	52.94	0.18

Figure 3.2 shows the representative size distribution of doxorubicin loaded PEGylated and targeted liposomes obtained by DLS analysis. PEGylated liposomal formulation had unimodal size distribution with narrow range. Polydispersity index of targeted liposomes was slightly higher than 0.1 indicating a broader size distribution. Main reason of higher PdI value was related to the antibody modification by post-insertion method. In this step antibody-PEG-PE micelles were forced into readily formed PEGylated liposomes by the help of a detergent molecule. Antibodies were attached to the end of PEG chains and created a larger liposome structure. A small second peak at 4809 nm was observed (Fig. 3.2b) probably due to aggregation of particles before or during size analysis.



a)



b)

Figure 3.2. Representative DLS result of size distribution for doxorubicin loaded liposomes **a)** PEGylated (HSPC:CHOL:DSPE-PEG) **b)** Targeted (HSPC:CHOL:DSPE-PEG)-Anti-CD20

In literature liposome stability was defined in three aspects; biological, physical and chemical. Biological stability indicates the in vivo life time of liposome which is related with the immune response and RES of the body. As mentioned above, when administered into blood stream, liposome will encounter blood proteins, opsonins which make them available for immune cells. For increasing in vivo life time of the liposome we applied two approaches that are commonly used in similar literature; 1) to disable opsonin binding, surface modification with hydrophilic PEG chain that

make them stealth by increasing hydrophilicity and 2) to overcome RES effect, extruding liposome to have an average size around 100 nm that will enable escape through fenestrated pores (100-150 nm) of liver tissue [64].

The liposomal stability is mainly defined by shelf-life which depends on physical and chemical properties. The deformation of liposome mainly occurs by oxidation of lipids and creation of shorter more soluble fatty acid chains which then leads to aggregation of liposomes [67]. In our study, stability and shelf life of liposome formulations were investigated by particle size measurements for 3 months period. Targeted liposome samples were stored at two different temperatures (4°C and 25°C) and particle size measurements were performed after each month (Table 3.3).

In an aqueous medium liposomes tend to aggregate into larger vesicles. Oxidation of lipid molecules destabilizes liposomes and together with attractive forces, aggregation rate increases. This deformation can be observed by increase in average diameter and PdI values of DLS analysis [68]. The time point at which maximum effective size is exceeded can be accepted as the shelf life of the liposome formulation. In Table 3.3 particle size distribution parameters of antibody targeted liposome that was stored at two different temperatures are presented.

Table 3.3. Change in particle size distribution of the anti-CD20 targeted liposome stored at 4°C and 25°C for 3 months

Month	Stored at 4°C		Stored at 25°C	
	z-average (nm)	**PdI**	**z-average (nm)**	**PdI**
0	131.6	0.037	131.6	0.03
1	142.0	0.044	176.4	0.08
2	166.8	0.056	183.1	0.09
3	168.4	0.074	217.1	0.19

When stored at 25°C liposome formulation was less stable than at 4°C. So, they were more susceptible to aggregation at room temperature. As one mechanism of aggregation, attractive hydrophobic forces were thought to make liposomes fuse. As the storage time increased, the size distribution and average diameter increased. When the liposomes were stored at 4°C, they were more stable and able to sustain narrow size distribution. At this storage temperature they had average diameter, 168.4 nm, after 3 months, which was slightly above the desired size interval (100-150 nm). Hence it can be suggested that targeted liposome have at most 3 months of shelf life at 4°C and 2 months at 25°C.

3.3. Encapsulation Efficiency and Release of Calcitriol from Liposome

Calcitriol has proved its antineoplastic activity on several tumor models [49, 73]. Antitumor activity of calcitriol upon its solitary administration was reported to be correlated with its ability to induce differentiation and growth arrest of myelomonocytic leukemia cells [72]. Synergistic effects of calcitriol with cytotoxic chemotherapy, radiation, and other cancer drugs have been reported. Among these, studies of the synergistic effect of calcitriol with DXR are also found [47, 50]. However, there was one drawback indicated for the calcitriol administration which is the hypercalcemia effect when administered systemically at high doses. Calcitriol level has to be below 50 pg/mL (0.12 nM) in serum in order not to have hypercalcemia effect which means a dose of 0.25 μg can be taken safely for a daily treatment [73]. Therefore, considering the additive antiproliferative effect when used with doxorubicin, it was decided to add calcitriol to doxorubicin loaded liposomes. By encapsulating calcitriol, it was aimed to overcome hypercalcemia while providing high doses of calcitriol together with doxorubicin to cancer cells.

Calcitriol is a lipophilic substance that is expected to be entrapped within liposomal membrane between fatty acid layers. In literature a few liposomal formulations were presented for calcitriol and they are mainly for topical delivery. A patent named “Liposome-based topical calcitriol formulation” was presented and calcitriol

encapsulation was achieved by adding calcitriol to lipid film [71]. A different study that targets prostate cancer used the same encapsulation method for calcitriol and compared 1,25$(OH)_2D_3$ antitumor effect with 1,25$(OH)_2D_3$-3-BE [74].

HPLC quantification was used to separate and detect calcitriol in order to eliminate lipid interference in measurements. Calcitriol peak was observed at 4.182 minutes. A representative HPLC peak is shown in Figure 3.3.

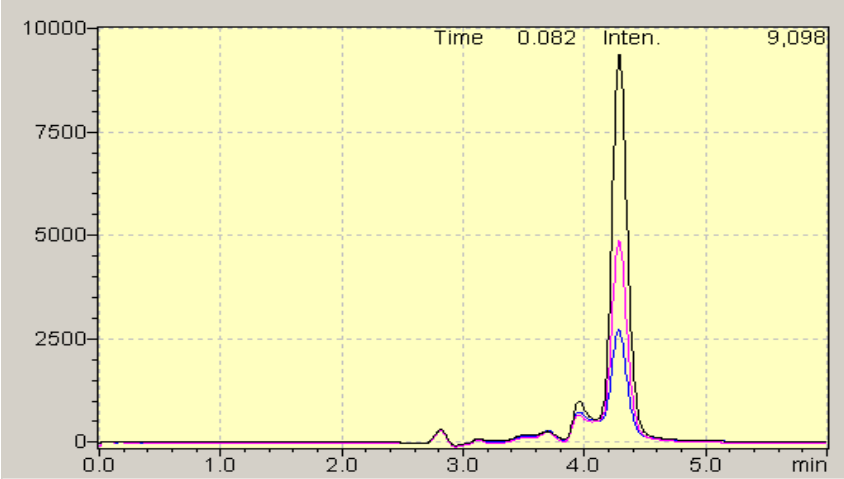


Figure 3.3. HPLC chromatograms of calcitriol at different concentrations (2, 1 and 0.5 µg). Retention time for calcitriol was 4.182 minute at 255 nm. C18 reverse-phase separation column (5 µm, 4.6x250 mm), MetOH:dH_2O (98:02)

Calcitriol encapsulation efficiency of liposome was 42.84%. The percent loading of calcitriol was calculated as 0.4%. In the study of Ray et al, [74], DMPC (20 µg) liposomes was prepared with 1 µg calcitriol which corresponds to 5% loading of calcitriol if encapsulation was occurred at 100% efficiency; no data was presented for encapsulation efficiency. Possible effect of encapsulated calcitriol on DXR encapsulation and release profiles were investigated with co-loading of calcitriol and DXR and no difference was observed on doxorubicin encapsulation which was over 95%.

Calcitriol release from doxorubicin loaded stealth liposomes was slow as expected from the hydrophobic nature of calcitriol. Figure 3.4 shows the release profile of calcitriol from doxorubicin loaded liposomes for two days. Release medium was a hydrophilic environment in which calcitriol has low solubility. Calcitriol as a hydrophobic molecule was thought to be retained in the lipid bilayer-liposomal membrane mostly and only 3.25% was released after 2 days. There was no data present in literature about the release profile of calcitriol but the results were expected and in accordance with above discussion. The encapsulation and released amounts of calcitriol were suitable for the antiproliferative effect and purpose of developed liposomal formulations. The slow release of calcitriol, supports a long term release at a concentration with anti-proliferative effect. An internalizing, targeted, calcitriol loaded liposome formulation will be designed in order to deliver calcitriol directly into target cell cytoplasm in the future work. It is thought that slow release of calcitriol will further enhance the anti-proliferative effect of targeted liposomal system.

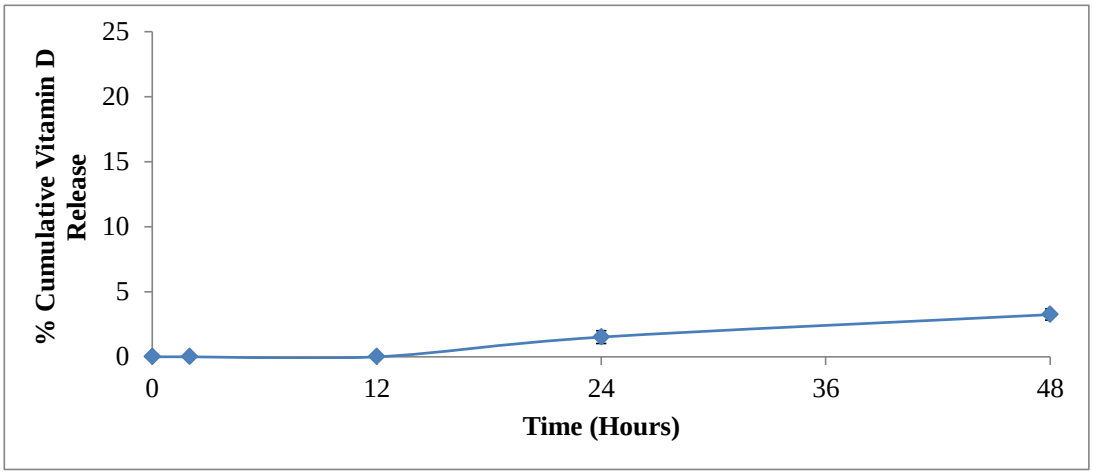


Figure 3.4. Calcitriol release from PEGylated liposome (HSPC:CHOL:DSPE-PEG) through 48 hours in PBS (0.1 M) at 37°C

Doxorubicin and calcitriol co-loaded liposomes were prepared with the same lipid content as single drug loaded liposome (HSPC:CHOL:DSPE-PEG). According to

calcitriol release rate from stealth liposome, calculated release amount of calcitriol will be around 1.61 µg in 72 hours.

Doxorubicin release from calcitriol loaded liposome is shown (Fig. 3.5). The calcitriol which was encapsulated to bilayer affected the release rate of doxorubicin. When liposomes were prepared only with doxorubicin, 26% of the drug was released after 48 hours. However, when doxorubicin was co-loaded with calcitriol 4.65% of the drug was released after 48 hours. The slower release rate of the drug showed that DXR permeability of bilayer was decreased when calcitriol was loaded into liposome membrane.

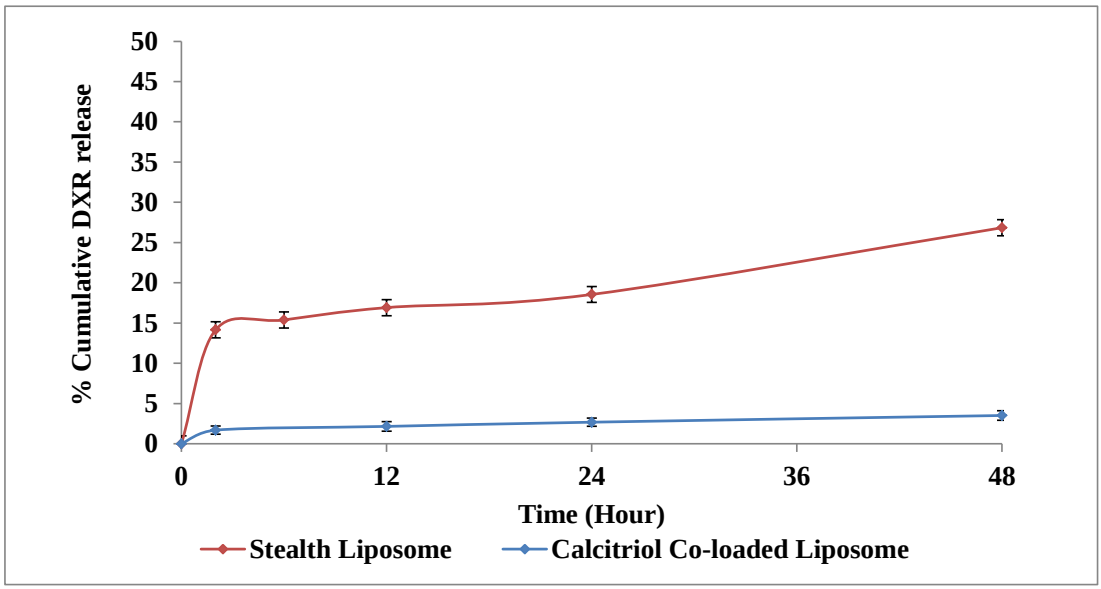


Figure 3.5. Doxorubicin release rate comparison of only doxorubicin loaded and doxorubicin/calcitriol co-loaded liposome through 48 hours in PBS (0.1 M) at 37°C

3.3. Liposome Modification with Antibodies

3.3.1. Coupling of pNP-PEG-DOPE Molecules with Antibody Molecule

For preparation of immunoliposome, CD20 or CD30, was reacted with pNP group of pNP-PEG-DOPE molecules (Fig. 3.6.). pNP group is reactive at basic pH so reaction proceeded around pH 8.0. Free pNP groups that did not react with the antibodies were promptly eliminated by spontaneous hydrolysis at pH 8.0 and turned into PEG-DOPE chain. The main question was if the reaction between pNP group and amino groups of antibodies was fast enough to overcome the fast hydrolysis of pNP at this pH. Lukyanov et al found out that around pH 9.3, 65% of pNP group bonded to antibody and remaining 35% was hydrolyzed to form normal PEG-Lipid unit in the bilayer which shows that pNP binding can overcome fast hydrolysis [37].

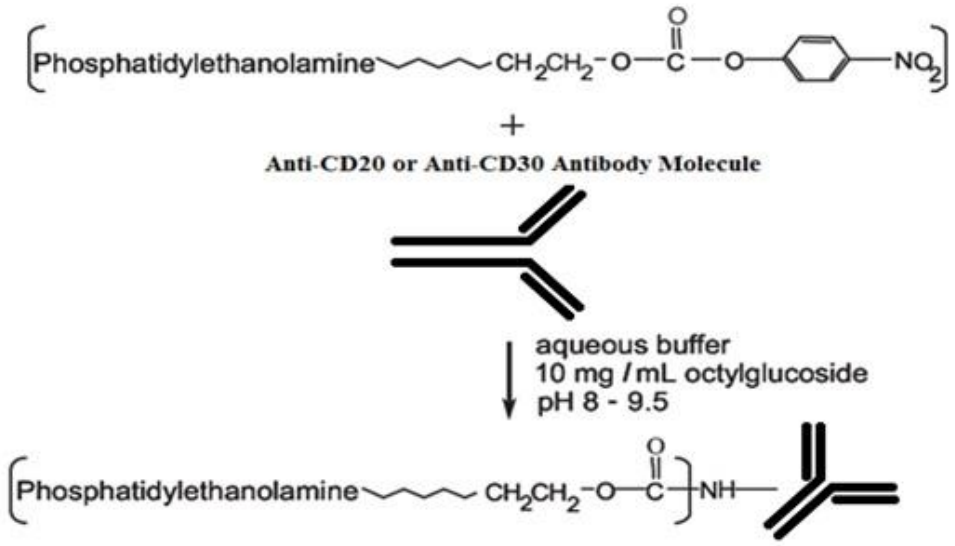


Figure 3.6. pNP-PEG-DOPE modification of anti-CD20 and anti-CD30 molecules. Urethane (carbamate) bond was formed between amino groups of antibody and PEG-PE molecules. (Modified from Lukyanov et al, [37]

The reaction efficiency of antibody and pNP-PEG-PE molecules was tested with an amine reactive dye called Fluorescamine which becomes fluorescent when reacted with primary amine groups as lysine. The amine groups were also the reaction site for "pNP" groups. The decrease in the absorbance of Fluorescamine showed that some of the primary amine groups were coupled with pNP-PEG-PE units. A 35% decrease in the absorbance was recorded when reaction occurred. The CD20 antibody molecule has a total of 49 lysine residues 36 of them being on heavy chain and 13 of them on the light chain. According to O.D. measurement and calculations, an average of 15 of the 49 lysine residues of antibodies was modified with pNP-PEG-PE which corresponded to 35% decrease in absorbance. When pNP-PEG-PE molecules were bound to Anti-CD30 Antibody, 65% of the lysine residues were found to be reacted. The exact number of amino groups reacted could not be calculated due to lack of amino acid chain information of Anti-CD30 in literature. The amount of modification was good enough to sustain an easy incorporation to the liposomal surface. It was reported that variable number of PEG-PE residues (10–32) per protein molecule were conjugated with different initial concentration of pNP-PEG-PE by Lukyanov et al, [37].

3.3.2. Liposome Incorporation of Antibody-PEG-DOPE Units

Synthesized Antibody-PEG-DOPE unit was ready to be incorporated into liposome bilayer by phosphoethanolamine (PE) end. DOPE lipid molecule was joined to lipid bilayer and antibodies were attached facing outwards from the surface. After the antibody molecules were labelled with Oregon green 488 dye. The number of antibody molecules conjugated to liposome was calculated with Oregon Green calibration curve after dissolution of Oregon green labelled liposomes in methanol. The liposome number in the solution was estimated from a calculation that uses amount of the lipid present and known liposome size. Basically, the phospholipid molecule number in a single liposome was calculated accordingly and expressed as μg, and then the total lipid amount (μg) was divided to this number [75].

$$N_{tot} = \frac{4\pi\left(\frac{d}{2}\right)^2 + 4\pi\left[\frac{d}{2} - h\right]^2}{a}$$

Where;

d is the diameter of liposome

h is the thickness of the lipid bilayer

a is the phospholipid head group area

N_{tot} is the total number of lipids per liposome

$$N_{lipo} = \frac{M_{lipid} \times N_A}{N_{tot} \times 1000}$$

Where;

M_{lipid} is phospholipid molarity in solution

N_A is the Avogadro number

N_{tot} is the total number of lipids per liposome

As a detergent molecule, Octyl-glucoside was used to create openings on lipid bilayer to make micelles able to integrate which also caused around 20% drug loss as explained in section 3.1.1. In literature attachment of around 100 antibodies per single 100 nm liposome was reported. In 1mL of solution, number of liposomes was calculated as 1.81×10^{12}. The total antibody number calculated was divided into total liposome number to calculate antibody attachment per single liposome. Our result was "92" for anti-CD20 and "31" for anti-CD30 antibodies per single 100 nm sized liposome. So, it was considered as enough for necessary cell interaction. Lukyanov et al, also concluded that all bound proteins completely preserved their specific activity by investigating specific binding by Elisa [37].

3.4. In Vitro Cytotoxicity Studies

Two different cell lines were used for in vitro studies; B Lymphoma (Namalwa) and B lymphocyte (Lymphoma). The morphology of Namalwa cells is shown in immunofluorescence microscopy image in Figures 3.7. B Lypmhoma (Namalwa) cells are CD20 positive cell lines that were used to compare cytotoxicity of anti-CD20 targeted and untargeted liposome formulations. In literature anti-CD20 antibody interaction with Namalwa cell line was preferred to study non-internalizing liposomes [40, 76, 77]. Anti-CD30 cytotoxicity studies were performed by interaction of liposomes with B lymphocyte (Lymphoma) cell line which are CD30 positive cell lines. Molavi et al, reported the enhanced cytotoxicity of anti-CD30 by its internalizing effect [80].

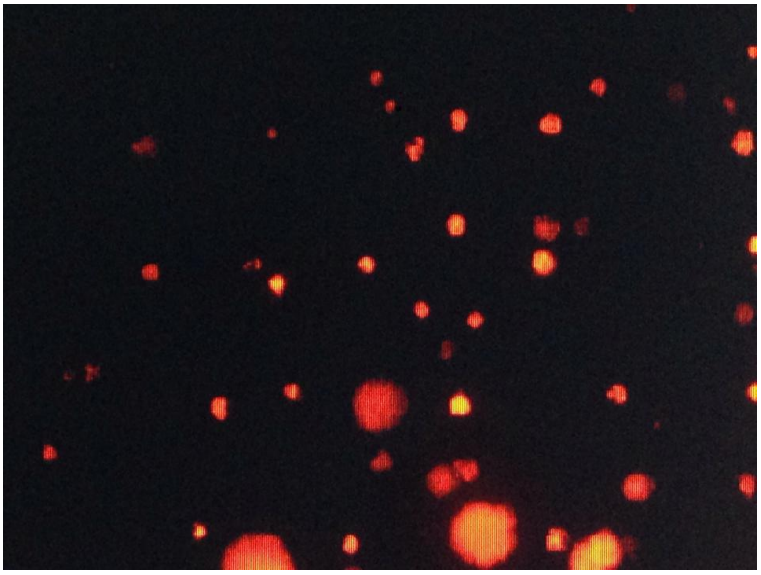


Figure 3.7. Morphology of floating Namalwa cells (RPMI 1640 medium, 300.000 cells) stained with ethidium bromide acridine orange dye observed under immunofluorescence microscopy (20X)

3.4.1. Cellular Toxicity of Doxorubicin

In order to study effective dose, different concentrations of doxorubicin (1.5, 1.125, 0.75 and 0.375 μg/well DXR) were added to B Lymphoma (Namalwa) cells and incubated for 24 and 48 hours. MTT assay showed that cell viabilities decreased with increasing concentrations of DXR after 24 hours. IC_{50} value for the doxorubicin was calculated as 11.47 μM (1.33 μg/well) for 24 hours incubation. This value was used as reference for further liposome studies and also in control groups with free drug. Lipid dose as 400 nmol/mL was used for drug loaded PEGylated liposomes to achieve 50% toxicity and in order to detect any improvement in toxicity with targeted liposomes.

Figure 3.8 shows the viability of DXR treated B Lymphoma cells (with respect to control) after 24h and 48h incubation in doxorubicin containing media. Cell viability decreased as drug concentration and incubation time increased. After 48 hours of exposure, the group with lowest DXR concentration showed no increase in cytotoxicity compared to 24h incubation. The average cytotoxicity of groups increased 9.47% and 6.06% for 1.13 and 0.75 μg/well DXR. The group with highest drug concentration showed a decrease in cytotoxicity after 48 hours (8.77%).

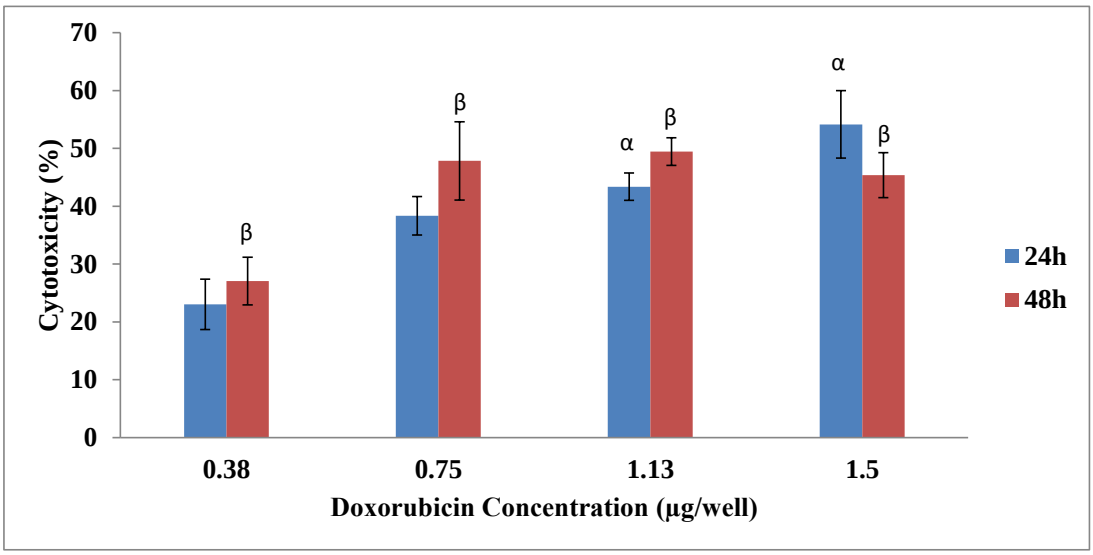


Figure 3.8. Dose dependent (0.38-1.5 µg/well) effect of DXR on Namalwa cells after 24 and 48 hours incubation. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

α: statistically significant difference between different dosage groups ($p<0.05$)
β: statistically significant difference between 0.38 µg/well doxorubicin and other groups ($p<0.05$)

3.4.2. Enhancement of Doxorubicin Cellular Toxicity by Calcitriol Pretreatment

The anti-proliferative activity of calcitriol over tumor cells was studied in literature as use of calcitriol alone and combination with cancer drugs for treatment like Cisplatin, Gemcitabine and doxorubicin [49, 73]. Enhanced DXR cytotoxicity over breast cancer cell line by calcitriol treatment (10 nM) was reported by Ravid et al [50]. In our study, calcitriol concentrations for pretreatment were selected according to literature. Study of Ravid et al, also concluded that 72h of calcitriol (10 nM) pretreatment blocks the production of antioxidant enzymes which improves oxidative damage caused by DXR cell interaction.

Namalwa (B Lymphoma) cells were pretreated with different calcitriol concentrations for 24, 48 and 72 hours and by 24 hours of DXR incubation. Control groups had free doxorubicin without pretreatment of calcitriol. Cytotoxicity enhancement effect of calcitriol pretreatment was investigated with MTT assay. Figures 3.10, 3.11 and 3.12 show cytotoxicity results of different (24, 48 and 72 hours) calcitriol pretreatment followed by 24 hours incubation with DXR. Calcitriol concentrations ranged between 25×10^{-5} and 10 μg/mL. After calcitriol pretreatment, groups were subjected to DXR (IC_{50}) for 24 hours. Cellular viability was between 55% and 65% for all groups when 24 hours of pretreatment was applied (Fig 3.10). When compared to free DXR group, no statistical difference was observed and no additional cytotoxicity was created by 24 hours calcitriol pretreatment. Highest cytotoxicity was detected when calcitriol concentration was 10 μg/mL (65.54±2.17%), which can be a sign for possible cytotoxicity enhancement. Percent cellular toxicity decreased for all groups when 48 hours of calcitriol pretreatment was applied. The main reason for this is the proliferation of Namalwa cells through pretreatment time, due to doubling time of the Namalwa cells being around 35h [82]. There was no statistical difference observed for toxicity between free DXR and calcitriol pretreatment groups. Calcitriol, 10 μg/mL pretreatment group has the highest cytotoxicity (54.02±2.02%). Until this point cytotoxicity was strongly related to IC_{50} DXR exposure and no considerable enhancement was observed upon calcitriol pretreatments. A meaningful increase in cellular toxicity was observed in two concentration groups when calcitriol pretreatment time increased up to 72 hours. When 5 μg/well and 10 μg/well calcitriol concentrations were used, pretreatment for 72 hours enhanced cytotoxicity up to 91.43% and 93.21%, respectively. Other calcitriol concentrations were not high enough to create any difference on DXR cytotoxicity and showed similar cellular toxicity with free DXR group. The enhancement of DXR cytotoxicity on Namalwa cells by 72h calcitriol pretreatment was shown by our study. The 72h pretreatment with calcitriol at concentrations of 5 and 10 μg/well were able to create over 90% cellular toxicity on Namalwa cells following 24h subjection to IC_{50} dose of DXR.

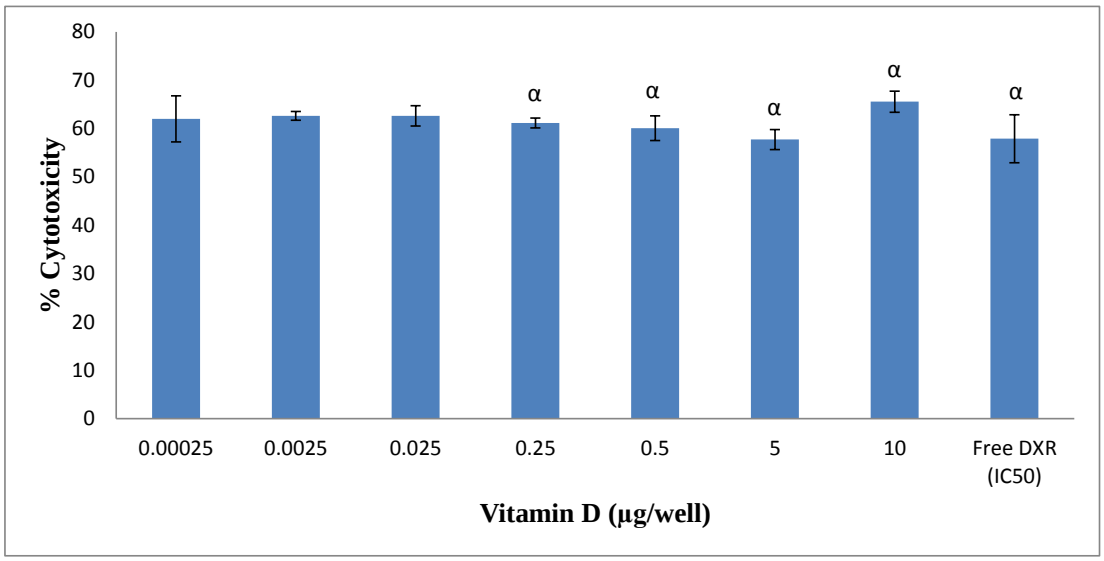


Figure 3.9. Viability of Namalwa cells after 24 hours of calcitriol pretreatment followed by 24 hours doxorubicin incubation at IC_{50} concentration. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

α: statistically significant difference between 10 µg/well calcitriol and other groups ($p<0.05$)

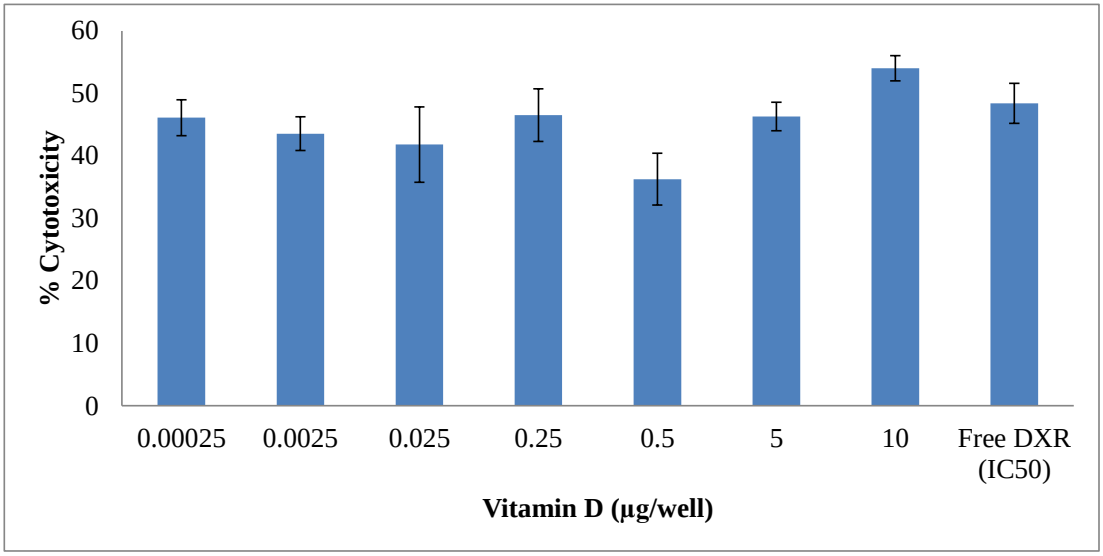


Figure 3.10. Viability of Namalwa cells after 48 hours of calcitriol pretreatment followed by 24 hours doxorubicin (IC_{50}) incubation. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

All groups were statistically significant different than 10 µg/well calcitriol ($p<0.05$)

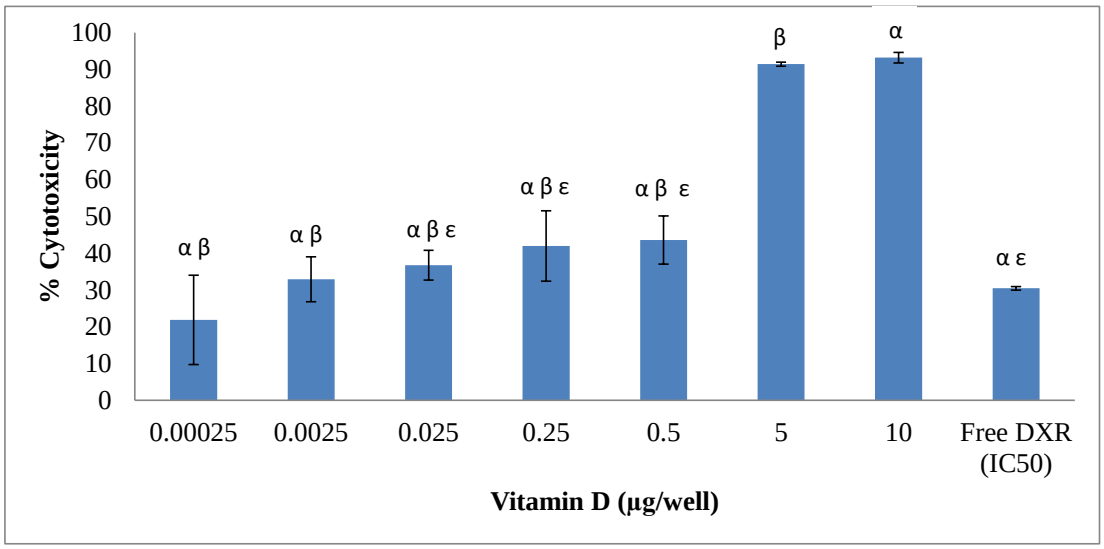


Figure 3.11. Viability of Namalwa cells after 72 hours of calcitriol pretreatment followed by 24 hours doxorubicin (IC_{50}) incubation. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

α: statistically significant difference between 10 µg/well calcitriol and other groups ($p<0.05$)

β: statistically significant difference between 5 µg/well calcitriol and other groups ($p<0.05$)

ε: statistically significant difference between free DXR (IC_{50}) and other groups ($p<0.05$)

3.4.3. Cellular Toxicity of Calcitriol and Doxorubicin Co-loaded Liposome

Calcitriol was encapsulated into hydrophobic lipid bilayer of DXR loaded liposomes. B Lymphoma (Namalwa) cells were incubated with both DXR loaded and DXR/Calcitriol co-loaded liposomes for 24, 48 and 72 hours. The main drawback oral or intravenous application of calcitriol is the need of super-physiological concentrations in order to create antineoplastic effects. However, these high concentrations cannot be achieved due to risk of hypercalcemia [49]. That is why we

developed DXR and calcitriol co-loaded liposomes that will create either a sustained release at the vicinity of the cancer cells having CD20 antigens on their surface or provide high intracellular concentration in cells especially for those having CD30 antigens on their surface, thereby eliminating the risk of hypercalcemia. In parallel to this aim, cellular toxicity of calcitriol/DXR co-loaded liposomes was higher than just DXR loaded liposomes after 24 hours. After 72 hours incubation with co-loaded liposomes, difference in cellular toxicity was more recognizable for 100, 200 and 400 nmol/mL groups which were parallel to pretreatment experiment results. Lower concentration groups of calcitriol co-loaded liposomes also showed higher toxicity then only DXR loaded groups.

Figures 3.13-3.15 show cellular toxicity of DXR loaded and DXR/Calcitriol co-loaded liposomes after 24, 48 and 72 hours of incubation. Here, liposome concentrations ranged between 25 and 400 nmol lipid/mL. Although calcitriol co-loaded groups showed enhanced cytotoxicity for all concentration groups, the effect was more pronounced when concentrations of 100 nmol/mL and higher were used. After 24 hours of incubation calcitriol co-loaded, 100, 200 and 400 nmol/mL groups, showed high cellular toxicities (83.74 ± 5.32%, 83.22 ± 3.77% and 82.89 ± 3.43%) which were superior to DXR loaded liposome group. Cellular toxicity decreased with decreasing liposome concentration when Namalwa cells were incubated with DXR loaded liposome. However, calcitriol co-loaded liposomes showed higher cellular toxicity at all concentrations. Upon 48 hours incubations, cytotoxicity of DXR loaded liposome increased up to the level of calcitriol co-loaded liposomes. The reason of this was thought as the DXR release from liposome in time. After 72 hours, liposomes loaded with DXR only lost their effect over Namalwa population and their cellular toxicity decreased to around 50% in all concentrations. The additive effect of calcitriol co-loading was more obvious through 72 hours incubation. After 72 hours, calcitriol co-loaded groups with 200 and 400 nmol/mL preserved their high cellular toxicity (83.55 ± 4.38% and 85.22 ± 4.16%) over Namalwa cells. Cytotoxicity of calcitriol co-loaded liposome groups were high relative to only DXR loaded liposomes.

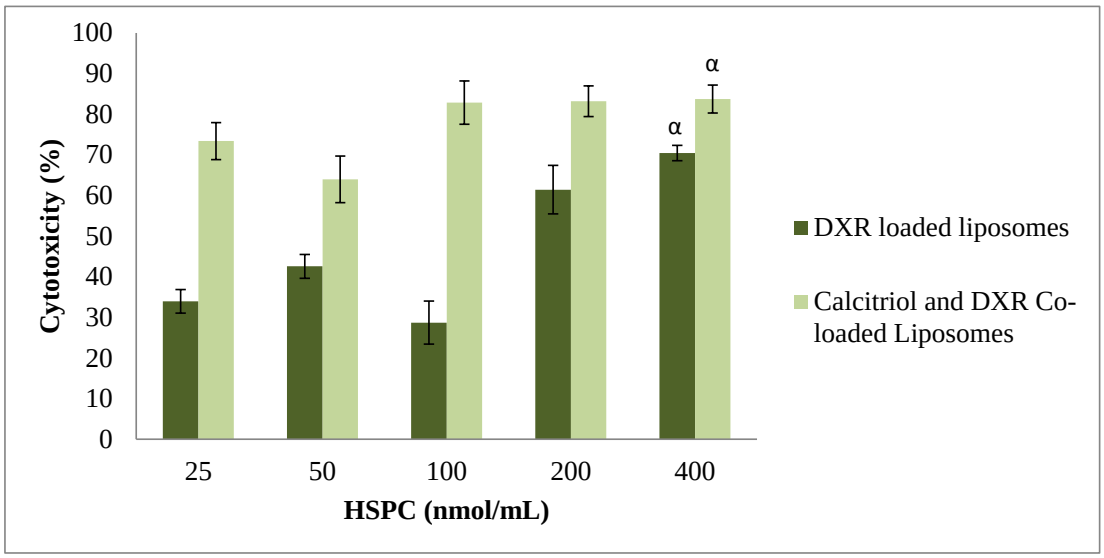


Figure 3.12. Viability of Namalwa cells after 24 hours incubation with doxorubicin loaded and calcitriol/doxorubicin co-loaded liposomes. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

All calcitriol and DXR co-loaded liposome groups were statistically significant different than DXR loaded liposome groups ($p<0.05$)

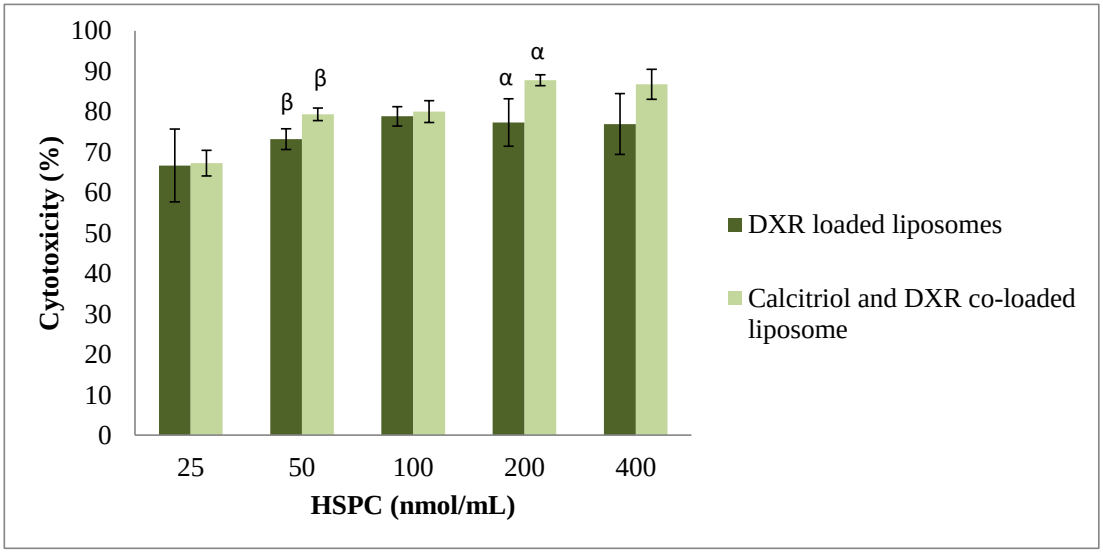


Figure 3.13. Viability of Namalwa cells after 48 hours incubation with doxorubicin loaded and calcitriol/doxorubicin co-loaded liposomes. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

α, β: statistically significant difference between different dosage groups ($p<0.05$)

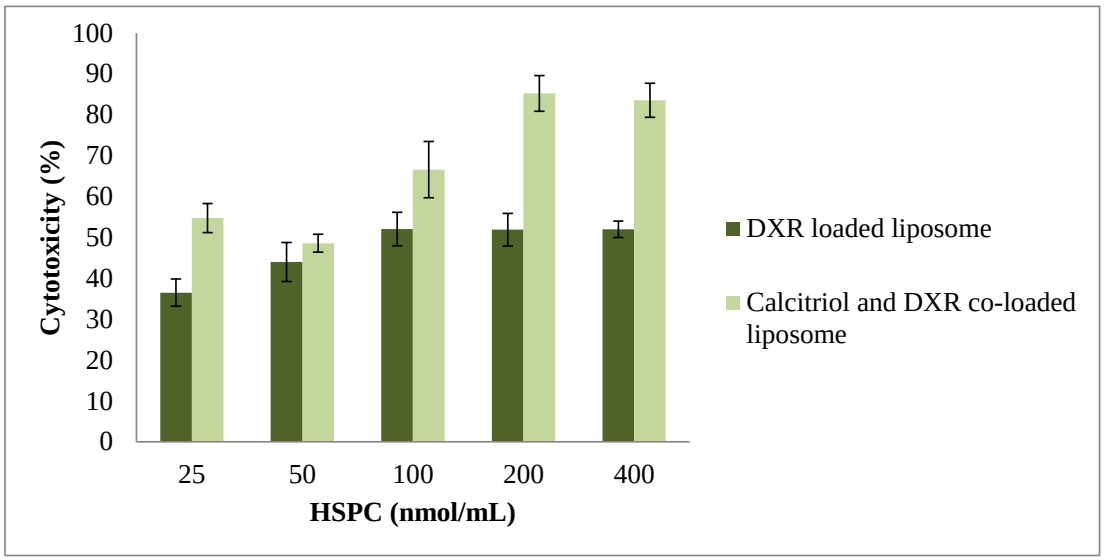


Figure 3.14. Viability of Namalwa cells after 72 hours incubation with doxorubicin loaded and calcitriol/doxorubicin co-loaded liposomes. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

Calcitriol and DXR co-loaded liposome groups with 25, 100, 200 and 400 nmol/mL HSPC were statistically significant different than DXR loaded liposome groups ($p<0.05$)

When calcitriol and DXR were co-loaded into liposomes, a faster anti-proliferative activity was observed than their free form dose groups (Fig. 3.10-3.12). In the free calcitriol experiment, DXR was introduced after 24, 48 and 72 hours of free calcitriol pretreatment and enhancement effect was observed after 72 hours. But in liposome formulations, cytotoxicity improvement compared to free forms was observed even after 24 hours. Liposomes might have nonspecifically interacted and fused into cell membrane by nonspecific endocytosis and released their content into cell which could be the main reason of higher toxicity. The calcitriol co-loaded 400 nmol/mL liposome group contains around 2.5 µg calcitriol and 25 µg DXR encapsulated. In the calcitriol pretreatment experiment, 2.5 µg calcitriol was enough to create cytotoxicity enhancement. So amount of calcitriol in liposome was high enough to create enhanced cytotoxicity. After a possible cell fusion, the release of calcitriol and

DXR to cell cytoplasm was expected. The dual delivery can thus provide enhanced cytotoxicity.

3.4.4. Cellular Toxicity of Anti-CD20 Targeted DXR Loaded Liposome

Anti-CD20 targeted doxorubicin loaded liposomal formulations were compared for cellular toxicity with untargeted liposomal doxorubicin. In order to determine cytotoxicity created by individual parts of the system, experimental groups were prepared as; free doxorubicin, free anti-CD20, PEGylated DXR loaded or empty liposome, PEGylated and anti-CD20 targeted DXR loaded or empty liposome. Anti-CD20 was reported to show therapeutic act against lymphoma types by its single and combinational treatments [78]. Cellular toxicity of anti-CD20 (Rituximab) and its combination with DXR loaded liposomes targeting were investigated in our study.

Cellular toxicity caused by liposomal formulations and non-liposomal drugs after incubation with Namalwa cells for 24 and 48 hours are presented in Figures 3.16 and 3.17 respectively. All groups were prepared with four different concentrations of HSPC phospholipid (100, 200, 300 and 400 nmol/mL) and quantities of non-liposomal drugs (DXR/anti-CD20) were adjusted to have same amounts with the corresponding liposomal formulations. After 24 hours of incubation, free anti-CD20 exposure created a cytotoxicity between 22.32% and 26.92% which shows its therapeutic act over Namalwa cells (Fig. 3.16). Rituximab (anti-C20) has therapeutic activity and synergistic effect with chemotherapy drugs against Lymphoma cells as reported by in vitro studies. Rituximab is a clinically approved drug and used for treatment of relapsed and refractory low-grade B-cell non-Hodgkin lymphomas [79]. Free DXR group was introduced at IC_{50} concentration which was also close to the DXR amount that is expected to be released in 48 hours from liposomes at 400 nmol/mL groups. The cellular toxicity of free DXR decreased with decreasing concentration. PEGylated DXR loaded liposome showed 54.69% cytotoxicity after 24 hours at 400 nmol/mL concentration which decreased to 36.96% at 100 nmol/mL concentration. However, PEGylated empty liposome caused relatively high toxicity

around 50%. Because of this, cytotoxicity created by PEGylated DXR loaded liposomes could not be related to DXR. The reason of the unspecific toxicity can be lipid toxicity. Anti-CD20 targeted and empty liposomes showed relatively high cytotoxicity that accomplished by additional cytotoxicity of Rituximab on PEGylated liposome. Anti-CD20 targeted DXR loaded liposome showed the highest cellular toxicity at all concentrations.

Cellular toxicity observed for free DXR decreased after 48 hours (Fig. 3.17). After 48 hours, cellular toxicity of most of the groups decreased compared to 24 hours incubation. The reason of this might be the fast proliferation rate of alive Namalwa cells and no addition of drug or liposome formulation. Free anti-CD20 showed increased cytotoxicity after 48 hours but cytotoxicity of anti-CD20 targeted empty liposomes decreased. Anti-CD20 is not an internalizing antibody which means liposomes attach to cell surface and stay on the surface. When a liposome attaches to cell surface, antibodies at only one side of the liposome will be able to interact with the cell which decreases the number of active antibodies. When free anti-CD20 was introduced, every antibody molecule can independently interact with cells that can explain the higher cytotoxicity created with same amount of free antibody. Anti-CD20 targeted DXR loaded liposome showed the highest cellular toxicity after 48 hours and caused over 80% cytotoxicity even at the lowest concentrations. Similar studies in literature supports our result. Sapra et al, showed that anti-CD19 and anti-CD20 targeted liposomes have higher cytotoxicity than untargeted liposomes on Namalwa cells [76]. They reported that after 1h incubation, IC_{50} values of PEGylated and anti-CD20 targeted liposomes were around 61 and 5 μM but they did not detect any cytotoxicity enhancement after 24h incubation for CD20 targeted liposomes. The enhanced cytotoxic effect of anti-CD20 targeted liposomes was explained with cell surface binding and release of DXR from the bound liposomes at the cell surface. However, when experiments performed in vitro all treatments can reach cells easily so a liposomal system that is targeted with a non-internalizing antibody could not cause more cytotoxicity than its' non-targeted form. Laginha et al, presented a similar study that was aimed to investigate possible effect of targeting liposomes

with two different antibodies at the same time which were anti-CD19 and anti-CD20. They reported increased cytotoxicity when liposomes were targeted. The IC_{50} values of PEGylated DXR loaded and anti-CD20 targeted liposomes were reported as 30.5 μM and 1.9 μM after 1 hours of incubation [81].

We have shown that free anti-CD20 (Rituximab) is able cause cytotoxicity by itself which should be the main reason of enhanced cytotoxicity. Moreover, we were able detect recognizable cytotoxicity increase when liposomes were targeted with anti-CD20 after 24h and 48h incubation (Figs. 3.16 and 3.17). The dual therapeutic act of DXR loaded and anti-CD20 (Rituximab) targeted liposome was determined and superior toxicity over untargeted liposomes was shown by our study.

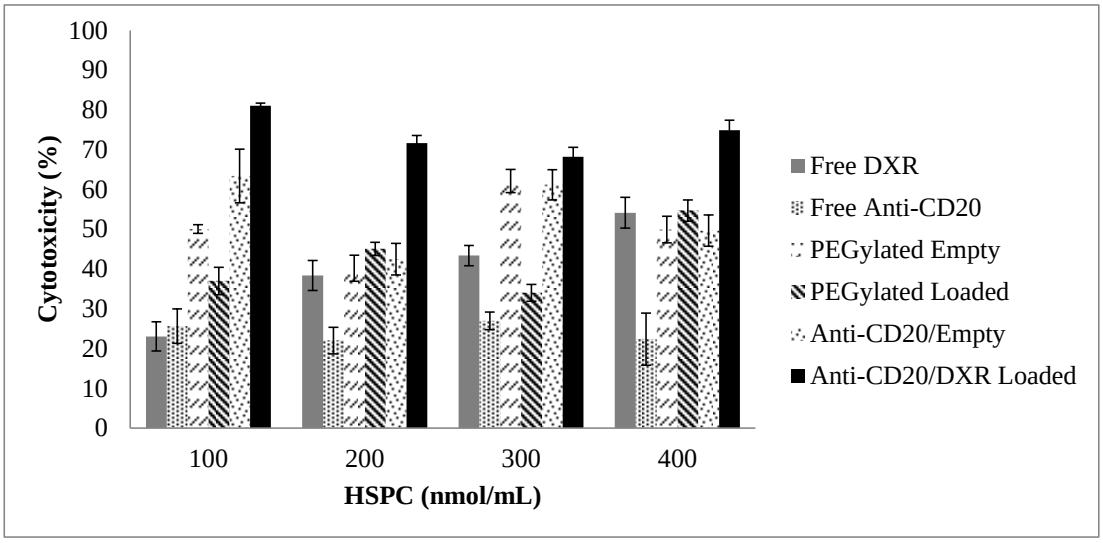


Figure 3.15. Viability of Namalwa cells after 24 hours incubation with non-liposomal and liposomal groups; free DXR, free Anti-CD20, DXR loaded or empty liposome, targeted DXR loaded or targeted empty liposome. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

All CD20 targeted DXR loaded liposome groups were statically significant different than other groups ($p<0.05$)

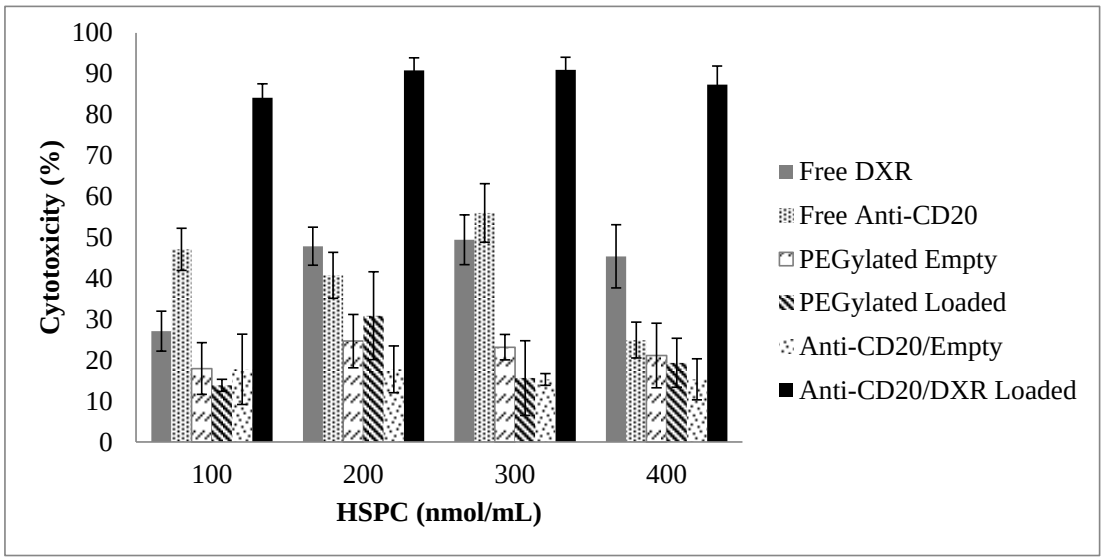


Figure 3.16. Viability of Namalwa cells after 48 hours incubation with non-liposomal and liposomal groups; free DXR, free Anti-CD20, DXR loaded or empty liposome, targeted DXR loaded or targeted empty liposome. Namalwa cells were seeded to have 300 cells/well concentration (n=6)

All CD20 targeted DXR loaded liposome groups were statically significant different than other groups ($p<0.05$)

3.4.5. Cellular Toxicity of Anti-CD30 Targeted DXR Loaded Liposome

Anti-CD30 targeted doxorubicin loaded liposomal formulations were compared for cellular toxicity with untargeted liposomal doxorubicin. In order to determine cytotoxicity created by individual parts of the system, experimental groups were prepared as; free doxorubicin, PEGylated DXR loaded or empty liposome, PEGylated and anti-CD30 targeted DXR loaded or empty liposome. Anti-CD30 targeted empty liposomes showed higher toxicity than untargeted empty liposomes which could be the reason of therapeutic act of anti-CD30 itself. The empty targeted and untargeted liposomes showed relatively low toxicities (9.54 ± 6.98% and 1.66 ± 13.13%). The cytotoxicity of DXR loaded groups increased after 48 hour which

shows the cell death with DXR release. Anti-CD30 targeted and untargeted doxorubicin loaded liposomes showed similar cytotoxicity after 24 and 48 hours. The target molecule was unable to show additional cytotoxicity. One reason could be the low number of anti-CD30 molecule per liposome which was not enough to show the enhancement effect. Therefore targeting, approach with anti-CD30 needs further optimization in this aspect.

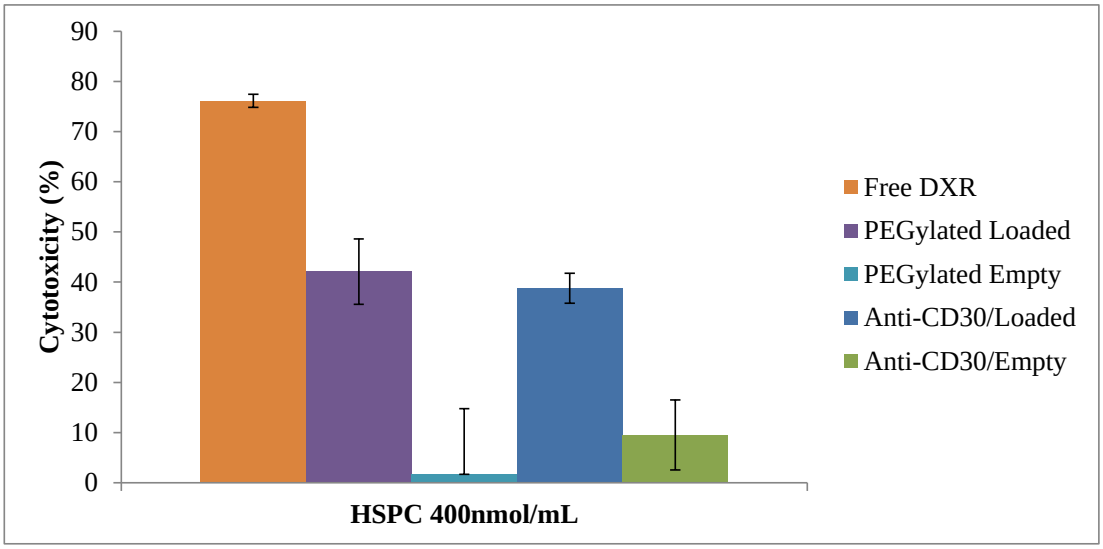


Figure 3.17. Viability of B lymphoma cells after 24 hours incubation with non-liposomal and liposomal groups; free DXR, DXR loaded or empty liposome, targeted DXR loaded or targeted empty liposome. B lymphoma cells were seeded to have 1500 cells/well concentration (n=6)

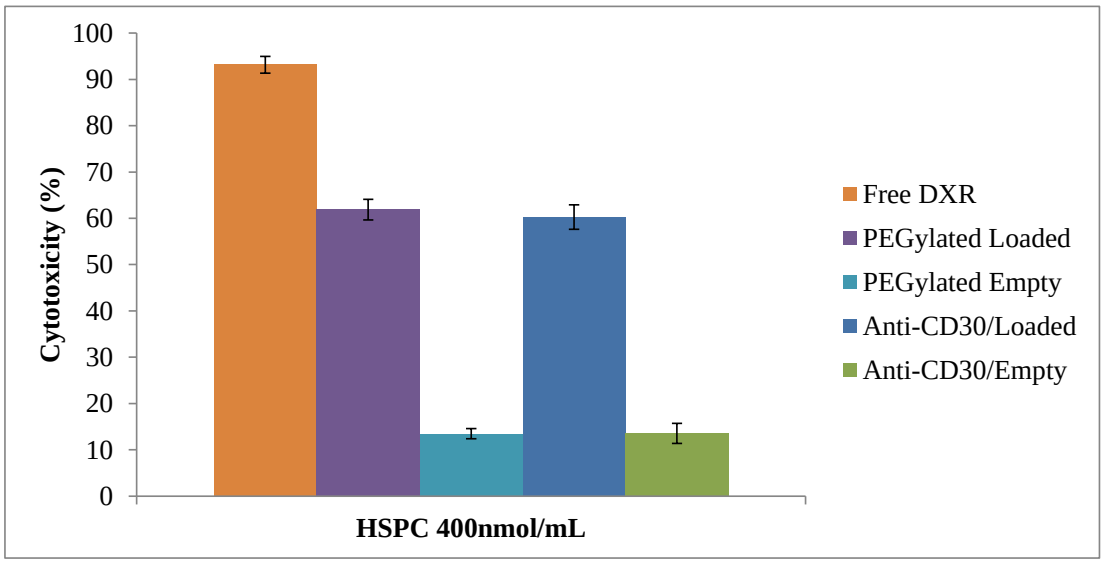


Figure 3.18. Viability of B lymphoma cells after 48 hours incubation with non-liposomal and liposomal groups; free DXR, DXR loaded or empty liposome, targeted DXR loaded or targeted empty liposome. B lymphoma cells were seeded to have 1500 cells/well concentration (n=6)

CHAPTER 4

CONCLUSION

In conclusion we have presented three modified doxorubicin encapsulated liposomal systems against Lymphoma. We have showed increased cytotoxicity when doxorubicin loaded liposomes targeted with anti-CD20 antibody to Namalwa cells. The MTT results of free anti-CD20 treatment revealed the therapeutic act of antibody itself over Namalwa cells. This study shows that non-internalizing anti-CD20 targeting improved cytotoxicity by two means; firstly by attaching liposomes to cells and providing DXR release very close to cell surface and secondly by dual therapeutic act created by DXR with anti-CD20 itself. Anti-CD30 MTT cytotoxicity results over B lymphocytes shows that the system needs to be further optimized. The number of antibodies attached to single liposome needs to be increased in order to obtain an improved cytotoxicity. Currently very low amounts of anti-CD30 stock, limits us to make any improvements.

We have also shown that free calcitriol pretreatment of Namalwa cells, enhances the cytotoxicity of DXR. By this aspect, a second novel system, liposome co-loaded with DXR and calcitriol was successfully presented in our study. Hydrophilic calcitriol molecule was successfully encapsulated to lipid bilayer without altering DXRs' high encapsulation. Calcitriol was encapsulated into liposomes with doxorubicin for the first time. The novel co-loaded liposomal DXR and calcitriol treatment caused higher cytotoxicity than their free treatment.

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APPENDIX A

CALIBRATION CURVES

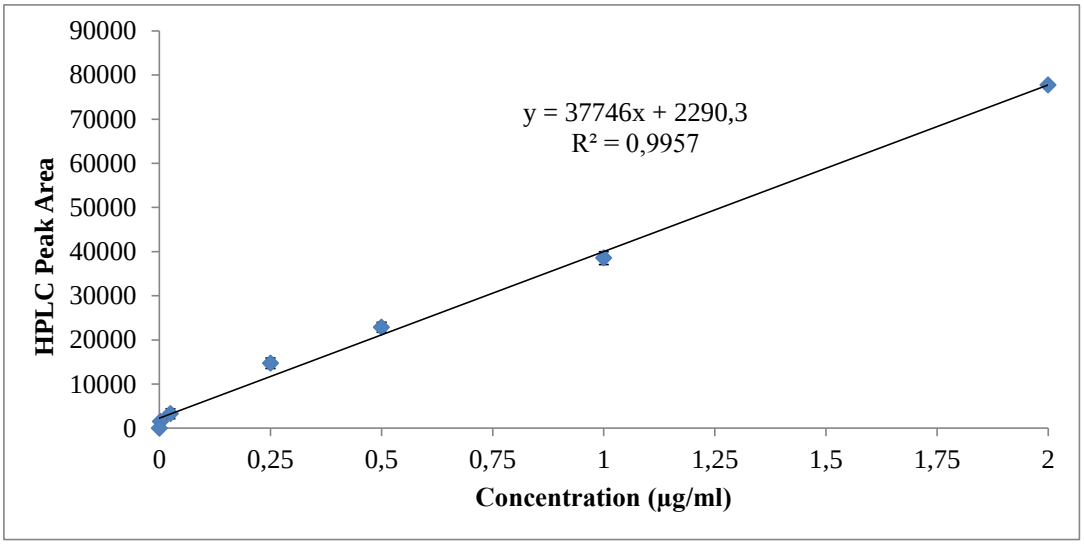

Figure A1 – Calcitriol calibration curve

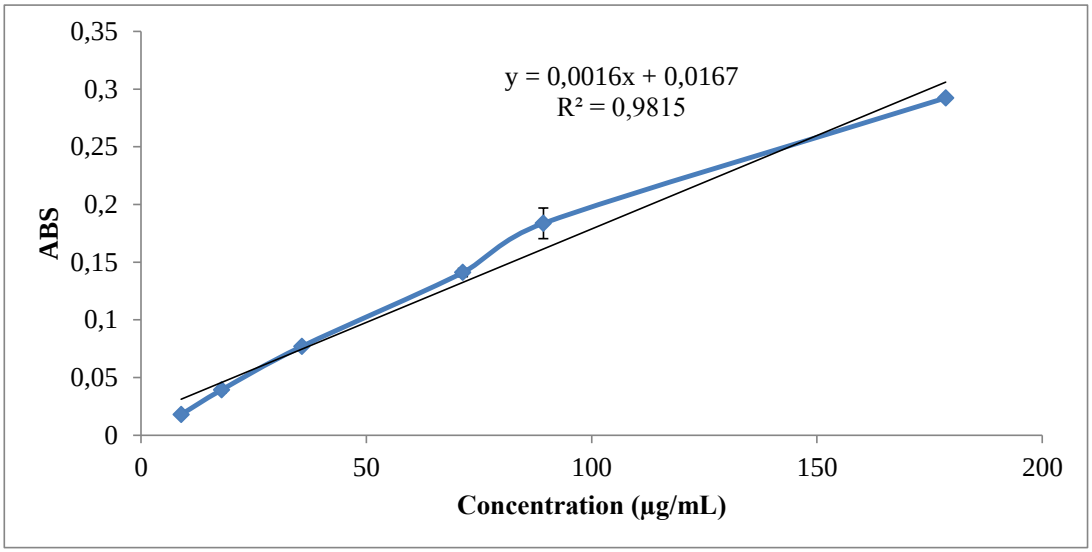

Figure A2 – Antibody-Oregon green calibration curve

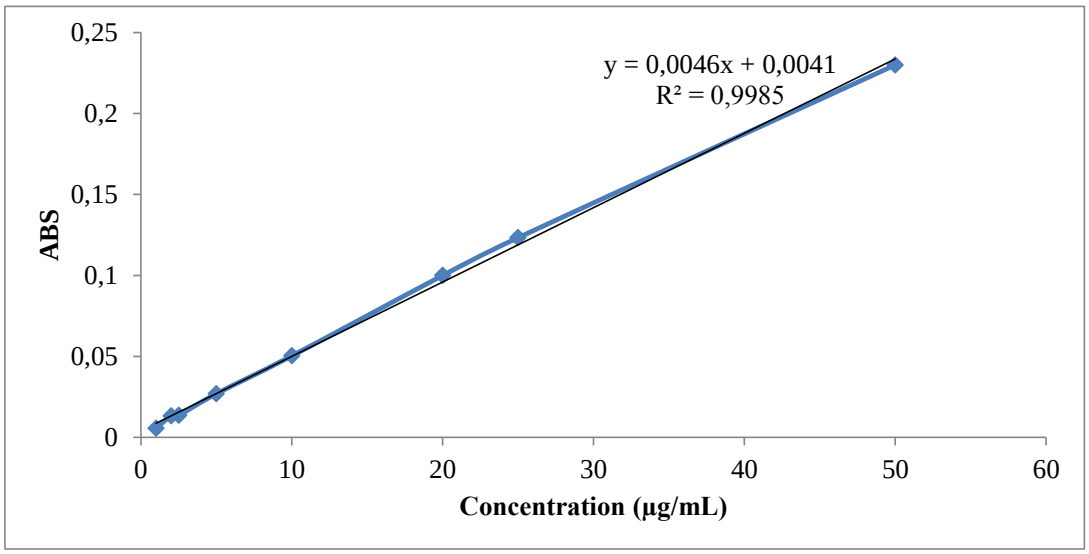

Figure A3 – HSPC calibration curve

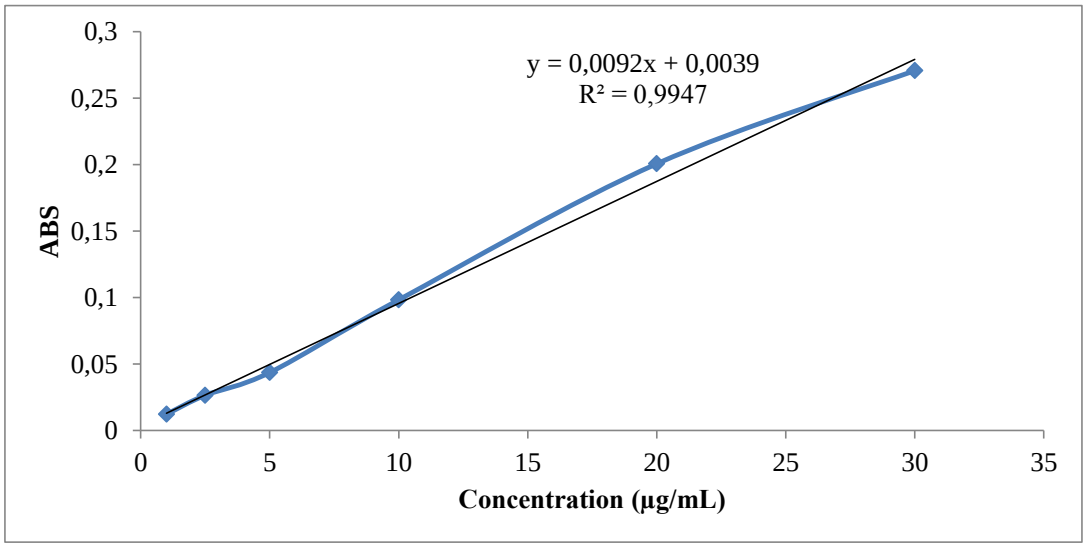

Figure A4 – Doxorubicin calibration curve (MetOH)

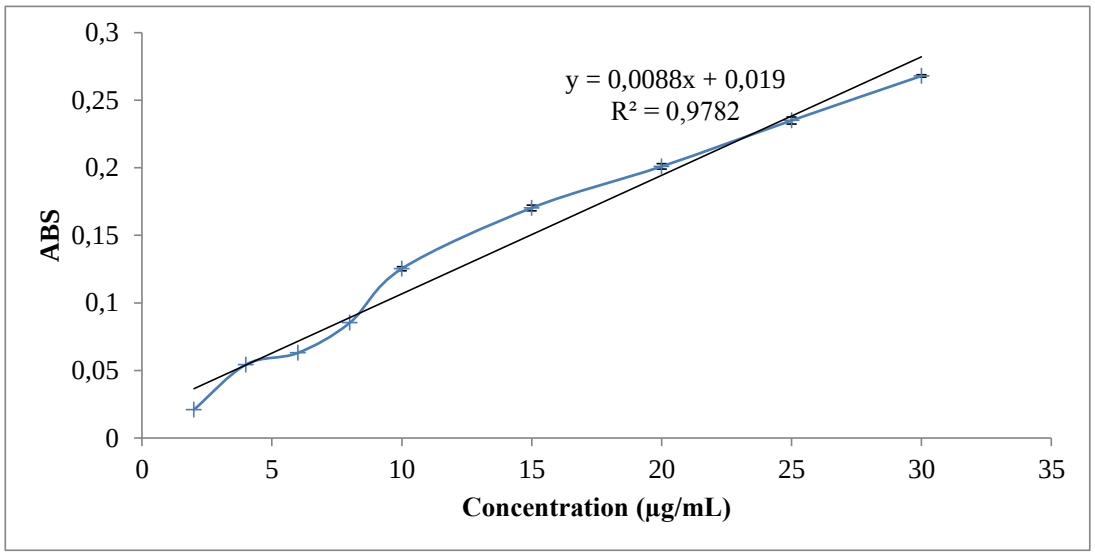

Figure A5 – Doxorubicin calibration curve (0.01M PB)