DESIGN AND PREPARATION OF ALKALI LIPOSOMES FOR DRUG DELIVERY

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ABSTRACT

DESIGN AND PREPARATION OF ALKALI LIPOSOMES FOR DRUG DELIVERY

Cancer is one of the deadliest diseases among other illnesses as an uncontrolled cell division. Liposomal technology has commonly been used in cancer therapy. Chemotherapeutical drugs, genetic materials, different imaging agents can be carried with liposomes. They are preferred by several important characteristics that selective passive targeting of tumors, increased stability and therapeutic index (reducing toxicity) via encapsulation and increased circulation life times with size adjustments. One of the indicator in cell cycle is intracellular pH. The aim of this study is to produce PEGylated alkali liposomes to provide cellular uptake in cancer cells and prevent cell division by changing of intracellular pH. Combination of liposomal technology and alkaline therapy in cancer cells may lead to the development of therapeutic strategies without using any drug to overcome chemoresistance and cell proliferation. For this purpose, alkali liposomes containing sodium carbonate (Na_2CO_3) solution were prepared and tested their effects on *4T1* breast cancer cell lines in vitro. The cell viabilities were evaluated using trypan blue and *WST-1* methods. Pictures were taken for cancer cells to differentiate live and dead cells under different alkali liposome conditions for 5 days. It was found that cell medium containing alkali liposomes up to 3% didn't affect cell growth. However, cell medium containing alkali liposomes greater than 7% significantly affected the *4T1* breast cancer cell growth and decreased the cell viability to about 40%. It was concluded that PEGylated alkali liposomes were prepared different concentrations to decrease or stop cell division of *4T1* breast cancer cell lines *in vitro*.

ÖZET

İLAÇ SİSTEMLERİ İÇİN ALKALI LIPOZOMLARIN TASARIMI VE HAZIRLANMASI

Kanser diğer hastalılar içinde en amansız hastalılardan biri olan kontrolsüz hücre bölünmesidir. Lipozomal teknoloji kanser tedavisinde yaygın olarak kullanılmaktadır. Lipozomlar ile kemoterapik ilaçlar, genetik materyaller, farklı görüntüleme ajanları taşınabilir. Onlar birkaç önemli özelliklerinden ki tümörleri seçimli pasif hedefleme, kapsülleme ile kararlılığının ve tedavi katsayısının (toksisitenin azaltılması) artırılması, boyut düzenlemeleri ile dolanım süresinin artırılmasından dolayı tercih edilirler. pH hücre döngüsünün göstergelerinde biridir. Bu çalışmanın amacı, uygun boyutta PEGli alkali lipozomlar üretip kanserli hücre içerisine alınmasını sağlamak ve hücre içi pH artırılması ile hücre bölünmesini engellemektir. Kanser hücreleri için lipozomal teknoloji ve alkali terapi birleştirilmesi ile herhangi bir ilaç kullanmadan ilaca karşı direnç ve hücre proliferasyonunun üstesinden gelinerek terapik stratejilerinin gelişmesine öncülük edebilir. Bu amaçla, sodium karbonat (Na_2CO_3) solüsyonu içeren alkali lipozomlar üretildi ve 4T1 meme kanseri hücre hatlarında in vitro olarak etkinlikleri test edildi. Hücre canlılıkları tripan mavisi ve *WST-1* metotları kullanılarak değerlendirildi. Beş gün boyunca farklı alkali lipozom koşullarında canlı ve ölü kanser hücrelerinin ayrımını yapabilmek için resimler alındı. 3% ‘e kadar alkali lipozom içeren ortamda hücre gelişmesini etkilemediği bulundu. Fakat 7%’den fazla alkali lipozom içeren ortamda önemli ölçüde 4T1 meme kanser hücre gelişimi etkilendi ve canlılık oranı 40% civarına düştü. Sonuç olarak, 4T1 kanser meme hücre hatlarının in vitro olarak bölünmesini azaltması ya da durdurması için farklı konsantrasyonlarda PEGli alkali lipozomlar hazırlandı.

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LIST OF ABBREVIATIONS

Abbreviation	Description
Abs.	Absorbance
ATP	Adenine Triphosphate
CMC	Critical micelle concentration
CMT	Critical micelle temperature
DLS	Dynamic Light Scattering
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG_{2000}	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
EGFR	Epidermal growth factor receptor
FDA	Food and drug administration
Fluo.	Fluorescence intensity
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
NPLD	non-PEGylated liposomal doxorubicin
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pH	Power of hydrogen
pH_i	Intracellular pH
pH_e	Extracellular pH
pK_a	Negative log of the acid dissociation constant (-log[K_a])
RNA	Ribonucleic acid
SUV	Small unilamellar vesicle
ULV	Unilamellar vesicle
US	Ultrasound
UV	Ultraviolet
V_m	Transmembrane potential

CHAPTER 1

INTRODUCTION

Cancer is a worldwide health problem to effect many people in increasing rates. The most common cancer types are lung, colon, breast, prostate, stomach, and liver in the world. There are 8.1 million new cases, 9.6 million cancer deaths, and 43.8 million people living with cancer in 2018(Global Health Organization, 2018). Thus, there are many drug delivery systems making in progress to decrease cancer deaths.

Liposomal drug delivery systems are of the most important biological carriers for living system [1] and they are mostly used for cancer therapeutic applications. Nano-sized liposomes are also easily accumulate at tumor area because of enhanced permeability and retention (EPR) effect, and reduce side effects of the encapsulated drugs [2]. Targeting, stability and therapeutic index of PEGylated liposomes are the main parameter for getting beneficial result to cure the cancer disease [3]. Therapeutic utility of liposomes can be increased with PEGylation by escaping reticuloendothelial (RE) system. PEGylation of liposomes improves the stability and circulation time. The 'passive' targeting ability on tumoral tissues (EPR effect) able to improve the therapeutic effects and reduce the toxicity of encapsulated drug by PEGylation.

Liposomes, due to their various forms can deliver both hydrophilic and hydrophobic drugs for cancer. Several chemotherapeutic drugs are encapsulated into the liposomes by their chemical characteristics and mechanism of actions. For example, Doxorubicin binds DNA inside the cancer cells and inhibits cellular division and grow. Paclitaxel behave mitotic inhibitors that stop cancer cells from making more copies of themselves. Gemcitabine is known as antimetabolites that interferes with the normal metabolism of cells, which makes them stop growing. Depending on the characteristics of the cancer, a targeted therapy medicine may be used in combination. One important outcome of any drug is to its increase the therapeutic index with minimum side effects. Nerve and muscle problems such as tingling, pain; skin and nail changes such as dry skin and color change; hair loss, fatigue, diarrhea and easy bleeding are the most common side effects of chemotherapeutical drugs. Encapsulation is one of the choice

for decreasing these side effects, but the other important problem is how to target the tumoral area. Because most of the drugs are accumulated at liver and kidneys, only small percentages are arrived to the tumoral area. As a result, more beneficial therapy should be found without using any drugs. Manipulation of intracellular and/or extracellular pH of tumors can be considerable potential for cancer therapies. Any small change of intracellular area can cause changing several signaling pathways and cellular metabolism. Concentration of H^+ ions can also be suggested for a role of pH in signalling.

There are several objectives in this study. Firstly, designing proper size and lipid/cholesterol ratio of the liposomes are important for enough circulation time and stability of biological carriers. The other objective is that decreasing side effects of cancer therapy by generation alkali liposomes without using any drugs. This objective is really important, because long term cancer therapy with chemotherapeutical drugs and radiation were too painful process for patients. In this study, it was done the characterization of PEGylated alkali liposome to decrease or stop cell division of cancer cells for *in vitro* applications.

This thesis was organized as five chapters. The chapter is the introduction that gives brief information about thesis content and objectives. In chapter two a literature survey was given for the connection between academic area and thesis background. Cancer and its treatment strategies were explained. Then, liposome and its application in cancer therapy were explained properly. Understanding of lipid mechanism and targeting parameters are also significantly important to generate proper size, surface charge and structure of nanoparticles for directly effects of cellular uptake mechanism [4]. Thus, uptake mechanism of liposomes and their effects of cell cycle mechanism by changing pH_i were reviewed in this part. In chapter three, materials and methods were explained about PEGylated alkali liposome contents and how they can are prepared step by step. Cell culture studies were also explained for *in vitro* studies. Their results were evaluated in chapter four in results and discussion part. Finally, experiment results were concluded with some suggestions in chapter five.

CHAPTER 2

LITERATURE SURVEY

2.1. Cancer

Cancer is a common health problem which can effects people in all gender and ages. The most common cancer types are lung, colon, breast, prostate, stomach, and liver in the world. There are 8.1 million new cases, 9.6 million cancer deaths, and 43.8 million people living with cancer in 2018 (Global Health Organization, 2018). Breast cancer is the most common cancer type for women and increasing cancer-related death in women in Europe and North America.

Cancer is defined as uncontrolled cell proliferation, loss of apoptosis (programmed cell death) ability and easily spread the other organ by angiogenesis (new blood vessels form from pre-existing vessels) (The Cell-2nd edition). There are six main hallmarks of cancer for development in humans as shown in Figure 2.1 [5]. Sustaining proliferative signaling is the most main feature of cancer cells. Normal tissues carefully control the production and release of growth-promoting signals. These signals are important for arranging cell number with conservation of normal tissue functions. Unfortunately, cancer cells takeover by deregulating these signals.

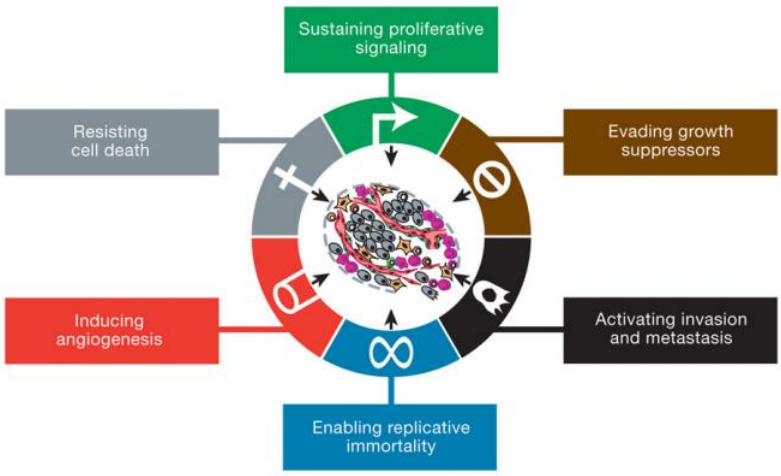


Figure 2.1.The hallmarks of Cancer [5]

Evading growth suppressors is another hallmark that cancer cells effect on RB (retinoblastoma-associated) and TP53 proteins by encoded tumor suppressors. The RB protein takes important role in cell division and growth, and TP53 protein stop cell cycle progression getting signal from stress or abnormal condition (for example genetic damage) until these conditions have been normalized. TP53 protein can also trigger apoptosis [5, 6]. Actually, apoptosis provides as natural barrier for cancer development, but apoptosis mechanism can be eliminated by some regulatory proteins. Bax, Bak, Bid and Bad are the members of Bcl-2 family that promote the cell death. Pro-apoptotic signaling proteins, cytochrome c was released by Bax and Bak proteins with disruption of the outer mitochondrial membrane. Enabling replicative immortality is significantly important feature because of unlimited replicative potential for cancer cells. The reason of this unlimited proliferation is telomerase activity that protects the ends of chromosomes [7, 8]. Telomerase is an enzyme that provides adding new repetitive nucleotides on chromosomes during cell division to ensure proper telomere length, because DNA loses some nucleotides during each cell division. Thus, encoding DNA was preserved during chromosome replication .Additionally, angiogenesis is a leading process that new blood vessel formation from the preexisting vasculature to supply of nutrients, oxygen etc. and clear out their metabolic wastes and CO_2 for normal or tumor cells. The angiogenic switch is activated under certain conditions low pO2, mechanical stress, injury and so on. Regulation of angiogenesis process is also controlled by some activators and inhibitors as shown in Table 2.1 [9] and balance its hypothesis as shown in Figure 2.2 [9]. Hypoxia is one of the major drivers of angiogenesis and hypoxia-induced genes play important roles in it. If angiogenesis was occurred in abnormal tissue or in abnormal position is called neovascularization [9].

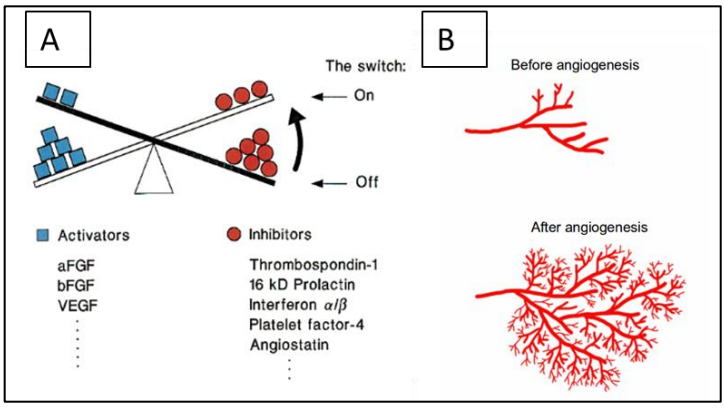


Figure 2.2.(A) Balance of Angiogenesis Switch and (B) Angiogenesis representation [9]

After all angiogenesis information, inducing angiogenesis is key hallmark for tumor cells to increase cell in number and spread out another tissues easily. Surprisingly, this process was occurred early during the multistage development of invasive cancers both in animal models and in humans [5].

Table 2.1.Modulators of Angiogenesis [9]

Activator	Inhibitor
Angiogenin	Angioarrestin
Angiopoietins	Angiostatin
Del-1	Anti-angiogenic antithrombin III
Endocrine Gland-VEGF	Arrestin
Fibroblast growth factors	Canstatin
Follistatin	Endostatin
Hepatocyte growth factors	Gro-beta
Leptin	Interferon alpha
Midkine	Interleukin12
Platelet-derived growth factor	Pigment epithelium-derived factor
Platelet-derived endothelial growth factor	Platelet Factor 4 Stanniocalcin-1 Thrombospondin
Pleiotropin	Tissue inhibitor of metalloproteinases
	Tumstatin
Proliferin	tRNA synthase
	Vasculostatin
	Vasohibin
Transforming growth factor	Vascular endothelial growth inhibitor
Vascular endothelial growth factors.	Vascular Endothelial-statin

The last important hallmark is activating invasion and metastasis that have mysterious background for understanding mechanism of tumor biology. Tumor metastasis is a multistage process that malignant cells spread from the primary tumor to different organs as shown in Figure 2.3 [10]. Cancer stem cells have also self-renewal capacity and multi-potency characteristics that cause tumor recurrence and initiating new tumor growth for important in metastatic process and invasion [10]. During these processes, cancer cells can be changed their shapes as well as their attachment properties for cell-to-cell and cell-to-ECM (extracellular matrix) adhesion to provide

invasion of different area. Epithelial-mesenchymal transition (EMT) is migratory cellular program that has become importance for cell invasion, resisting apoptosis and metastasis [5].

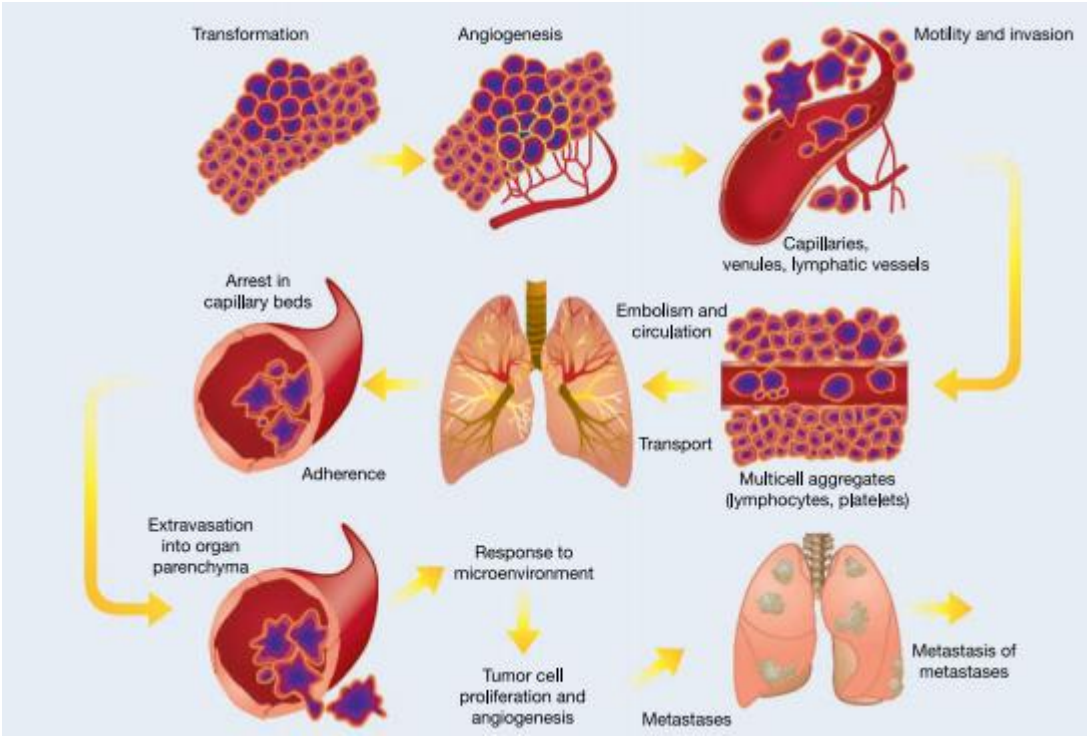


Figure 2.3.The sequential process of metastasis [10]

In the last decade, there were also added two hallmarks that are re-programming of energy metabolism and evading immune destruction. Cancer tissues are also composed of multiple cell types and have heterotypic interactions with one another [5]. In addition, every cell and cancer cells need energy for cell growing, cell proliferation, increasing cells in number and so on. Thus, this required energy is generated from cellular respiration. It is a set of processes that take place in the cells of organisms to convert biochemical energy from nutrients into adenosine triphosphate, and then release their waste products. There are two important cellular respirations in eukaryotic cells: Aerobic and Anaerobic. Glycolysis is an important metabolic pathway for both of them. High levels of ATP and CO_2 were produced under aerobic conditions by entering pyruvate to tricarboxylic acid (TCA) cycle producing citrate. But, less amount of ATP is produced under anaerobic conditions by converting pyruvate into lactate. If there wasn't enough oxygen in the environment, normal cells prefer to produce energy anaerobically through glycolytic fermentation. In contrast, proliferating cancer cells prefer glucose consumption and lactate production even if under aerobic conditions is

called Warburg effect, because anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD^+). This situation causes acidic tumor microenvironment as shown in Figure 2.4 [11-13]. Hypoxia condition is controlled by hypoxia inducible factor 1 (HIF1α). The results of HIF1α activation is up-regulation of glycolysis and hence the production of lactic acid. Over 40 genes are known to be regulated at the level of transcription by HIF1α activation [14]. Thus, Acidic pH_e effects several important signals (SREBP2 (sterol regulatory element-binding protein 2 and ACSS2 (acyl-CoA synthetases short-chain family member 2)) which take role in transcriptional upregulation of cholesterol synthetic enzymes and growing cancer cells [15].

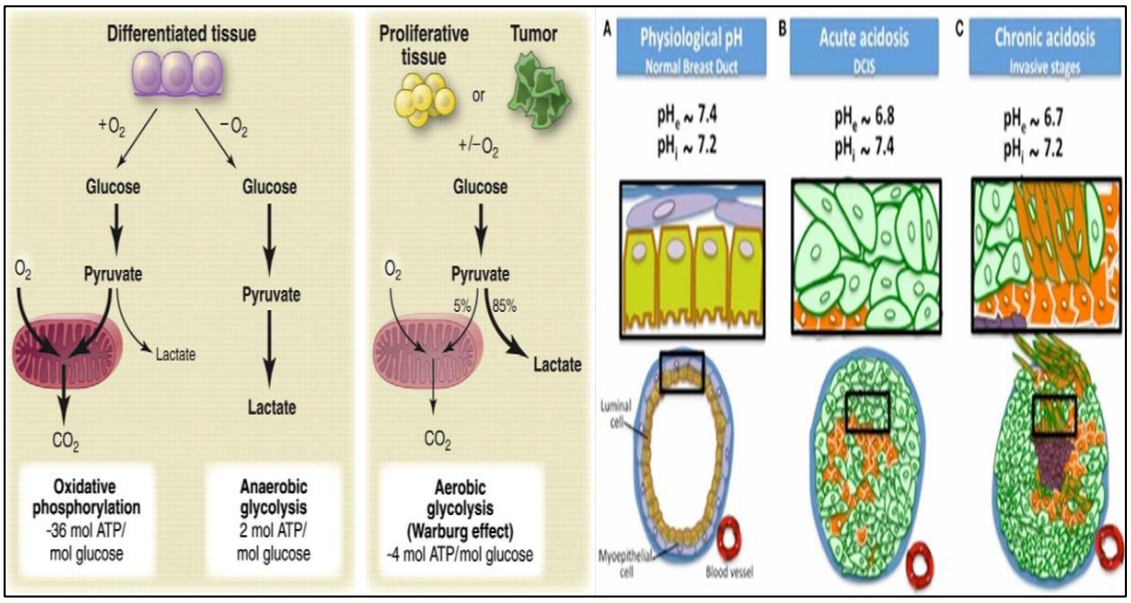


Figure 2.4.Differentiated tissue and tumor cell preferences for cellular respiration[12] and pH differences between normal and tumor tissues of breast[11]

Enzyme carbonic anhydrase 9 (CA9) is also membrane-bound isozymes characterized by the highest H^+ transfer rate and the protein can be upregulated under hypoxic conditions [14, 16]. Expression of the enzyme CA9 on the tumor cell surface catalyses the extracellular trapping of acid by hydrating cell-generated CO_2 into HCO_3^- and H^+. pH_e is held constant through the fine tuning of plasma CO_2 partial pressure (PCO_2) and bicarbonate concentration HCO_3^- by the lungs and kidneys for healthy cells as shown in Figure 2.5 [14].

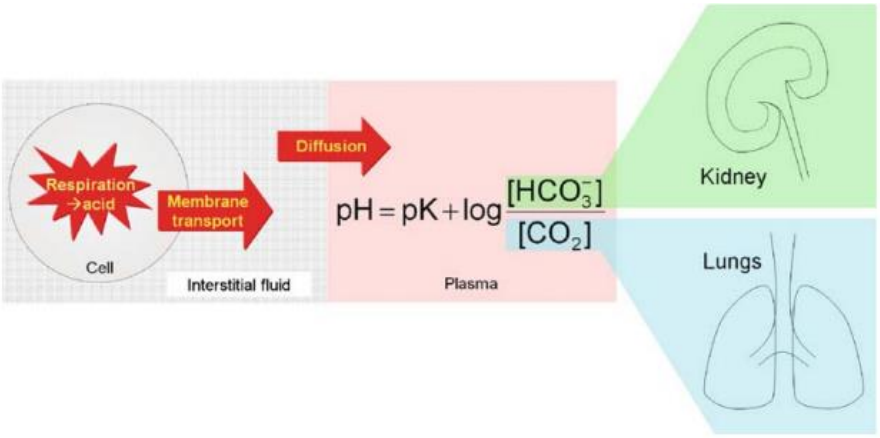


Figure 2.5.Representation of pHi changes that effect on pHe [14]

Overall of cancer metabolism, cancer cells utilize both conventional oxidative metabolism and glycolytic anaerobic metabolism, but their proliferation profiles were demonstrated glycolytic metabolism preference in the presence of O_2. This situation causes acidic microenvironment of the tumor cell. Thus, Hypoxia (chronic acidosis) is one of the major drivers of angiogenesis and invasive stages getting alkaline pH_i leads to epigenetic and genetic alterations with changing new cell phenotypes [13]. As a result, acidic extracellular milieu favors for tumor growth, invasion and development [14].

2.2. Cell Cycle and pH_i Changes

Cell cycles are strictly controlled by several internal and external signaling pathways. G1 and G2 checkpoints are also important security area for proper cell division. If there is any small problem during cell cycle phases, these checkpoints undergo cells for apoptosis as programmed cell death [17]. The mitogenic signaling in cancer cells is better understood than normal cells. Cancer cells may produce growth factor ligands and send signals to normal cells for helping tumor-associated stroma with different growth factors [5]. If cancer cell signaling pathways and cycle phases can be understood properly, cell mitosis will be stopped easily.

Early ionic changes during cell proliferation or differentiation were first reported in 1979. There are three distinct ionic mechanisms were involved in pH_i regulation:

(i) a sodium-independent Cl^-/HCO_3^- exchanger

(ii) a Na^+/H^+ exchanger

(iii) a voltage-dependent Na^+/HCO_3^-cotransporter

Changes of pH_i were proposed for possible signal for cell proliferation or differentiation. Electrogenic Na^+/HCO_3^- cotransporters and an electroneutral Na^+/H^+ exchangers are involved in pH_i regulation at all stages of maturation (progenitor cells, pro-oligodendrocytes and mature oligodendrocytes). Comparison of flux values can give information about cellular membranes and membrane areas. These fluxes increased during cell differentiation and pH_i regulating mechanism increases during oligodendrocyte maturation [18]. Only increase in 0.2 unit of pH was caused somatic cell development, but more internal alkalization should be studied to analyze pH_i mechanism for cell survival and differentiation.

Table 2.2.pH_i recovery rates and corresponding H^+ efflux [18]

	OLP			Mature oligodendrocytes[a]		
	Recovery rate (pH unit · min^{-1})	H^+ efflux (mMol · L^{-1} · min^{-1})	H^+ efflux per membrane surface area[b]	Recovery rate (pH unit · min^{-1})	H^+ efflux (mMol · L^{-1} · min^{-1})	H^+ efflux per membran surface area[b]
Bicarbonate/CO_2	0.16 ± 0.01 (15)	5.38 ± 0.54 (15)	5.38	0.21 ± 0.01 (22)	11.87 ± 0.84 (22)	35.61
Bicarbonate/CO_2 + Amiloride	0.08 ± 0.01 (15)	2.69 ± 0.37 (15)	2.69	0.12 ± 0.01 (10)	6.60 ± 0.71 (10)	19.80
HEPES	0.15 ± 0.08 (13)	2.44 ± 0.35 (13)	2.44	0.13 ± 0.01 (23)	3.88 ± 0.23 (23)	11.64

*OLP:immature oligodendrocyte progenitor
*Efflux is a mechanism responsible for moving compounds out of cells

The other study said that intracellular alkalization reduced the epidermal-growth-factor (EGF) induced proliferative signal, tyrosine phosphorylation and immediate-early-gene expression. When pH_e changes from 6.7 to 7.3, the pH_i was effected from this stimulation and changed from 6.87 to 7.18 as shown in Table 2.3 [19]. Two major proteins of 91 kDa and 85 kDa of tyrosine phosphorylation weren't also expressed in intracellular alkalization. C-fos gene is an immediate-early gene that its expression was reduced approximately 50% by intracellular alkalization at the time of EGF mitogenic stimulation as shown in Figure 2.6 [19]. 67% reduction of proliferation in internal alkalization was also observed during EGF stimulation.

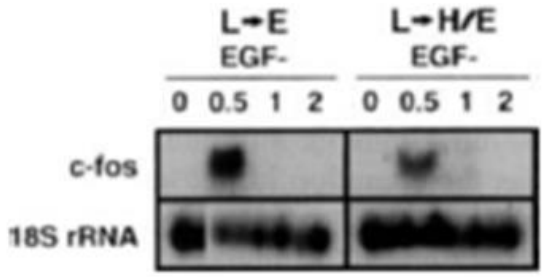


Figure 2.6.c-fos expression changes with internal alkalization (18S rRNA gene probe was used for gel loading checking, L: pH 6.7; H: pH 7.3) [19]

Na^+/H^+ antiporter system takes role in pH_i regulation in a variety of cells, such as fibroblasts, skeletal systems etc. that was activated by extracellular alkalization. Re-initiation of DNA synthesis is also highly depended on pH_i [20].

Table 2.3.pH_e changes effect on pHi [19]

Stimulation	Intracellular pH	
	before stimulation	after stimulation
Extracellular pH 6.7→7.3	6.87 ± 0.06	7.18 ± 0.07
Extracellular pH 7.3→6.7	7.07 ± 0.07	6.87 ± 0.06
Fetal calf serum (pH 7.3)	7.11 ± 0.09	6.99 ± 0.07
Fetal calf serum (pH 6.7)	6.87 ± 0.07	6.77 ± 0.07

Mesenchymal Transition (EMT), a migratory cellular program associated with development and tumor metastasis, is fueled by upregulation of ribosome biogenesis during G1/S arrest [21]. The Warburg effect favors also an intracellular alkaline pH which is a driving force in many aspects of cancer cell proliferation (enhancement of glycolysis and cell cycle progression) and of cancer aggressiveness (resistance to various processes including hypoxia, apoptosis, cytotoxic drugs and immune response) [13]. In addition, cell metabolism and cycles should be analyzed to understand of pH_i effects on cell survival. Cell metabolism is composed of several chemical reactions and dynamic exchanges between a cell and its microenvironment. NADH and NADPH are important for cellular metabolism that carrier energy. DNA decompaction, RNA and protein synthesis (in late G_1 phase) followed by DNA replication (in S phase) and lipid synthesis (in G_2 phase) occur after resting cells (in G_0) are provided to cell proliferation.

There are several important events in both transmembrane potential and intracellular pH oscillation during cell cycle. One of them is cellular redox transitions and nutrients are used to produce energy for cellular metabolic events. Glucose, protein and fatty acids as main energy supplier to support primary reactions such as amino acids and nucleotide synthesis and gene transcription. pH_i was changing between 6.8 and 7.2 during cell cycle phases and mitosis as shown in Figure 2.7(C) [17]. For example in G_2, both free ATP concentration and pH_i reach their maximal value, high and alkaline and in early mitosis, ATP was hydrolyzed, pH_i was high and alkaline. pHi was also decreased and reached 6.8 value at the end of mitosis and it conserved its value during stationary phase (G_0) [17]. This changing pH_i values are important parameter to effect cell cycle phases and mitosis by designing drug delivery system.

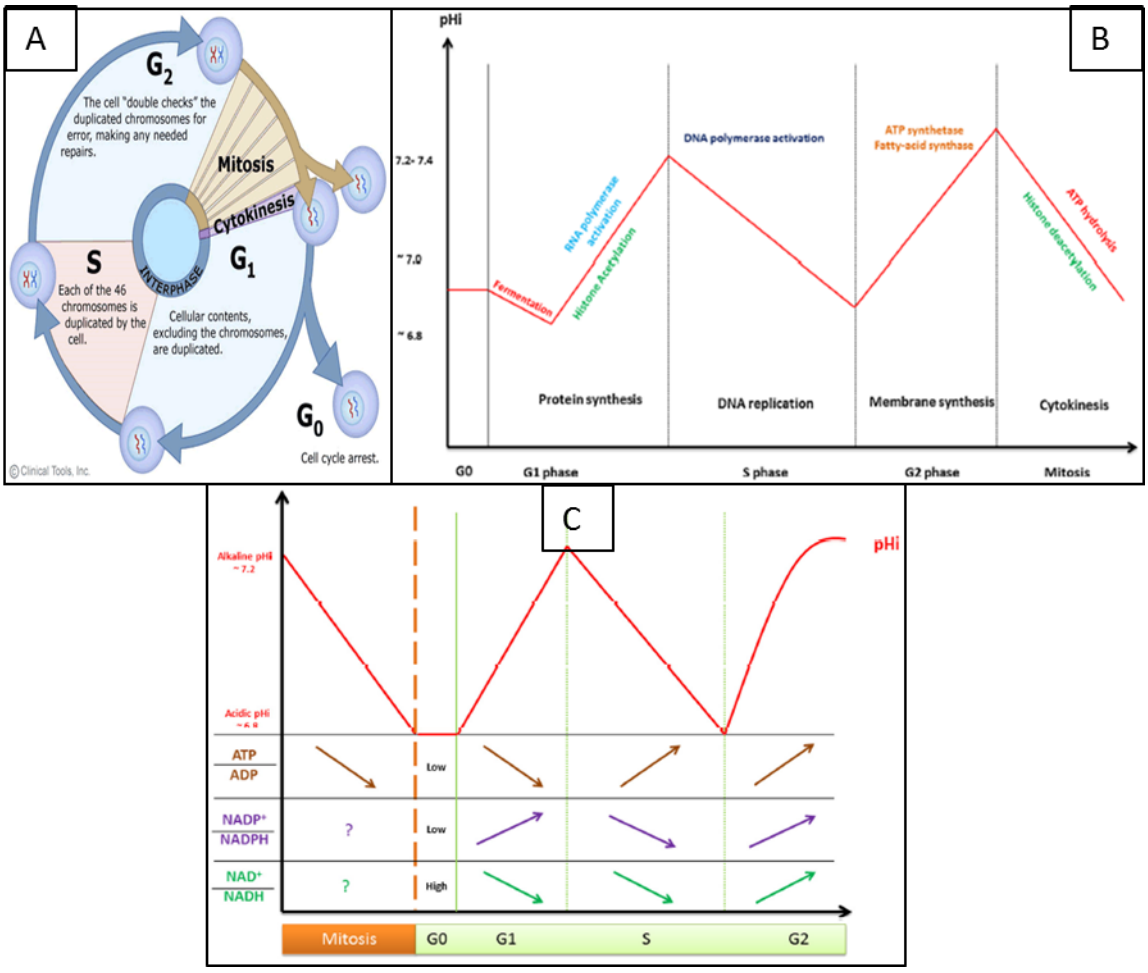


Figure 2.7.(A) Cell cycle phases representation [41] (B) pH_i changes through cell cycle phases and mitosis[17] (C) pH_i, ATP, NADPH and NADH changes during mitosis and cell cycle phases [17]

2.3. High pH Therapy

Conditions of the tumor microenvironment, such as hypoxia and nutrient starvation, play critical roles in cancer progression [15]. Manipulation of intracellular and/or extracellular pH of tumors can be considerable potential for cancer therapies. Because, healthy cells have more alkaline pH_i than cancer cells, and this situation causes tumor acidity tends to correlate with cancer aggressiveness and activates some important signals which promote invasiveness and angiogenesis. Hypoxic conditions are also known to be associated with resistance to chemotherapy and radiotherapy, and with poor cancer prognosis [16]. Thus, there are four different therapies for changing pH_i and pH_e. Alkalizer therapy that increases the extracellular milieu; proton pump inhibition that decreases the pH_i while increases the pH_e; intracellular acidification therapy that decreases both pH_i and pH_e; extracellular acidification therapy that decreases the pH_e and useful for pH-sensitive nanoparticles [22].

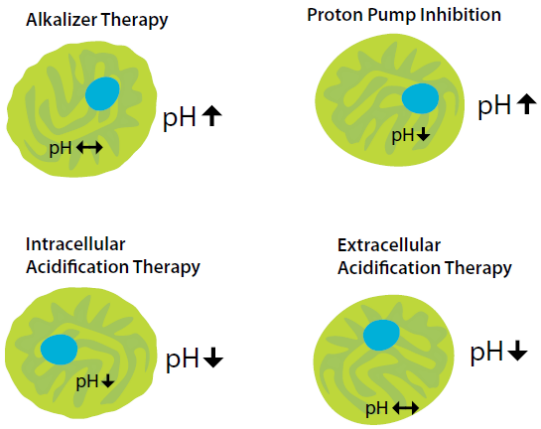


Figure 2.8.Different types of therapeutic strategies [22]

Alkalizer therapy is good enough for changing pH_e and combination of this therapy with liposomal technology which can provide pH_i changes. This is an important parameter for preventing cell mitosis of cancer cells by increasing pH_i with alkali liposomes. Before this information, there are some alkaline therapies and their effects which were investigated in several studies. These therapies were also used for management of metabolic acidosis for multiple organ failure. There are some buffer examples and mechanism of actions of it was shown in Table 2.4 [23].

Table 2.4.Several buffers with pH values and mechanism of actions [23]

	1000 ml of the respective buffer contains:	pH of buffer	Mechanism of action of buffer
Sodium bicarbonate	50 g $NaHCO_3$	8.0	$Na^+ + HCO_3^- + H^+ \Leftrightarrow H_2CO_3 + Na^+ \Leftrightarrow H_2O + CO_2 + Na^+$
Ringer's acetate	5.8 g Sodium chloride, 298 mg potassium chloride, 294 mg calcium chloride, 203 mg magnesium chloride, 4.1 g sodium acetate	6.0	Acetate is metabolised to bicarbonate
Carbicarb	35 g Na_2CO_3, 27.7 g $NaHCO_3$	9.6	The carbonate ion (CO_3^{2-}) abstracts protons mostly from proteins and to a lesser extent from dissolved carbon dioxide
Addex®-THAM	400 g Trometamol	9.2	Hydrogen ion acceptor titrating H^+ derived from carbonic acid (H_2CO_3), thereby reducing P_{aCO_2} and increasing blood pH
Tribonat®	36 g Trometamol (Tris), 13 g sodium bicarbonate, 2.8 g disodium phosphate 12 g acetate buffering	8.1	A combination of the effects derived from trometamol (reduced P_{aCO_2}) and sodium bicarbonate (increased P_{aCO_2}) results in constant P_{aCO_2} in the spontaneously breathing patient. The buffering capacity of phosphate is small, but the addition of phosphate counteracts the hypophosphatemia often seen in conjunction with buffering of metabolic acidosis.

Especially, NaCl, $NaHCO_3$ and Carbicarb (equimolar solution of sodium bicarbonate and sodium-carbonate) treatments were investigated for in-vitro and in-vivo conditions because of their buffering effect. Composition of NaCl, $NaHCO_3$ and Carbicarb were also evaluated in Table 2.5 [24]. The lowest PCO_2 value was in Carbicarb solution and it had the greatest alkalizing potential. It means that it is more stable since it loses CO_2 more slowly when exposed to room air. At the same time, increases in dissolved carbon dioxide leads to solution increase bicarbonate anion and hydrogen cation. This situation affects the greater buffering capacity of Carbicarb [25, 26]. The other study concluded that Carbicarb and Bicarbonate also have similar buffering capacity except CO_2 generation. Carbicarb produces less amount of CO_2 [24].

Table 2.5.Compositions of NaCl, $NaHCO_3$ and Carbicarb [24]

	NaCl	Carbicarb	$NaHCO_3$
Na^+ mmol/L	1,000	1,000	1,000
Cl^- mmol/L	1,000	0	0
HCO_3^- mmol/L	0	333	1,000
CO_3^{--} mmol/L	0	333	0
pCO_2 (mm Hg) 37°C	0	3	>200
Osmolality/kg	2,000	1,667	2,000

There was another study which was investigated these treatments in Mongrel dogs. Lactate is a normal end product of glycolysis and blood lactate levels are also frequently used to monitor tissue hypoxia. If we compare these treatments, there was significant increase in arterial pH without increase in lactate concentration with

Carbicarb therapy as shown in Figure 2.9 [27]. Thus, Carbicarb can be preferred for in vivo studies without causing metabolic acidosis.

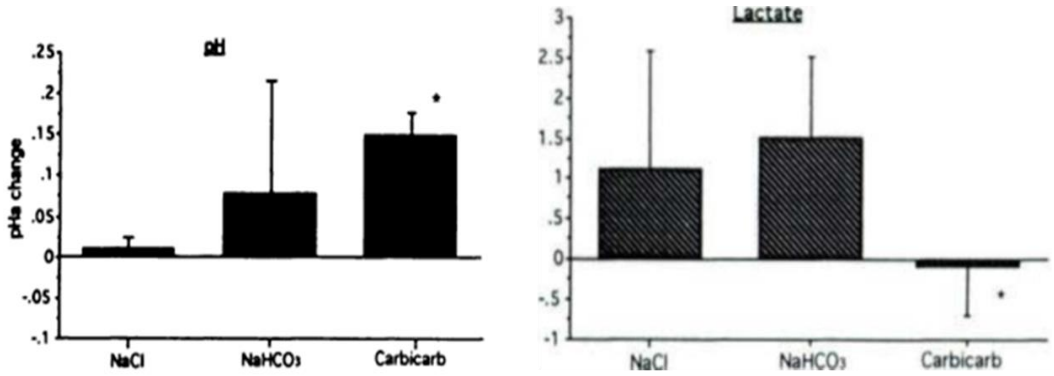


Figure 2.9.Arterial pH and Lactate changes with NaCl, $NaHCO_3$ and Carbicarb administrations for Mongrel dogs [27]

Carbicarb therapy has more advantages than bicarbonate therapy, because it decreases systemic blood pressures when bicarbonate therapy increases. This case is good enough for successful defibrillation (the stopping of fibrillation of the heart) by buffering of excessive H^+ ions [28]. The timing of treatment may affect the elimination of CO_2 [25]. In addition, effective amount of buffer or drug is significantly important parameter for living system, because several dermatologic side effects were observed in vivo study with patients as shown in Figure 2.10 [29]. When pH increases excessive amount in the tissue microenvironment that causes reducing the ionized fraction and malfunction of cardiovascular system by binding calcium ions to circulating proteins [29, 30].

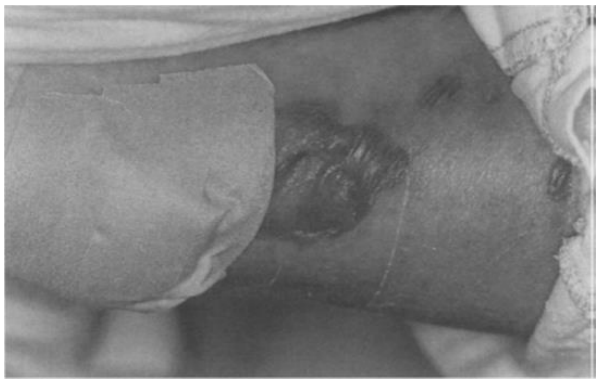


Figure 2.10.The example of blister (vesicates) caused with Carbicarb treatment [29]

2.4. Liposome and its Applications in Cancer Therapy

There are many types of cancer treatment such as surgery, radiation, hormonal therapy, chemotherapy, targeted therapy, and immunotherapy. Nowadays, surgery and radiation provide a basis of all cancer types. Radiation working principle is based on killing dividing cells via DNA damage [31]. But, they are difficult and painful processes for patients. If chemotherapeutic drug delivery system can be improved very well, it paints a promising picture for cancer patients. Drug delivery systems provide potential to decrease toxic effects of chemotherapeutic drugs by using encapsulation property in normal tissues [32].

Liposomes were discovered as closed bilayer structures by A. D. Bangham in 1965, and they have accepted the oldest nanocarrier system. They have been used for drug delivery systems in 1970s. They are composed of 2- distearoyl-sn-glycerophosphocholine (DSPC), Cholesterol and DSPE-PEG_{2000} (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] to produce phospholipid bilayers which can be provided drug encapsulation to protect from degradation. They can be used many clinical applications because of their biocompatibility and biodegradability features [21, 33].

Phospholipids are found in stable membranes composed of two layers as known a bilayer. In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. When preparing liposomes with mixed lipid composition, the lipids should be dissolved and mixed in an organic solvent to make sure that homogeneous mixture of lipids [34].First process of lipid preparation is the lipid film hydration. It is a simple process that adding aqueous medium to the container of dry lipid. The most important point of this process is lipid suspension which was maintained above the melting temperature (T_m) during the hydration period. Maintenance of temperature above the T_m of the lipid suspension allows the lipid to hydrate in its fluid phase with adequate agitation. Hydration time may differ slightly among lipid types and structures, but 1 hour can be enough for proper shaking of lipid suspension. T_m gets different value for different phospholipids structures, for example the most higher value is DSPC composition that is 55^0C. There are three types of liposomes: multilamillar vesicles (MLV), Small Unilamellar Vesicles (SUV) and Large Unilamellar Vesicles

(LUV) as shown in Figure 2.11(A) [21]. These are used to deliver different types of drugs. MLV are composed of a number of concentric phospholipid bilayer membrane separated by aqueous phase. These are big in size and may be up to 5 μm. LUV are composed of a single lipid bilayer surrounding aqueous compartment. The size of these liposomes is in the range of 100- 250 nm. SUV are composed of aqueous compartment enclosed by a single lipid bilayer. The size of these liposomes is range between 20-100nm [21, 33]. And also, ionic contents of these liposomes depend on the adding buffer during hydration process. For example, If Na_2CO_3 buffer is used during hydration process, alkali ionic contents of liposomes can be produced as shown in Figure 2.11(B). Different buffers are preferred for different drug encapsulation that depending on pH values of drug.

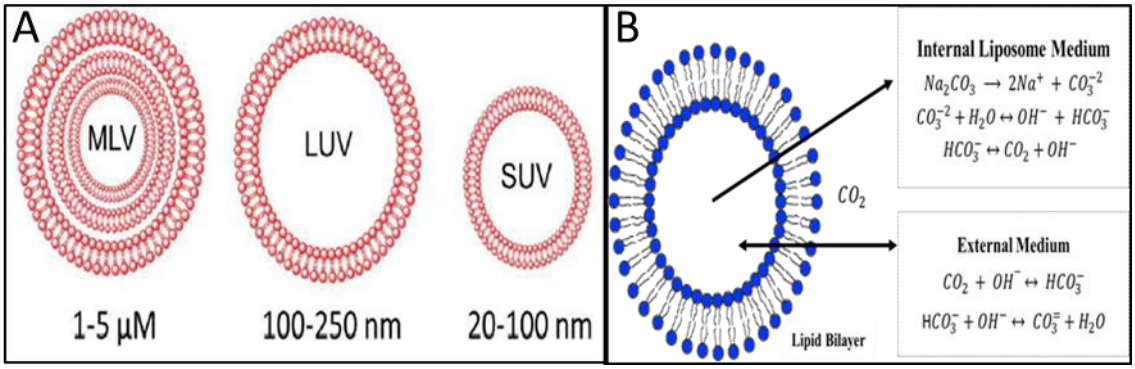


Figure 2.11.(A)Classification of liposomes based on the lamellarity [21] (B)Schematic representation of alkali liposome with ionic contents

There are several types of artificial phospholipids that DPPC, DMPC, DSPC, HSPC are the most common ones. The major components of liposomes are phospholipids and cholesterol that determined the behavior of liposomes. The most common phospholipid component of liposomes is DSPC. The head portion can be modified by attaching a functional group. The 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) is an example of a functional phospholipid used to conjugate other polymers like polyethylene glycol (PEG). The type, molar percentage and packing orientation of phospholipids determine the ultimate shape and size of the liposomes. Because of this, type of phospholipids should be select by their chemical compositions and physical properties to generate proper liposomes. Cholesterol is also important for rotational freedom by flip-flop movements that generates liposomes leaky

properties. It is the main component of the liposomes that stabilize the bilayer structures.

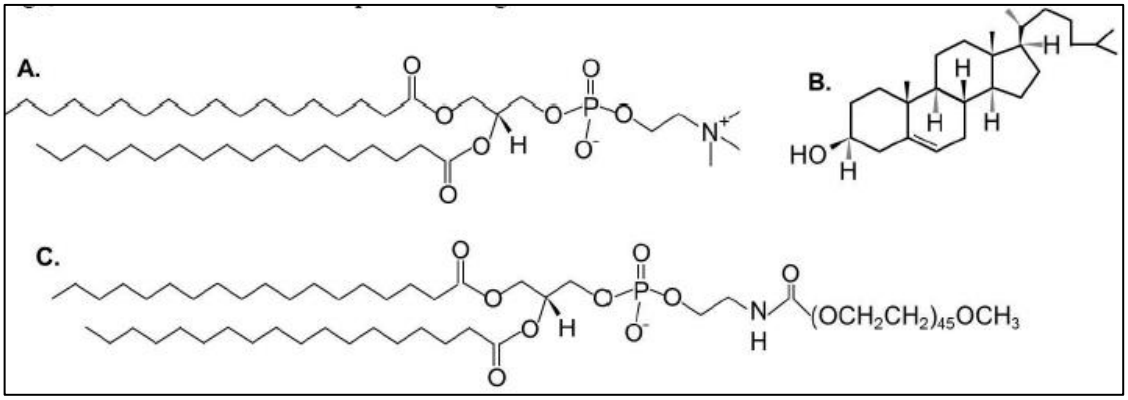


Figure 2.12.Chemical structures of common liposomal components: (A) DSPC (B) Cholesterol and (C) DSPE-PEG [21]

Targeting is also important issue for proper treatment after nanocarriers designing and preparation. They can extravasate into the tumors through the gaps between endothelial cells and accumulate there due to poor lymphatic drainage as known as enhanced permeability and retention (EPR) effect. This process is also passively targeted to tumoral area. The nanoparticle size and surface characteristics determine the effectiveness of passive targeting via EPR effect as shown in Figure 2.13 [35]. Longevity of the nanocarrier in circulation is very important for passive targeting that is size and coating surface characteristics depending [35]. PEG coated liposomes is preferred from its properties. PEG act as steric barrier and is effective in preventing rapid elimination of RES. Increasing circulation time gives an advantage for extravasation through the leaky vasculature and slow accumulation in the tumoral area via EPR effect. There are also several active targeting ways of nanoparticles: Antibody, Aptamer and Ligand based targeting. They are preferred depending on increasing uptake mechanism of nanoparticles. Targeting nanoparticles to receptors or other surface membrane proteins overexpressed on target cells are selective properties for this targeting [36]. The size, encapsulation efficiency, and degradation rate of the liposomes also affects the release rate of the therapeutic agent.

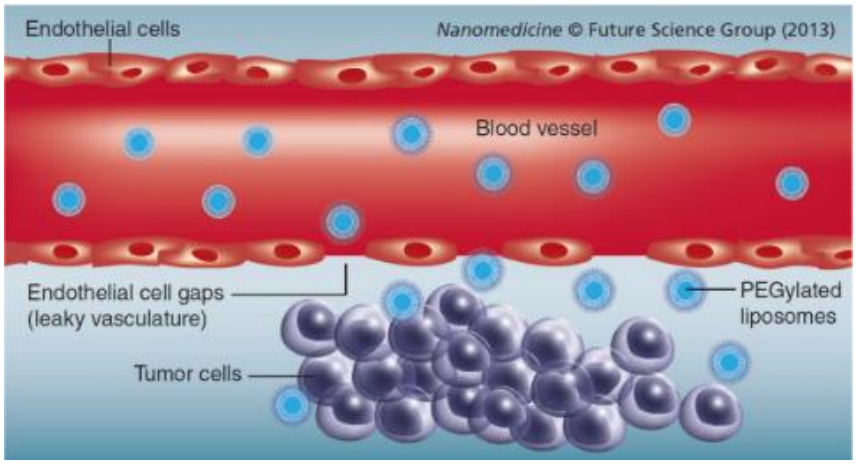


Figure 2.13.Passive targeting of nanoparticles by enhanced permeability effect (EPR) [35]

There are several uptake mechanisms of nanoparticles: Fusion (simple adsorption), Endocytosis (by phagocytic cells of the reticuloendothelial system) and Facilitated diffusion. The interactions of nanoparticles with the soft surfaces of biological systems like cells play key roles in understanding their properties and make progress in therapy. One important concern during designing nanoparticles is that ensuring easily enter the cells. Surface charges, shapes and sizes of the liposomes effect their penetrating the cell membrane. Each nanoparticle exhibits a preferred pathway for cellular internalization. For example, the 100-150 nm size ranges can be taken inside of the cells by Clathrin mediated endocytosis (vesicles containing plasma membrane proteins with receptor sites specific to the nanoparticles). It is also energy dependent process [4].

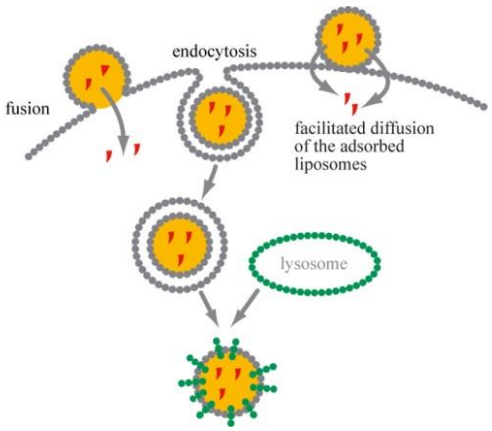


Figure 2.14.Ways of delivery of liposome content [4]

CHAPTER 3

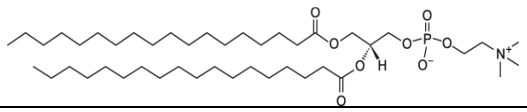
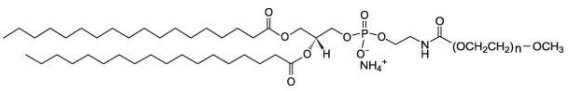
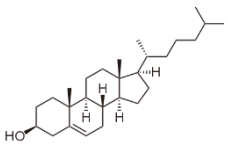
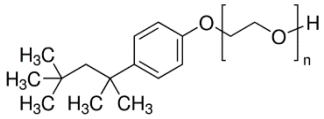
MATERIALS AND METHODS

3.1. Materials and Devices

During liposome making, DSPC, DSPE-PEG_{2000} and Cholesterol were weighed certain amounts to prepared liposome shell structure. The used materials were listed in Table 3.1. DSPC, DSPE-PEG_{2000}were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol was purchased from Fluka, chloroform and the other chemicals were purchased from Sigma-Aldrich®, Inc. (St. Louis, MO, USA). For dialysis process, dialysis membrane was purchased from and used pre-wetted RC tubing (MWCO: 10 kD, Spectra/Por® 6 Dialysis Membrane).

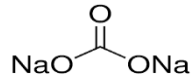
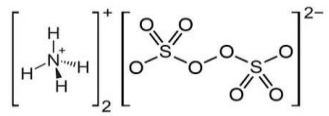
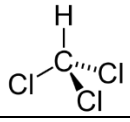
For *in vitro* studies, Fetal bovine serum (FBS), Phosphate buffered saline (PBS) and Trypan Blue were purchased from Gibco, Cell proliferation colorimetric reagent (WST-1) from BioVision. *4T1* cell line was donated by Dokuz Eylül University (DEU) and used for cell culture experiments.

Table 3.1. Chemicals and their structures

Chemicals		Chemical Structure
DSPC	1,2-Distearoyl-sn-Glycero-3- Phosphocholine	
DSPE-PEG_{2000}	1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (polyethylene glycol)-2000]	
Cholesterol	(5-Cholesten-3β-ol)	
T_{X-100}	Triton X-100	

(Continued)

Table 3.1 (Cont.)

Na_2CO_3	Sodium Carbonate	
$(NH_4)_2SO_4$	Ammonium Sulfate	
$CHCl_3$	Chloroform	

A mini-extruder device was used during liposome making with polycarbonate membrane of 200 nm pore size (Whatman Nucleopore Track-Etch filtration product), filter supports, and the extrusion materials. They were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, ABD). A Longer Syringe Pump (LSP02-1B Dual Channels Syringe Pump, Longer Precision Pump Co., Ltd. Halma Group) was also used to generate same size of liposomes without damaging the membrane.

Figure 3.1.Longer pump and Mini-extrusion device

Liposome characterization was made by Dynamic Light Scattering (DLS) device. The liposome size distribution was easily determined by Zetasizer Nano Series (ZEN 3600, Malvern Instruments) and also, Leica Light Microscope was used during cell visualization and counting. Microplate reader (product model:AMR-100) was used for determining of cell viability percentages. WST-1 colorimetric assay was used during this process. Tetrazolium salt WST-1 was converted to formazan by cellular mitochondrial dehydrogenases so that this leads to increase in the amount of formazan dye formed. As a result, the formazan dye produced by viable cells and it was determined by measuring the absorbance at 440 nm using microplate reader as shown in Figure 3.2. During alkali liposome releasing measurements, Thermo-Scientific pH

probe and its device were used. Software program was used to observe pH changes continuously when added alkali liposome and triton-x.

Figure 3.2.Microplate reader for measuring absorbance value

3.2. Alkali Liposome Preparation

Thin Lipid Film Formation:

The liposome formulation has 57-38-5% ratios of DSPC, Cholesterol and DSPE-PEG_{2000}. These chemicals were put into a vial and then added enough amount of chloroform to dissolve the chemicals with each other. After chloroform was evaporated from lipid film structure by nitrogen gas system as seen in Figure 3.3, the film was put into a vacuum for 4 hours to get rid of any chloroform. Finally, the film was stored at -20^0C in a freezer.

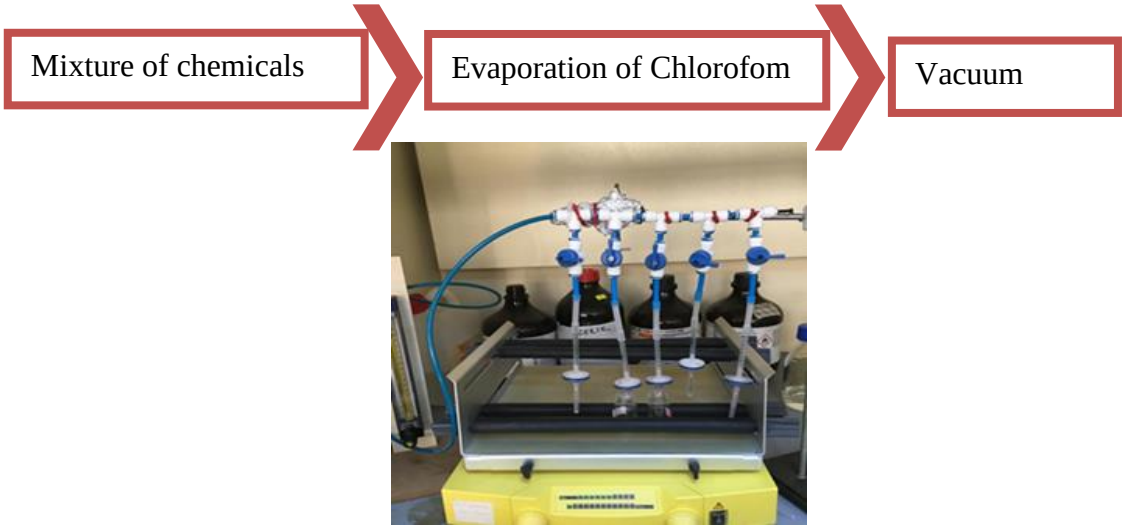


Figure 3.3.Evaporation of chloroform by nitrogen gas system

Thin Lipid Film Hydration:

Hydration time and temperature are important for effective liposome formation, because these parameters effect the liposomal shape, size and stability. The temperature must be 10°C above the phase transition 55°C of phospholipid DSPC. Cholesterol also provides rigidity onto the bilayer membranes when the temperature is above the phase transition (T_m). According to this information, the water bath temperature was set as 65°C. After a while, 1 ml of $(NH_4)_2SO_4$ or Na_2CO_3buffer was put into the lipid film which was prepared before, and then it was incubated into the shaking water bath at 65°C for an hour to form bilayer lipid structure.

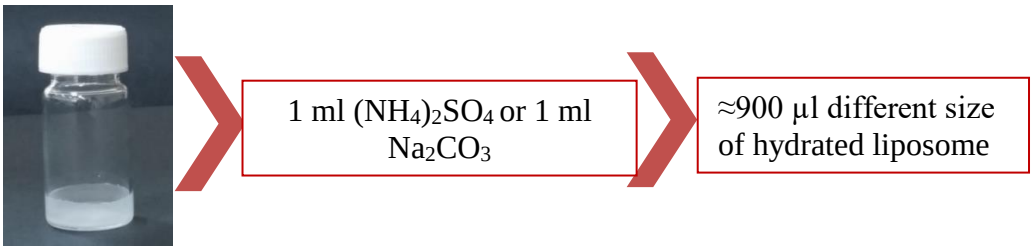


Figure 3.4.Thin film hydration steps

Thin Lipid Film Extrusion:

Firstly, mini-extrusion devices were assembled as shown in Figure 3.4 and 200 nm polycarbonate membrane was put between filter supports. Before all assembling, the membrane and the supports were wet to stick each other properly. Then, this completed part was put on hot plate block to warm it. When all parts were come to determined temperature, which is important for bilayer liposome formation, it was passed through several times with the $(NH_4)_2SO_4$ or Na_2CO_3buffer into Hamilton syringes to prevent the gaps between the supports and the membrane. Then, this hydrated lipid film was pulled into the syringe, and the syringe was placed to the mini-extruder device. Also, the syringe pump device was adjusted to a flow rate of 119,8 mm/ml and an injection volume of 1.0 ml. After that, the hydrated lipid film was passed 11 times through the syringes as shown in Figure 3.5. Number of passes are important for homogeneous liposome formation and determined passes were optimized by another study. At the end of the process, hydrated lipids were put into a DLS cuvette, and the size distribution of

lipids was measured with DLS method. Finally, the liposome sample was poured into a clean vial, and it was put into the +4°C refrigerator.

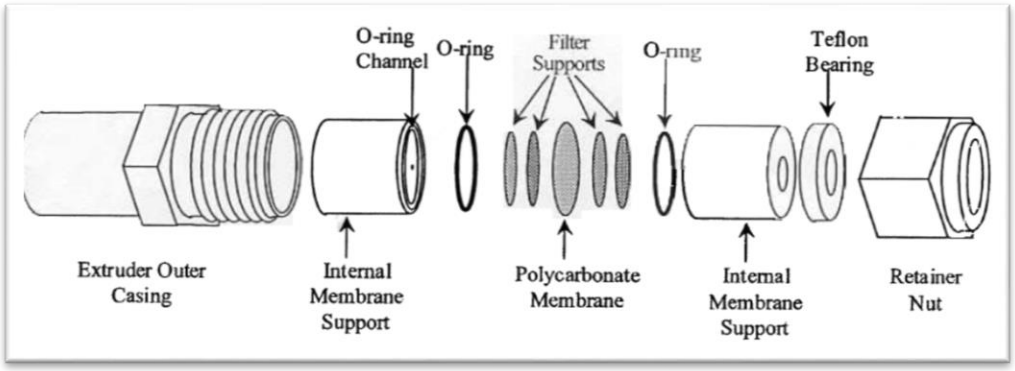


Figure 3.5.Assembling parts (Avanti)

Figure 3.6.Mini-Extrusion parts, Filter supports and Polycarbonate membrane, Hamilton syringes and Pump (Avanti)

3.3. Dynamic Light Scattering Measurement

After the liposome hydration process, the liposome sample was put into disposable cuvette to measure particle sizes using dynamic light scattering as shown in Figure 3.6. The principle of the DLS method is that for measuring the size and size distribution of molecules and particles which are dissolved in a liquid. The Brownian motion of these particles or molecules causes the intensity of the light they scatter to fluctuate rapidly. As a result, temporal fluctuations were analyzed by means of the intensity.

Figure 3.7.Zetasizer Nano Series (ZEN 3600, Malvern Instruments)

3.4. Liposome Dialysis

Dialysis is an effective method of delivering large quantities of base [37]. After DLS measurement, the liposome sample was dialyzed in a 0.9%NaCl solution. Firstly, enough dialysis membrane was cut and put into distilled water to get rid of some hazardous chemicals for 15 min. Then, one side of the membrane was closed properly with green clamp as shown in Figure 3.7 and the liposome sample was poured into the membrane with micropipettes. After that, the other side of the membrane was also closed with green clamp and this sample was put into the 0.9%NaCl solution. The solution was changed with fresh 0.9%NaCl solution for three times and the liposome was waited for overnight in the solution to get rid of some charges which are located outside of the liposomes.

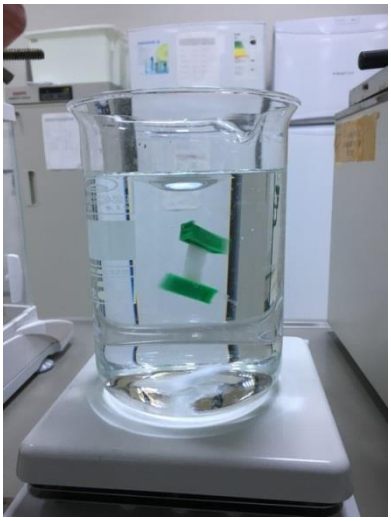


Figure 3.8.Dialysis of the liposome in 0.9%NaCl solution

3.5. Continuous pH Measurements

After all these processes, which were mentioned before, stability of the liposome was measured by pH meter (Thermo-Scientific). Firstly, 10 ml of 0.9%NaCl solution was added in a clean vial and the vial was placed onto magnetic stirrer. Then, magnetic fish was put in the vial and pH probe was plunged into a solution. Thermo-Scientific computer program was used to check continuously pH differences of the solution as shown in Figure 3.8. Top of the vial was also closed properly to provide solution equilibration (prevent any gas or material entering). After 2 minutes, 10µl of alkali liposome was added into the solution and measured pH for approximately 4 min to observe any changes. Then, 30µl of Triton-X was added to lyse the liposomes and observe the pH changes. Alkali liposomes contain high amount of basic ions inside of the bilayer structure, so high amount of the liposomes causes more pH changes into the solution.

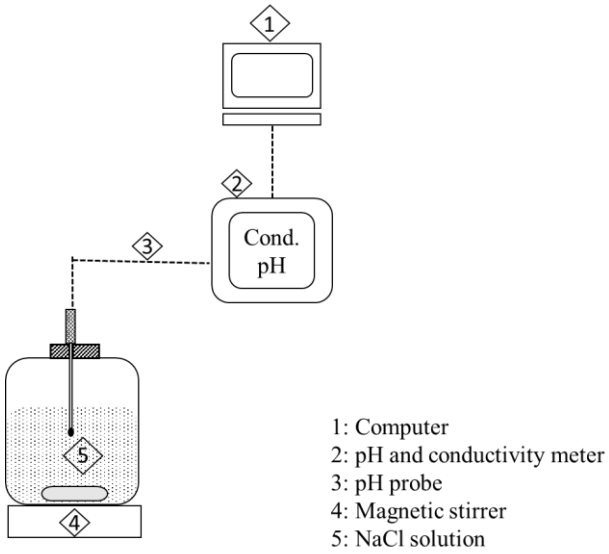


Figure 3.9.Experimental setup of continuous pH measurements

Additionally, same steps were applied for different amount of the liposome additions as shown in Figure 3.9.

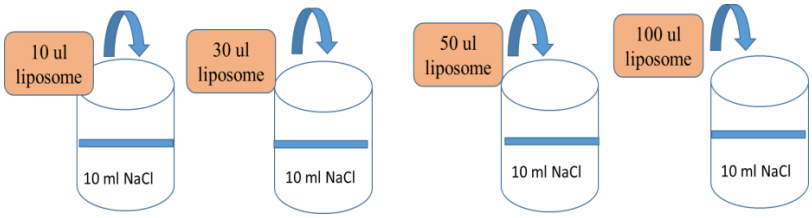


Figure 3.10.Different amounts of liposomes for continuous measurement

3.6. Effect of Alkali Liposomes on *4T1* Breast Cancer Cells *in vitro*

Cultured *4T1* Cells into Petri Dishes:

4T1 Cell lines were taken from Oncology lab in Dokuz Eylül University (DEU), and were grown under controlled conditions. A medium supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, O_2 and CO_2, hormones and temperatures to provide cellular maintenance. They were cultured into a flask and passaged several times. Then, they were transferred in a 96 well plate to observe and measure cell viability. They were incubated in 2 days to reach enough confluence, normally 80% (Animal Cell Culture Guide). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Figure 3.11.Cell passages with PBS and planting 96 well plates

Preparing Different Alkali Liposome Concentrations:

Different alkali liposome concentrations were prepared to visualize cell viability behavior. There are control sample with no liposome and 1-3-5-7-10% liposome samples as shown in Table 3.2. Each concentration was added in *4T1* cells for six same wells (2 of them for microscope images and 4 of them for cell proliferation assay). Normally, culture cells (0.1 – 5 x 10^4/well) in a 96-well microplate in a final volume is 100 µl/well culture medium for 3 days of cell viability, but 200 µl/well culture medium was prepared for 5 days of cell behavior visualization.

Table 3.2.Liposomal concentrations of cell medium

Control(200 µl Medium)
1%Liposome(2 µl Liposome +198 µl Medium)
3% Liposome(6 µl Liposome +194 µl Medium)
5% Liposome(10 µl Liposome +190 µl Medium)
7% Liposome(14 µl Liposome +186 µl Medium)
10% Liposome(20 µl Liposome +180 µl Medium)

Checking *4T1* Cells Viability Under Different pH conditions:

All determined amount of samples were added each well plate and cells were waited for 1,2,3,4 and 5 days inside an incubator with different pH conditions. Then, they were checked day by day measuring cell viability with WST-1 and hemocytometer. Intracellular pH (pH_i) is very important for cell cycle processes. It can be any small changes inside of the cell, cell cycle checkpoints will be affected in this condition. For example, pH_i decreases during mitosis process. If we hold pH higher, cell won't divide in this manner [17]. As a result, we could control cell division by changing pH_i.

Counting Live and Dead Cells by Hemocytometer:

Counting cells using a hemocytometer is simple and getting accurate determination results. Cells were taken from incubator to count each well plate properly. Firstly, all medium (inside of control, 1-3-5-7-10% liposome samples) were transferred to another empty well. And, 2 µl trypan was added each well to get microscope images for all samples. Then, 30 µl of medium was poured backward in each sample and each well was scratched gently with micropipette one by one. After, each sample was transferred in eppendorf tube and same amount of trypan blue, 30 µl of it was added to make Trypan Blue-treated cell suspension. Then, they were mixed very well to prevent precipitation of cells and 10 µl of sample was put onto thoma lam. Using a glass hemocytometer should be gently filled both chambers underneath the

coverslip, allowing the cell suspension to be drawn out by capillary action. As a result, live and dead cells were easily counted focus on the grid lines of the hemocytometer with a 10X objective under Leica light microscope. Because, unstained cells as live cells do not take up trypan blue and dead cells stained with trypan blue can also be counted for viability estimation.

Table 3.3.Calculations for Trypan Blue Counting

Cell Number Calculations	
#of viable cells/total #of cells*100	%of viable cells
Viable cells/squares	Average #of cells/square
Final volume/volume of the cells	Dilution factor(DF)
(Average #of cells/square)*DF*10^4	Concentration(Viable cells/ml)

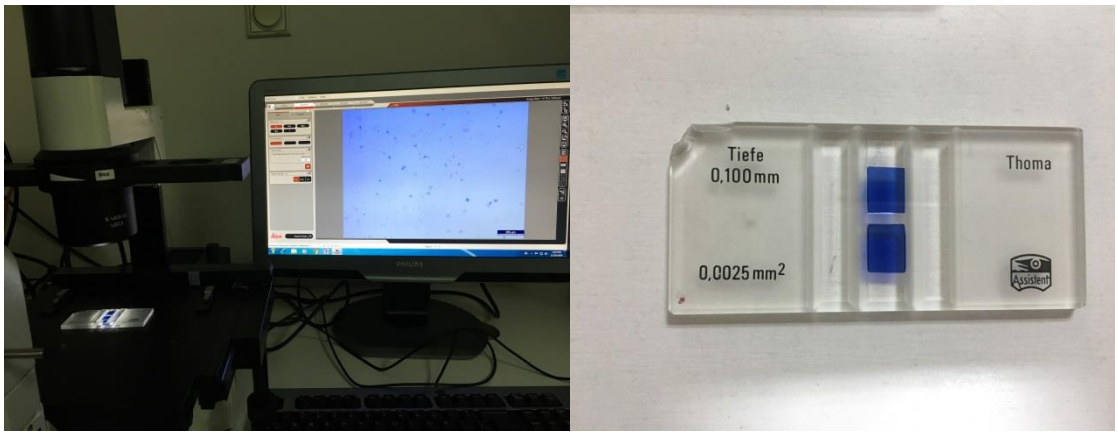


Figure 3.12.Light Microscope and Thoma lam for cell counting

Checking Cell Viability by *WST-1* Cell Proliferation Assay Kit:

WST-1 provides a simple and accurate method to measure cell proliferation by cellular mitochondrial dehydrogenases as shown in Figure 3.12. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 440 nm. The all assay can be performed in the same micro-well plate and does not require any extra steps for example washing, harvesting and cell solubilization. There are several applications to use this method:

- Measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients, etc.

- Analysis of cytotoxic and cytostatic compounds such as anticancer drugs, toxic agents and other pharmaceuticals.
- Assessment of physiological mediators and antibodies that inhibit cell growth.

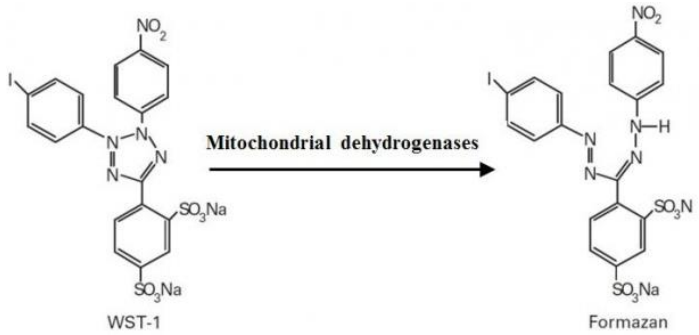


Figure 3.13.Chemical structure changes *WST-1* to Formazan

Firstly, 10 µl wst-1 reagent was added in each well and microplate was covered with aluminum foil to protect from light. Then, it was incubated to 4 hours in standard culture conditions. Incubation time changes also from 0.5 to 4 hours, because the appropriate incubation time depends on the individual cell type and cell concentration used. If adherent cell type can be used in the study, incubation time should be approximately 4 hours (Biovision datasheet). After incubation period, microplate was adjusted in microplate reader to measure cell absorbance at 440nm and 650 nm (reference wavelength).

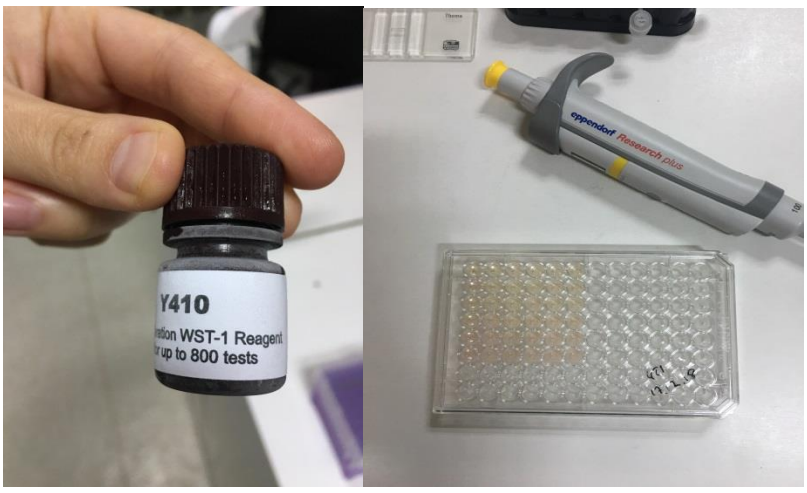


Figure 3.14.*WST-1* Reagent and all samples with different pH conditions

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Alkali Liposome Preparation and Characterization

For drug delivery systems, liposome sizes should be between 150-200 nm. One reason of that circulation time is significantly important for effective cancer therapy. Liposomes which have 150-200 nm size range, have circulation time in the blood is higher than the small (less than 70 nm) and large particles (more than 300 nm). The other reason is to escape from reticuloendothelial system. Macrophages destroy small and large particles from the blood; this process is called 'accelerated blood clearance' (ABC) [33]. Cell membrane can also allow only small molecules pass through inside of the cell. The particle size significantly influences their endocytosis processes and cellular uptake properties [4]. As a result, appropriate size of liposome should be produced to increase therapeutic index for cancer applications. As a result, proper liposome size is important for drug delivery system.

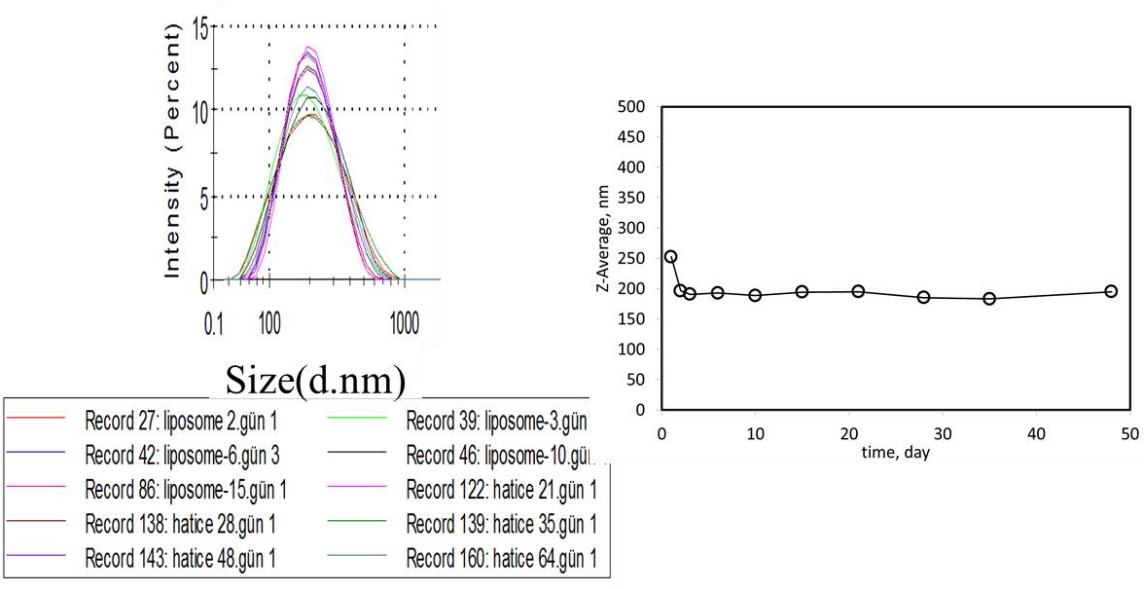


Figure 4.1. Sizes of Alkali Liposome stored at 4^0C in refrigerator for 48 days

Stability and average sizes of alkali liposomes as the Figure 4.1 show the intensity size distributions for 48 days. Alkali liposomes protect their sizes over this

time ranges. There were seen small differences between first day measurement and the others. Temperature may be the cause in this situation because first size result was measured after dialysis at room temperature. The subsequent measurements were performed on liposomes stored in refrigerator at 4^0C. In conclusion, alkali liposomes can be stored at 4^0C without their size variations for approximately 2 months and they are stable.

Preparation of Alkali and Acidic Liposomes

Preparation methods were provided to generate liposomes with different surface charges and internal ion contents. If ammonium sulfate buffer was used before hydration process, liposomes which have acidic internal ion contents were produced. It is useful for weak base chemotherapeutic drug encapsulation. Because it concentrates on more acidic side of the membrane, that is inside of the liposome [38]. If sodium carbonate was also used, liposomes which have basic internal ion contents were produced. Both alkali and acidic liposomes were produced in this study and their ionic contents were also evaluated by continuous pH changes using triton-x. Firstly, 100 µl alkali liposomes was added in 0.9%NaCl solution and 30 µl triton-x was added in this solution 5 min later. Same process was performed for acidic liposomes to visualize their ionic contents by pH changes. Approximately 1 unit increases after alkali liposomes degradation and 2 unit decreases after acidic liposomes degradation was observed in continuous pH measurements as shown in Figure 4.3.

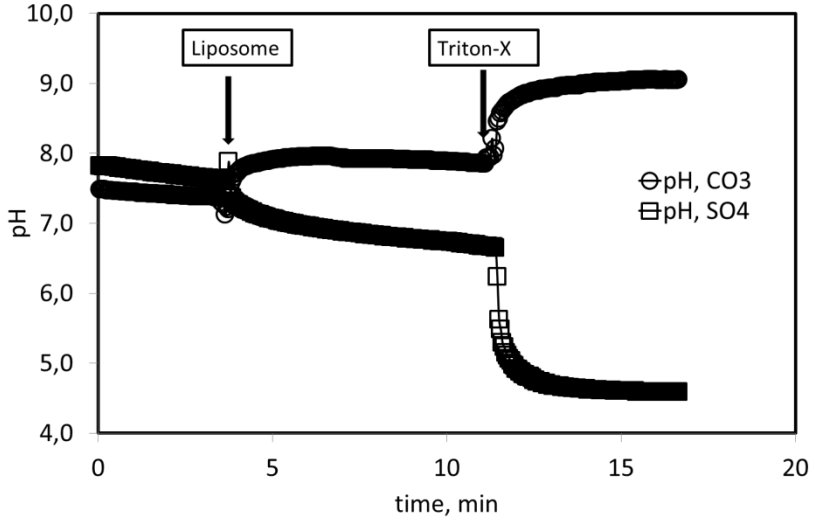


Figure 4.2. Continuous Measurement of Alkali and Acidic Liposomes

Stability of Alkali Liposomes

Encapsulation efficiency and drug leakage are important cases in vesicular drug delivery system [37], so that the stability is an important parameter that can affect the performance of liposomes. There is any change in particle size of carriers can affect targeting, safety and efficacy of liposomes. If liposome releases their contents before reaching targeted area, this will cause unwanted situations for healthy cells.

Alkali liposomes still protect their ion contents after 48 days as shown in Figure 4.1. As mentioned before in methods part, first 100 µl alkali liposomes were added in 0.9%NaCl solution and then triton-x was added in the solution. When adding alkali liposome inside of the solution, there weren't significant pH changes, but after adding triton-x, there were 1-1.5unit pH changes in the solution. The reason of this situation is that triton-x was lysed the alkali liposomes and their contents were released in the solution. Only triton-x effect was also evaluated for pH changes in the solution. There was added 30 µl of triton-x without adding any alkali liposome. There wasn't significantly effect on pH changing for it, only 0.3-0.4 unit differences. As a result, liposome size and their contents (higher basic ion concentrations) are still conserved after 48 days as shown in Figures 4.1 and 4.2.

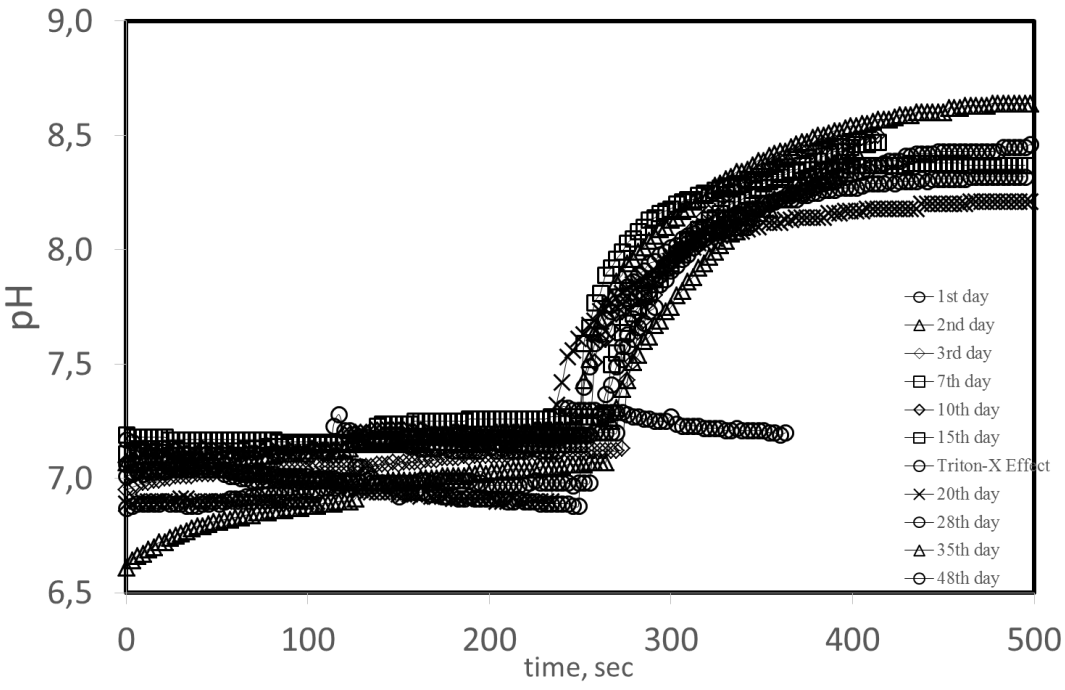


Figure 4.3. Continuous Measurement of Alkali Liposomes for 48 days

Capacity of Alkali Liposomes

Nanoparticles structure and surface charges play an important role in penetration and cellular internalization. Positive and negative nanoparticles can be utilized for different endocytosis pathways [4]. In these reasons, nanoparticle characterization is important issue for cellular uptake. In this study, there were observed pH differences after adding alkali liposomes in 0.9%NaCl solution as shown in Figure 4.4. The reason is that there are some charges out of alkali liposome surfaces. Surface charges should be eliminated by increasing dialysis process time for proper liposome internalization, because nanocarriers with neutral surface charges can easily passes from cell membranes. More pH differences were also evaluated after adding of triton-x, because there are more basic charges inside of the liposomes than out of the surfaces. As a result, if we can add high amount of alkali liposomes, we can get high pH differences in the solution. Approximately 1unit pH increases were observed after 100 µl alkali liposomes degradation.

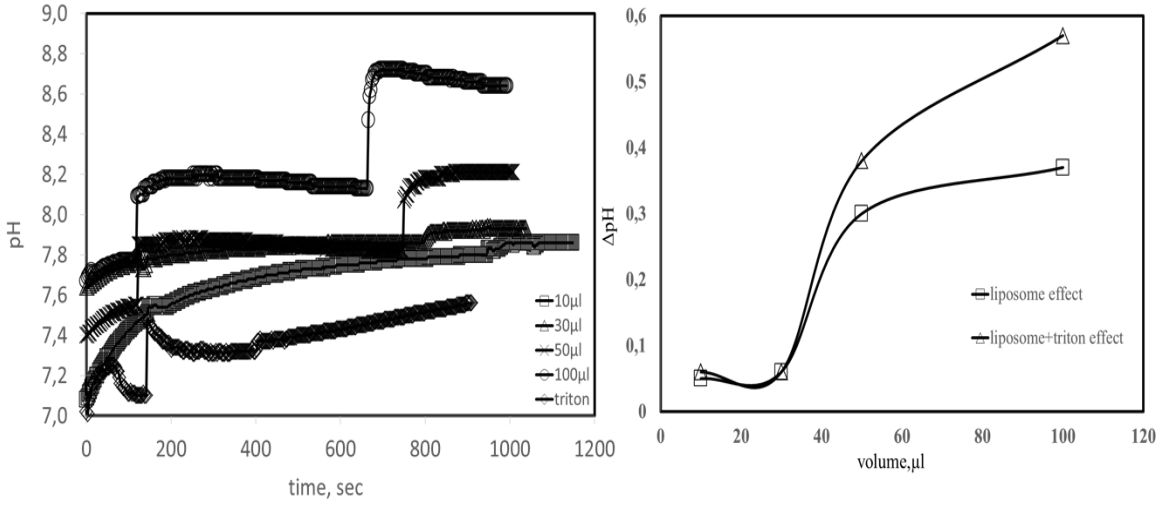


Figure 4.4. pH Changes of adding different Alkali Liposome concentrations

4.2. Effect of Alkali Liposomes on *4T1* Breast Cancer Cells *In Vitro*

4.2.1. Observation with Microscope Images

The *4T1* mammary carcinoma is a preferred tumor cell line because of highly tumorigenic and invasive properties [39]. When they transplanted in the cell culture petri dishes, they are easily adhered on the surface of the petri dishes and proliferated in the shortest time, 24 hours.

The cells were allowed to adhere on the surfaces and reach enough confluence of them for 24 hours. Predetermined concentration of alkali liposomes were also added each well to observe cell behavior and calculate cell viability number. Some images were taken after 24hour treatment of 0-1-3-5-7 and 10% alkali liposome concentrations. Trypan blue dye was used to determine number of dead cells and viable cells. There were observed many viable cells with clear color except 7 and 10% liposome treatment cells as shown in Figure 4.5. Confluence of 7 and 10% liposome treatment cells was also less than the other treatment cells. Intracellular and extracellular pH are important for maintaining normal cell reactions. If there can be any small changes of ionic content of the cells, several signaling pathways and cellular metabolism are effected in this situation. High amount of alkali liposomes means that more interactions of nanoparticles with cancer cells, so that metabolism and number of the cancer cells will be effected with uptaking of alkali liposomes into the cells.

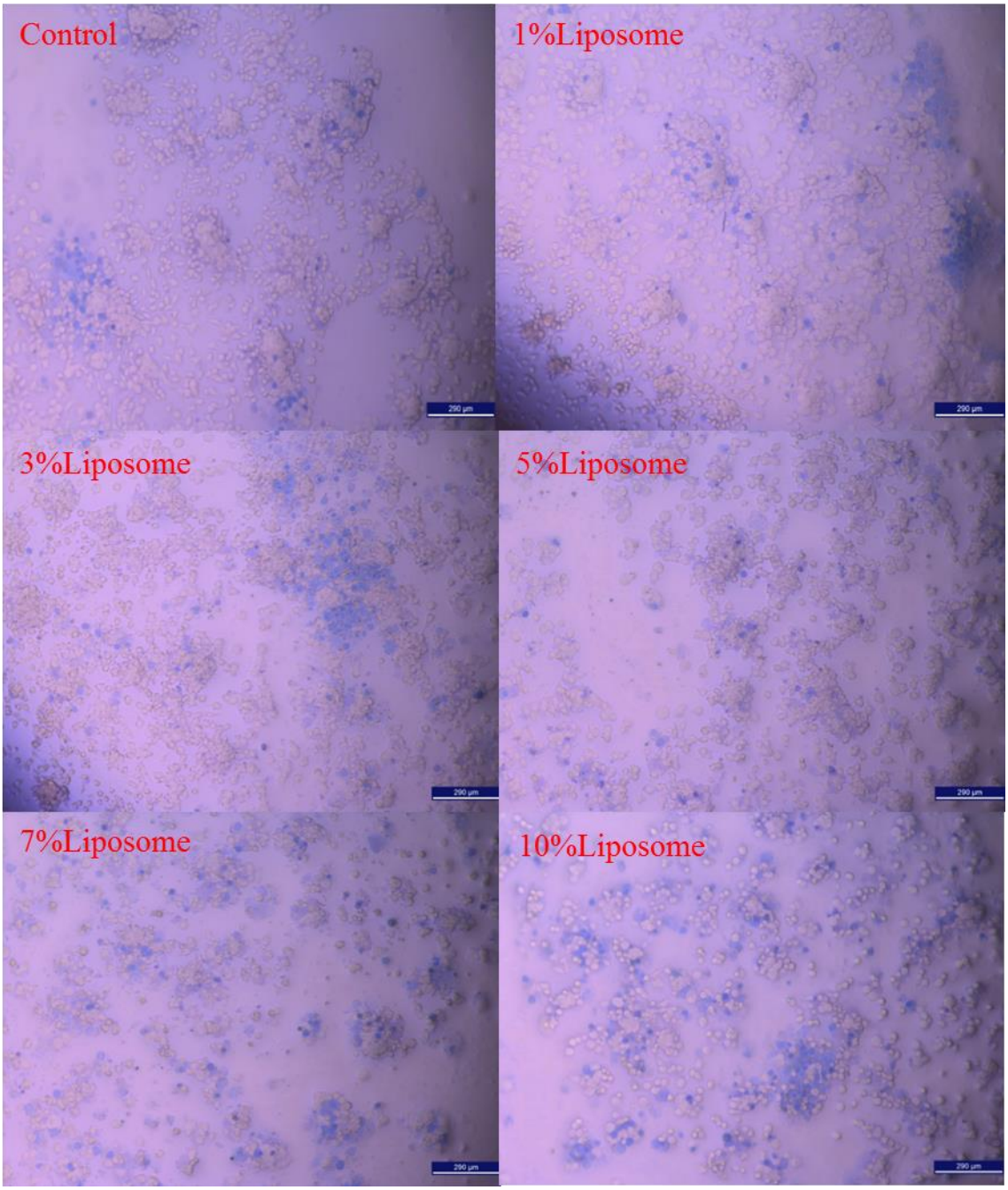


Figure 4.5. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-3-5-7-10 % alkali liposome treatments 10 X magnification, 24 h later

Confluence of 7 and 10% liposome treatment cells were still decreasing after two days as shown in Figure 4.6. There were also more dead cells with blue color in 7 and 10% liposome treatment wells. In addition, confluence of 5% liposome treatment cells was decreasing according to 24hour result. Treatment time and concentration are important parameters to decide effective therapy conditions.

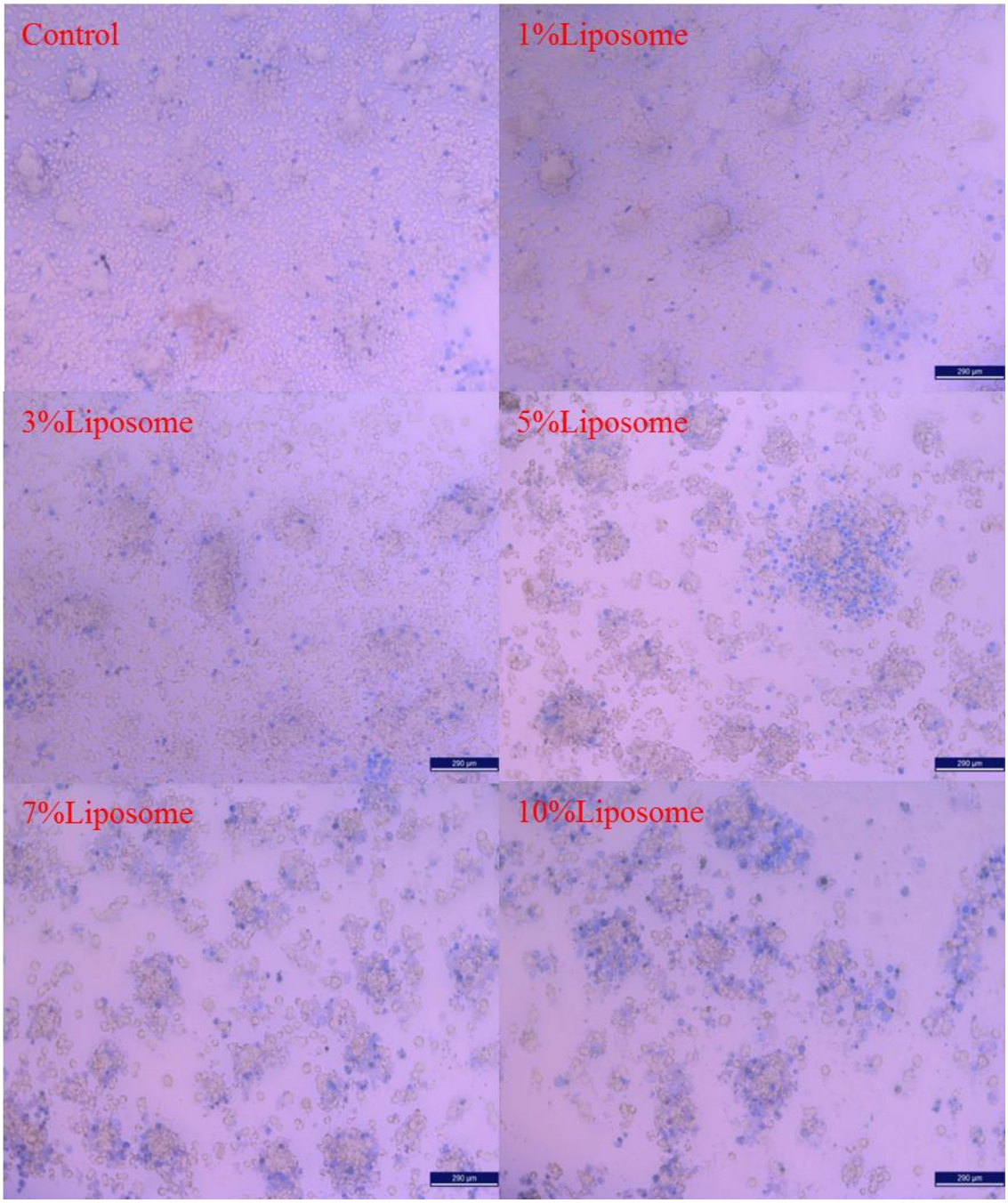


Figure 4.6. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-3-5-7-10 % alkali liposome treatments 10 X magnification, 48 h later

Control had similar confluency with 1 %liposome treatment and good proliferation after 3 days as shown in Figure 4.7. 5, 7 and 10% liposome treatment cells were decreasing day by day according to their confluency results. At the same time, some cell agglomeration were seen in 10%liposome treatment well and the most confluency reduction was also seen in it.

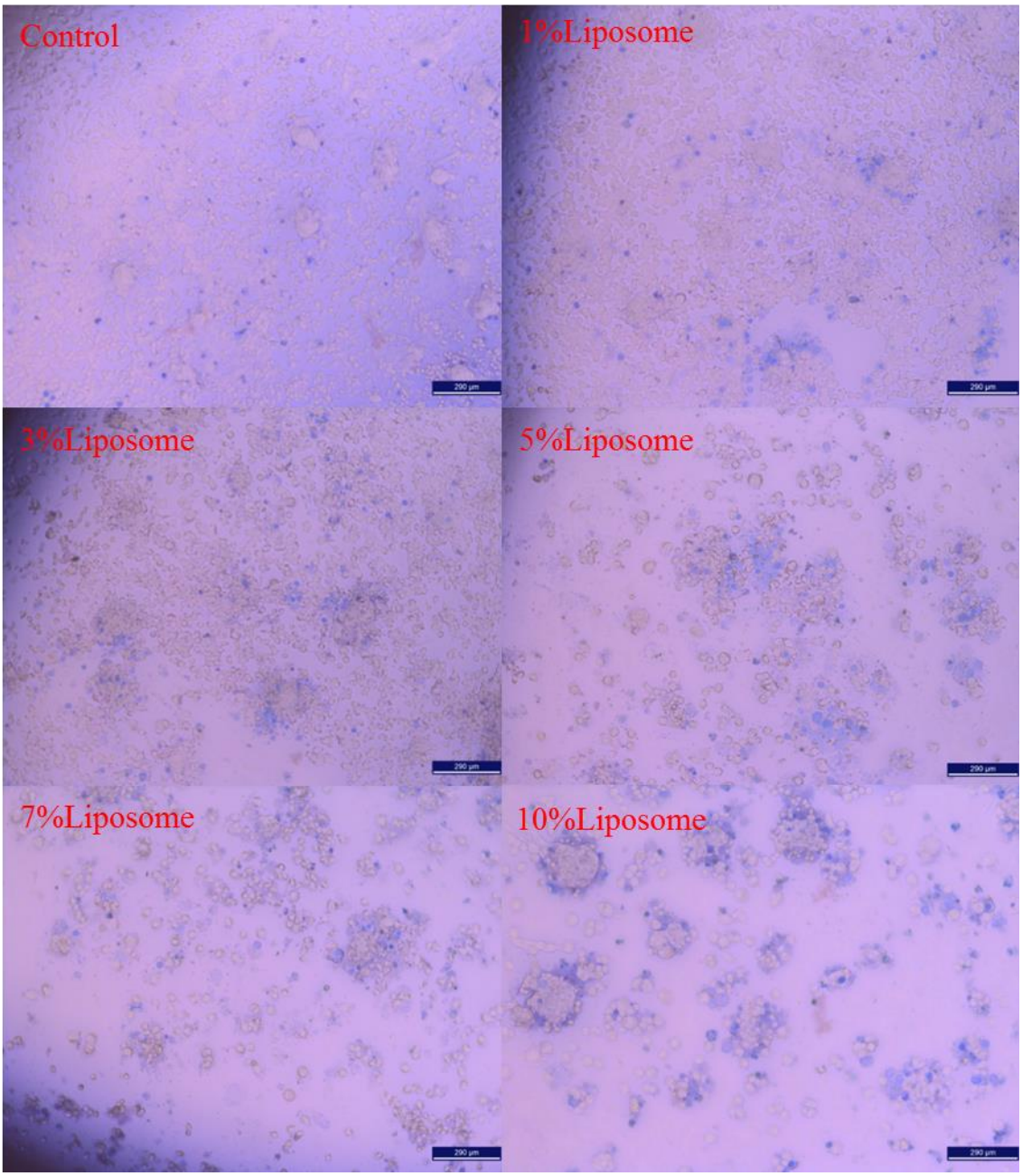


Figure 4.7. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-3-5-7-10 % alkali liposome treatments 10 X magnification, 72 h later

There some cell agglomerations were seen in the 3 and 5% liposome treatment wells, but more in the 5% treatment as shown in Figure 4.8. Fortunately, cell number significantly decreased in 7 and 10% liposome treatment wells. Both of them reached in the lethal dosages means that half of the cells were dead. Our aims in this study were to effect cell cycle stages and shut down cancer cell metabolic pathways. Cells have 4 main stages during cell cycle: two gap phases (G1 and G2); an S (for synthesis) phase;

and an M phase (partitions of genetic material and cell division). Intracellular pH (pH_i) is about 7.2 during M phase and it decreases to 6.8 till the end of the M phase. It changes between 6.8 and 7.2 during the other phases [17]. When alkali liposomes were added in the medium, we think that they could enter the cells by endocytosis or fusion and effect cell cycle phases by increasing pH_i. If pH_i is more than 7.2, the cells won't divide anymore to effect internal pathways. As seen in Figure 4.8 (7 and 10% liposome treatment wells), connections couldn't be observed between cells and most of them were seen dead.

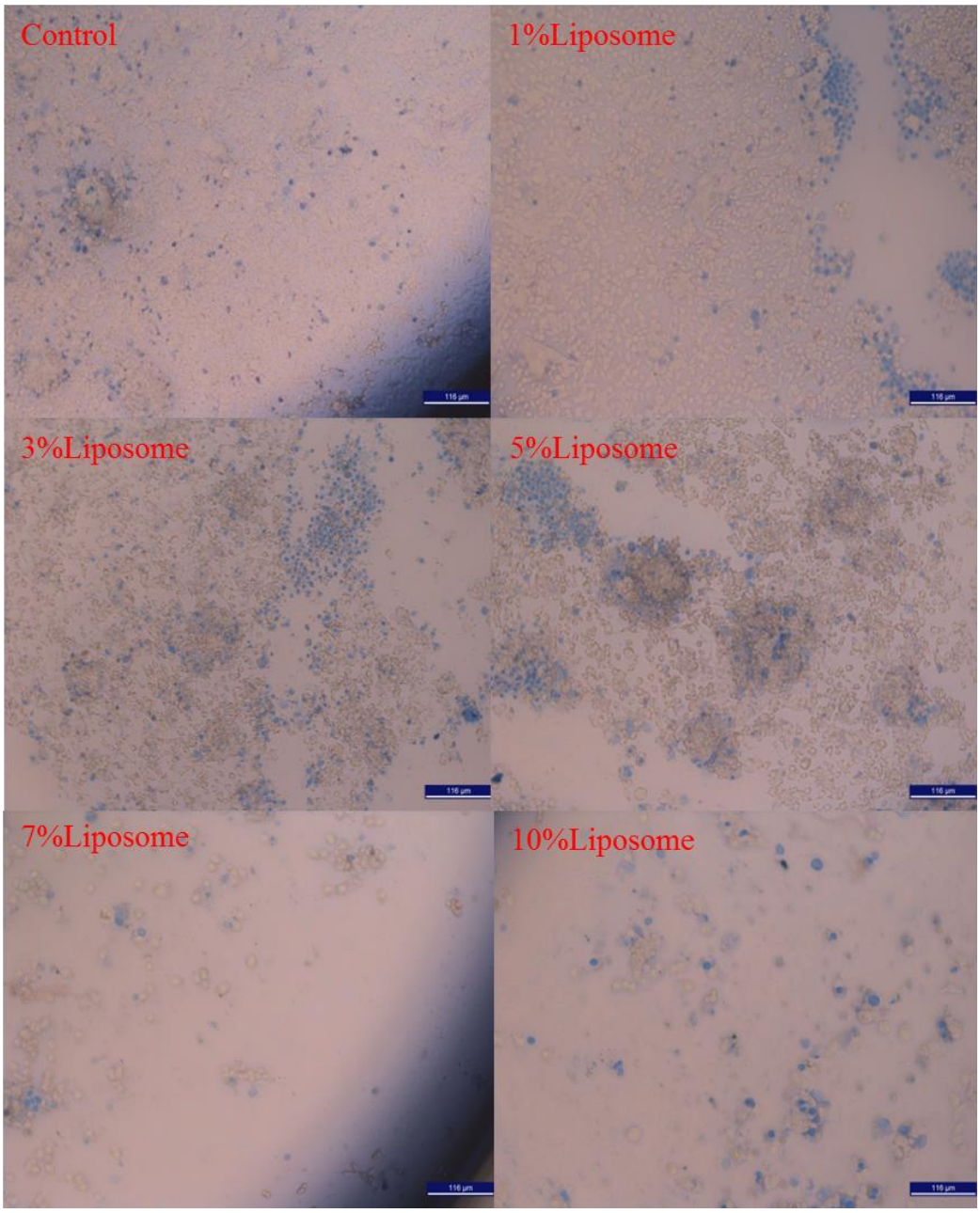


Figure 4.8. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-3-5-7-10 % alkali liposome treatments 10 X magnification, 96 h later

As shown in Figure 4.9, it seems that cell reproduce and increase in the number of control and 1-3-5% alkali treatments. The viability results showed that cells increase in number after 5day treatment, but the viability results decreased.

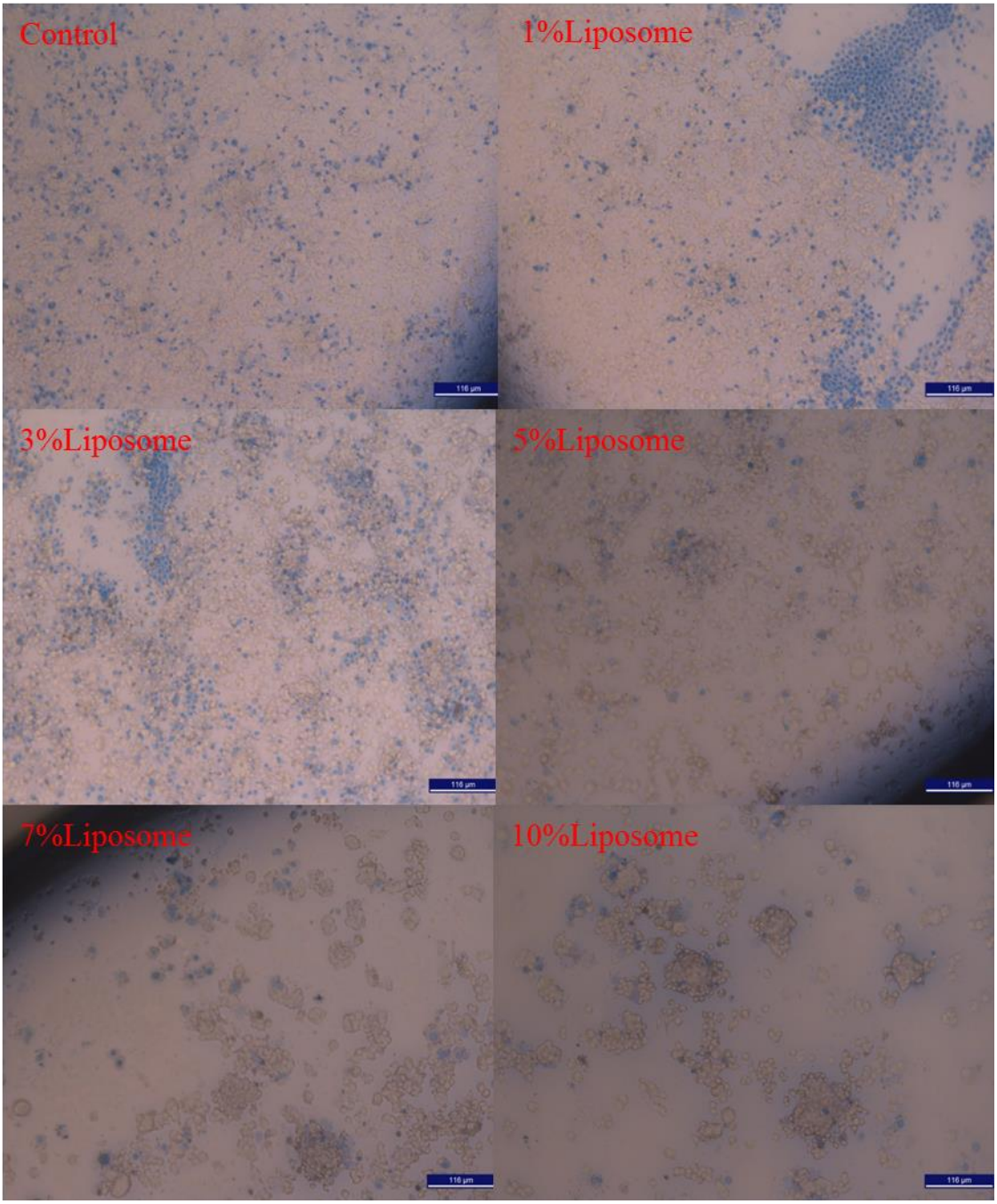


Figure 4.9. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-3-5-7-10 % alkali liposome treatments 10 X magnification, 120 h later

As shown in Figure 4.10, cell attachment on the surfaces and confluence are higher in the control and in 1% alkali treatment but they are lower in 10% alkali

treatment. This result shows us alkali liposomes therapy can be succeed in decreasing cancer cell number.

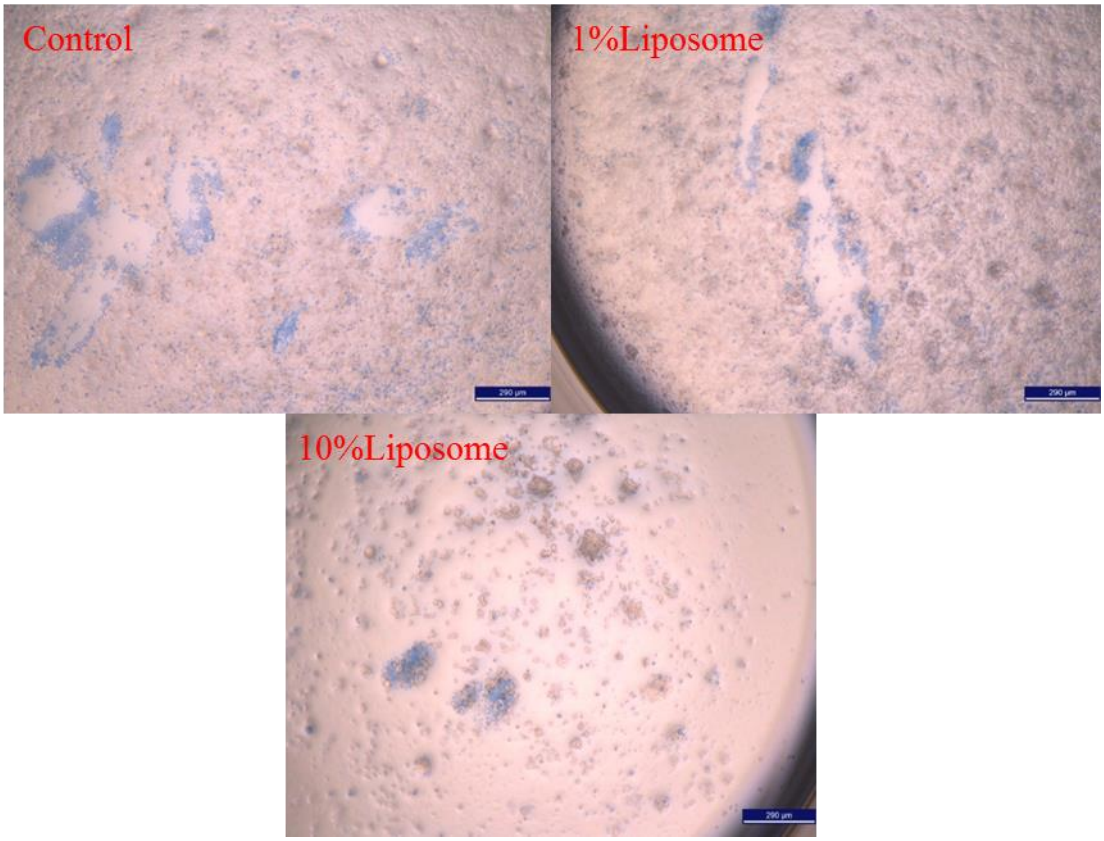


Figure 4.10. *4T1* Cell Images Under Different Alkali Liposome Concentrations: Control with no alkali liposome and 1-10 % alkali liposome treatments 4 X magnification, 120 h later

4.2.2. Effect of Alkali Liposomes on Cell Growth and Cell Viability

4T1 Cells were investigated for 24, 48, 72 and 96 h. Trypan blue and *WST-1* methods were used for getting cell viability results. As shown in Figure 4.11, cell viability is decreasing with increasing alkali liposome concentrations for both methods. It means that our study successfully stopped cell division by effecting internal pH changes, but there were small interferences with 5 and 10%liposome treatment after 24 h results. There weren't enough cells in both wells. It should be checked in wells with microscope if cell planted in the wells equally. One well didn't include enough cells for evaluating viability results, at least two wells should be reserved for trypan blue method. For the other days, two wells were evaluated for trypan method and 4 of them for *WST-1* method.

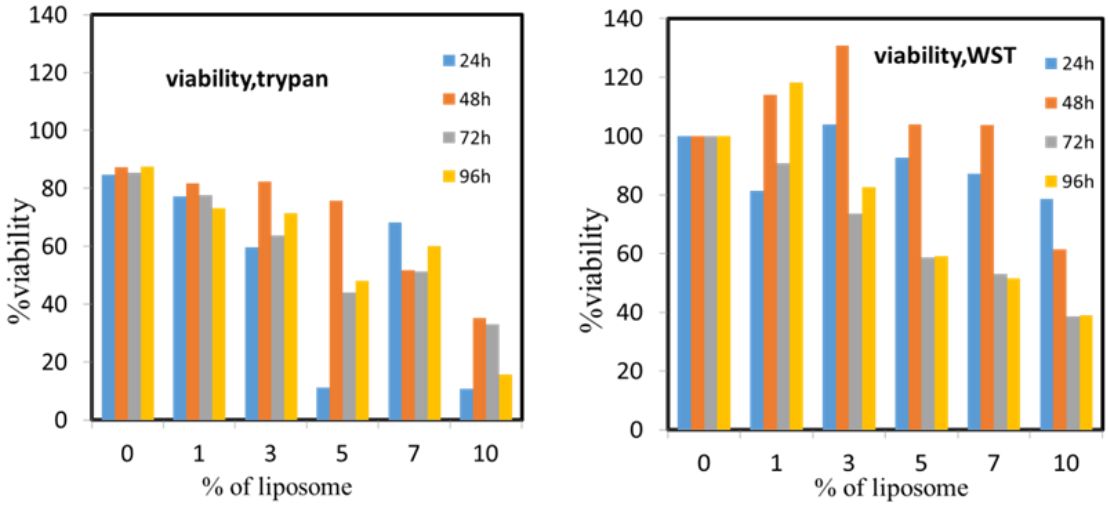


Figure 4.11. Viability percentages using different methods: Trypan Blue Staining and *WST-1*

Effective liposome concentrations and duration time are important parameter for visualizing tumor cell behaviors for good therapy. There were significantly decreasing in cell number for 7 and 10% alkali treatments after 4day and also, minimum cell viability results for 10% alkali treatment, but there were sharp increases in cell number for control-1-5% alkali treatments and also small increase in 7 and 10% treatment after 5day. Selection of tumor cell lines is also important variable for understanding nanoparticles mechanism. The mouse mammary carcinoma *4T1* which used in this study was isolated from BALB/cfC3H mice. It grows progressively and cause disease uniformly, and also good model for human metastatic breast cancer because of its characteristics [40]. For this reason, it is hard to get rid of all tumor cells and stop cell division of these cells.

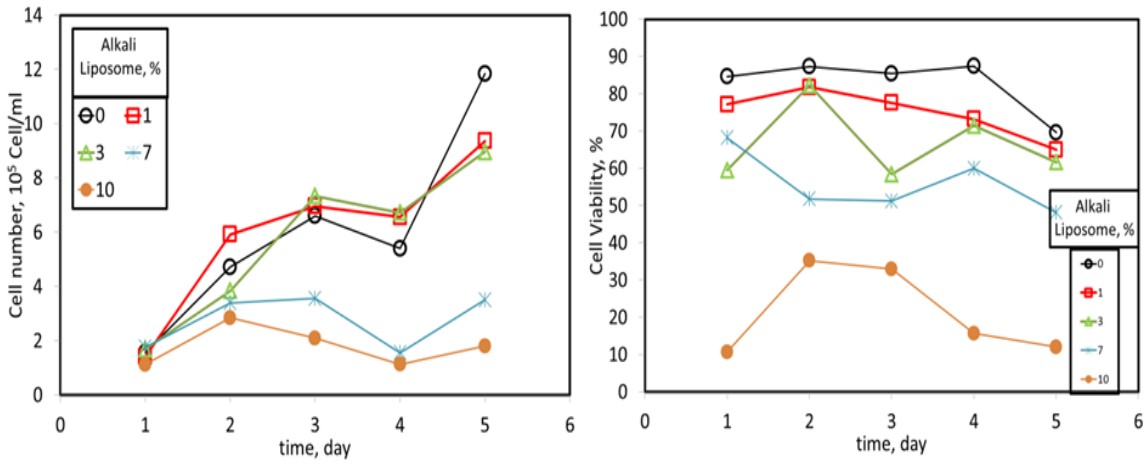


Figure 4.12. Cell number and viability results with trypan blue staining method

There is positive correlation between *WST-1* and Trypan method for cell viability as shown in Figures 4.12 and 4.13. There were decreasing cell viability according to increasing alkali liposome concentrations. Cell viability reached LD_{50} value means that the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test cells or animals. But, there were seen increasing viability results after 2day treatment. One of the reason is that cells tends to increase in number for adding new fresh medium, enough oxygen supply and 5%$C0_2$conditions. If suitable conditions are provided for cancer cells or healthy cells, they will easily proliferate and increase in number by maintaining cellular reactions. The other reason should be cellular uptake mechanism of alkali liposomes. 24 hours may be required for intake of alkali liposomes by fusion or endocytosis. There are lots of channels and transporters on cell membrane for getting any nutrients, ions, oxygen etc. according their surface charges, sizes, shapes. There are also several signaling pathways that effect cell cycle phases during any changing of intracellular conditions. In this study, internal alkalization was effected on cell cycle phases and different signaling pathway was activated for this conditions. The last reason can be cellular behavior of *4T1* cancer cells. Because they aren't easily effected any changes. They have also aggressive, highly tumorigenic and invasive characteristics.

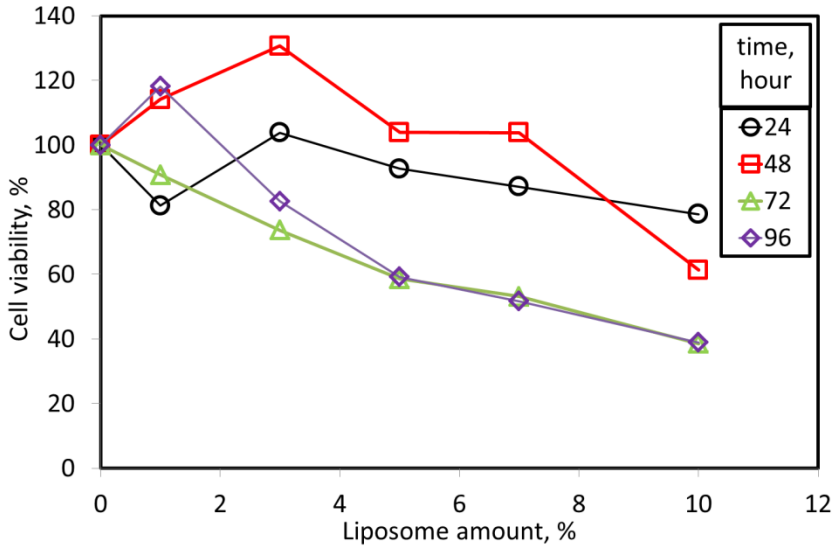


Figure 4.13. Cell viability percentages using *WST-1* method

CHAPTER 5

CONCLUSIONS

Liposomes are widely used in drug delivery systems because of their biocompatibility and biodegradability properties. In this study, PEGylated alkali liposome was prepared and used for decreasing the number of *4T1* cancer cells. Selection of preparation methods has also significantly importance, because their size, surface charge, shape, stability and loading efficiency are affected from type of preparation methods.

There are many chemotherapeutic drugs which are used in clinical development for drug delivery vehicle. For example, Doxorubicin is the most popular chemotherapeutic drugs. In clinical results show that only small amount of the drug (Dox) through the blood vessel can reach to the tumor site and most of the drug distributed to the whole body. Therefore, one important parameter of any drug is to increase the therapeutic index of the drug with minimum side effects. Unfortunately, most drugs don't have enough therapeutic property. This means that chemotherapeutic concentration is as same as the toxic one. In several studies are concerned about this issue and they want to reduce toxicity effects or increase the efficiency of suitable drug carrier. Because of this, there wasn't used any chemotherapeutic drugs during preparation of PEGylated alkali liposome in this study. Na_2CO_3 buffer was used for formation of the liposome, and this can be provided to encapsulate high basic ions inside of the liposome structure. In this way, when the liposome can be intake from the cells by fusion (simple adsorption) or endocytosis (by phagocytic cells of the reticuloendothelial system), metabolic pathway of the cells can be affected from changing pH_i of the cells.

Cell proliferation, survival and differentiation were controlled by several growth factors acting on cellular signaling cascades. pH_i changes effect also different potassium and calcium channels for importance of cell differentiation and maturation. In this study, pH_i can be changed by alkali liposomes and cell survival fraction was investigated during more internal alkali conditions. Approximately 200 nm alkali liposomes were produced and their size conservation was investigated through 50 days.

They conserved their contents and sizes for at least 48 days. When triton-x was added in liposomal solution, more basic ions were released into the solution by liposomal rupturing. Different ionic contents of liposomes were also produced by using ammonium sulfate and sodium carbonate buffers, and their ionic content differences were demonstrated with adding detergent. After preparing proper size and having basic ions content of liposomes (alkali liposomes), different amount of alkali liposomes and their ionic contents were investigated by continuous measurement set up with adding triton-x. High pH differences were appeared when rupturing high amount of alkali liposomes into the NaCl solution. Approximately 0.6-0.8unit pH changes were observed after adding 100 µl of alkali liposomes. These ranges were important for effecting intracellular cell metabolism and cell signaling pathways.

Alkali liposome influences were also investigated on *4T1* cancer cells with preparing different alkali liposome concentrations (0-1-3-5-7-10%) for 5day period. Fortunately, cancer cell viability decreased day by day according to increasing alkali liposome concentrations. Half of them were dead after 4day treatment and cell number was also decreased with increasing pH conditions as shown in Figure 4.12. Trypan blue and *WST-1* methods were used for getting cell viability results and getting microscope images. In addition, a key parameter of cancer therapy is also specific target of the cancer cells, because healthy cells can be affect from physiological pH_i changes when alkali liposome fused in the cells. The chemotherapeutic drugs used in the cancer therapy treatment also affect both cancer and healthy cells. The mechanism of targeted therapy based on the avoiding normal cells and going directly effect to the cancer cells. Targeted to tumor cells should be thoroughly investigated for both cases chemotherapeutic treatment and pH_i changing with alkali liposomes.

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