PREPARATION AND EVALUATION OF POLYMER BASED MICROCARRIERS FOR HYDROPHOBIC ANTI-CANCER DRUGS

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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 work.

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ABSTRACT

PREPARATION AND EVALUATION OF POLYMER BASED MICROCARRIERS FOR HYDROPHOBIC ANTI-CANCER DRUGS

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Chemotherapy is one of the most important treatments for cancer. However, systemic toxicity, drug resistance and unstable kinetics of the drug in the blood are serious problems of chemotherapy. The use of biodegradable polymers for controlled release of anticancer drugs has gained popularity in recent years. Controlled release of drugs from polymeric carriers has some advantages such as improvement in the efficiency of treatment, reduction in systemic toxicity and prevention of the drug resistance that is developed by the cancer cells.

In this study, poly(D,L-lactide-co-glycolide) microparticles were used as carriers for the controlled release of all-*trans*-Retinoic acid, tamoxifen, tamoxifen citrate and idarubicin. It was aimed to prepare a drug carrier system for controlled release of hydrophobic anticancer drugs.

The empty and drug loaded poly (D,L-lactide-co-glycolide) microparticles were prepared by solvent extraction/evaporation technique with single emulsion

(oil/water). Optimized microparticles were characterized by using inverted light microscopy and scanning electron microscopy to examine their morphology and sizes. Drug content of microparticles and the amount of released drug were determined spectrophotometrically. *In vitro* toxicity of the microparticles on MCF-7 human breast cancer cells was investigated.

It was revealed that the microparticles were smooth and spherical in shape. Their sizes differed in the range of 2-20 μm. atRA-loaded microparticles showed approximately 90% encapsulation efficiency and it was confirmed that changing in drug/polymer ratio affected the extend of drug content. Increase in drug content caused a slower release pattern. Moreover, although the empty microparticles caused some toxicity, atRA-loaded PLGA microparticles showed slight cell growth inhibition.

Key words: Controlled drug release, anticancer drugs, all-*trans*-Retinoic acid, poly(D,L-lactide-co-glycolide).

ÖZ

HİDROFOBİK ANTİKANSER İLAÇLAR İÇİN POLİMER TABANLI MİKROTAŞIYICILARIN HAZIRLANMASI VE İNCELENMESİ

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Birçok kanser türünde kullanılan en önemli tedavilerden biri kemoterapidir. Fakat, sistemik toksisite, ilaç dirençliliği ve kandaki değişken ilaç kinetiği kemoterapinin ciddi problemleridir. Biyoyıkımlı polimerlerin antikanser ilaçların kontrollü salımı için kullanılması son yıllarda önem kazanmıştır. Antikanser ilaçların polimer taşıyıcılar yardımıyla salımının, tedavinin etkisini artırması, sistemik toksisiteyi azaltması ve kanser hücreleri tarafından geliştirilen ilaç dirençliliğinin önlenmesi gibi özellikleri incelenmektedir.

Bu çalışmada all-*trans*-Retinoik asit, tamoksifen, tamoksifen sitrat ve idarubisin gibi suda az çözünen antikanser ilaçların kontrollü salımı için bir ilaç taşıyıcı sistemin geliştirilmesi planlanmıştır. Suda az çözünen antikanser ilaçların kontrollü salımı için ilaç taşıyıcı sistem olarak poli(laktid-ko-glikolid) (PLGA) mikroküreleri kullanılmıştır.

Bu çalışmada önce, boş ve ilaç yüklü poli(D,L-laktid-ko-glikolid) mikroküreler tek emülsiyonlu (yağ/su) çözücü ekstraksiyon/buharlaştırma metodu ile

hazırlanmıştır. Optimize edilen boş ve ilaç yüklenmiş mikroküreler karakterize edilmiştir. Mikroküre morfoloji ve büyüklüğünü incelemek için ters ışık mikroskobu ve taramalı elektron mikroskobu kullanılmıştır. Mikrokürelerdeki ilaç miktarı ve mikrokürelerden salınan ilaç miktarı spektrofotometrik olarak belirlenmiştir. Daha sonra, toksisite çalışmalarında mikrokürelerin MCF-7 meme kanseri hücreleri üzerindeki *in vitro* toksisitesi incelenmiştir.

Hazırlanan mikrokürelerin düzgün yüzeye ve küresel biçme sahip olduğu gözlenmiştir. Mikroküreler 2-20 µm aralığında değişen boyutlarda elde edilmiştir. atRA yüklü mikrokürelerde yaklaşık 90% oranında hapsolma etkinliği gözlenmiş ve ilaç/polimer oranının partiküllere yüklenen ilaç miktarını etkilediği doğrulanmıştır. Mikrokürelere yüklenen ilaç miktarındaki artışla, mikrokürelerden daha yavaş bir salım profili elde edilmektedir. Ayrıca, boş mikrokürelerin hücrelere karşı toksik etkisi olmasına rağmen atRA yüklü mikrokürelerde gözlenen toksik etkinin daha düşük olduğu görülmüştür.

Anahtar Sözcükler: Kontrollü ilaç salımı, antikanser ilaçlar, all-*trans*-Retinoik asit, poli(D,L-laktid-ko-glikolid).

To My Dear Family

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ABBREVIATIONS

DDS	Drug Delivery System
ESE	Emulsification Solvent Evaporation
PGA	Poly-glycolide
PLA	Poly-lactide
PLGA	Poly(lactide-co-glycolide)
PVA	Polyvinyl Alcohol
RA	Retinoic Acid
atRA	all-*trans*-Retinoic Acid
9cRA	9-*cis*-Retinoic Acid
13cRA	13-*cis*-Retinoic Acid
TMX	Tamoxifen
TMX-Cit	Tamoxifen Citrate
IDA	Idarubicin
5-FU	5-fluorouracil
SEM	Scanning Electron Microscopy
MP	Microparticle
O/W	Oil in Water

CHAPTER I

INTRODUCTION

Cancer occurs as a result of loss of some genetic regulations in the cell. The loss of control in cell division regulation may depend on factors such as the activity changes of enzymes which are the keys of most reactions in the cell, mutations in the genetic material, the blockage of activity of tumor suppressor genes and activation of oncogenes.

Due to the continuous investigation in this area, there are also many developments achieved. New cancer therapies, novel drugs or chemotherapeutic methods and finding new cell regulation mechanisms are some of them. However, there is no definite answer for cancer cure. Chemotherapy used in cancer treatment has serious side effects including the toxic effects of drugs on normal cells together with the tumorous ones. Sometimes the protection systems in cancerous cells result in pumping out all the drug molecules from the cancer cells. Therefore, the drug resistance in cancer cells causes a decrease in efficiency of chemotherapy.

Material scientists are trying to formulate new methods for the delivery of anticancer drugs to target tumor tissues. They look for new materials and ways to manipulate existing ones in order to fulfill needs in medical sciences. Current needs include reducing the toxicity of drugs, increasing their absorption, and improving their release profile [1]. For the delivery of anticancer drugs, polymeric biomaterials are being investigated for the last several years because of their controllable properties.

CHAPTER II

LITERATUR REVIEW

2.1. Biology of Cancer

Cancer is a genetic disease, arising from accumulation of certain mutations in cells. The fundamental abnormality in cancer cells are the uncontrolled and continual proliferation because cells lack the necessary regulations for growth and division, thus cancer can be defined as "the uncontrolled proliferation of cells" [2,3].

A tumor may be either benign or malignant. Benign tumor remains confined in its original location because the cells lack the capability to invade normal tissues. However, malignant tumors are capable of both invading surrounding tissue and spreading throughout the body via the circulatory or lymphatic systems. This is called metastasis. Metastasis describes the stage at which the cancer cell gains the ability to invade normal tissue, so that it can move away from the tissue of origin and establish a new colony elsewhere in the body [2,4,5].

In cancer cells, the cell-cell and cell-matrix interactions are less than normal cells. Reduced expression of cell surface adhesion molecules causes the cells become less adhesive. This loss of adhesiveness allows cancer cells to leave a tumor mass, migrate and invade other sites in the body [2,5].

Moreover, cancer cells secrete growth factors that promote the formation of new blood vessels around the tumor. This process is defined as angiogenesis. Angiogenesis, which supply the nutrient and oxygen to the proliferating cells causes the growth of tumor and metastasis due to high permeable walls of new blood vessels [2,6].

Another characteristic of most cancer cells is that they fail to differentiate normally. In most differentiating cells, proliferation ceases or divide only rarely [2]. Differentiation program of many cells includes programmed cell death (apoptosis) in the case of failure while cell differs from progenitor cell. If cells fail to enter the apoptotic pathway, the mutation/failure results in defective cells and may contribute substantially to tumor development.

Cancer cells are seemingly immortal because they continue to divide indefinitely. This growth potential is often attributed mostly to the presence of telomerase in cancer cells. Telomerase prevents the shortening of the chromosomes by replicating the single stranded ends of DNA (telomer) after each DNA replication. In normal cells, telomers at the end of linear chromosomes are shortened after each DNA replication due to absence of telomerase. Telomer shortening causes the aging of cells and after many division, telomeric repeat number decrease and cells die [5,6].

2.1.1. Causes of Cancer

A variety of agents increase the conversion frequency of cells (or animals) to the transformed condition. These agents can be chemical, physical or biological [3]. Carcinogens which are substances that cause cancer may include many chemical agents, radiation and viruses. They may cause epigenetic changes or may act, directly or indirectly, to change the genotype

of the cell. Carcinogens may be classified into two according to their ability to "initiate" or "promote" tumor formation, indicating different stages in cancer development [4].

Some other carcinogens contribute to cancer development by stimulating cell proliferation, rather than by inducing mutations. The phorbol esters and hormones are example of those proliferating agents. They are called "tumor promoters" [2].

Radiation and many chemical carcinogens damage DNA and induce mutations. Those are also called initiating agents since the initial event leading to cancer development is the induction of mutations in key target genes. For instance, the major cause of skin cancer is ultraviolet radiation from the sun, the potent liver carcinogen is aflatoxin and the major elements of lung cancer are the carcinogens in tobacco smoke such as benzo(*a*)pyrene, dimethylnitrosamine and nickel compounds [2,6].

In addition to initiating and promoting agents, some viruses also may induce cancer. There are many classes of tumor viruses, including both DNA and RNA viruses. At least five types of human cancers are accepted to be caused by viruses. For instance; lymphoma and anogenital cancers is associated with Epstein-Barr virus and Papilloma (wart) virus respectively and liver cancer may be initiated by Hepatitis B virus [2,4,7].

Mutations in DNA are preliminary causes of cancer. Oncogenes and tumor suppressors are two classes of genes in which mutations cause transformation of cells. The inactivation of tumor suppressors and the activation of oncogenes may lead to tumor initiation. Moreover, many cancers are associated with genetic instability which is revealed by increases in the frequency of genomic

changes. Chromosomal reorganizations or alterations involving deletions and duplication may result in changes the activity of genes [4].

2.1.2. Cancer Chemotherapy

Cancer is currently treated by surgery, chemotherapy and radiation. Several other strategies are being investigated such as immunotherapy, gene therapy and inhibition of angiogenesis [2,5].

Cancer chemotherapy is one of the most important and commonly applied therapy for the treatment of cancer. Surgery is often performed with the aim of curing cancer and, chemotherapy may be given to reduce the risk of the remission.

There are various types of drugs used in cancer chemotherapy according to type and stage of cancer. The available anticancer drugs have distinct modes of action on different types of normal and cancerous tissues.

The most commonly used anticancer agents aim to:

- Damage the DNA of the cancer cells (e.g. cisplatin, doxorubicin, daunorubicin, idarubicin).
- Inhibit the synthesis of new DNA strands to stop the cell from replicating (e.g. methotrexate, fluorouracil, mercaptopurine, tamoxifen).
- Stop mitosis so that the cell division and the progression come into a halt (e.g. vinblastine, vincristine, paclitaxel) [8].

Figure 1 describe a summary about mechanisms of action for some of the anticancer drugs.

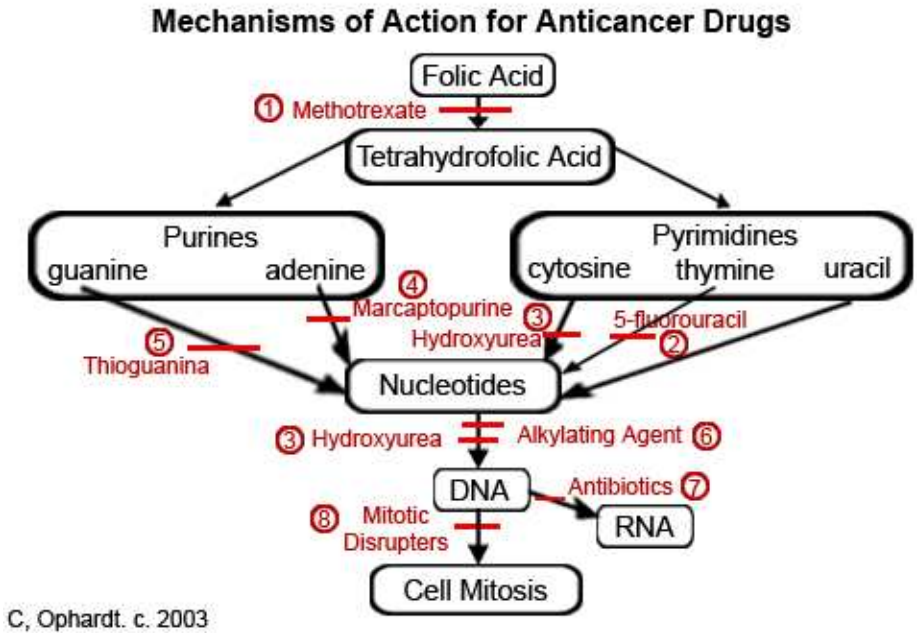


Figure 1. Mechanisms of action for some of the anticancer drugs [8].

The effectiveness of many anticancer drugs is limited by their toxicity to normal rapidly growing cells especially in the intestinal and bone marrow areas. Anticancer drugs are highly toxic compounds and they have no selectivity against cancer cells. Another problem with cancer chemotherapy is that cancerous cells which are initially suppressed by a specific drug may develop a resistance to the drug. For this reason cancer chemotherapy may consist of using several drugs in combination for varying lengths of time. Use of drug combination in chemotherapy enhances the efficacy of treatment.

In addition, chemotherapeutic drug kinetics are problematic. The key point with drug administration in traditional chemotherapy is that the blood level of the agent should remain between a maximum value over which drug may represent toxicity, and a minimum value below which the drug is no longer effective. When first dose is administered into the blood, drug concentration increases suddenly and exceed the toxic level. Then, it decreases slowly below the minimum effective concentration until second dose is administered [6,9]. Illustrated in Figure 2.

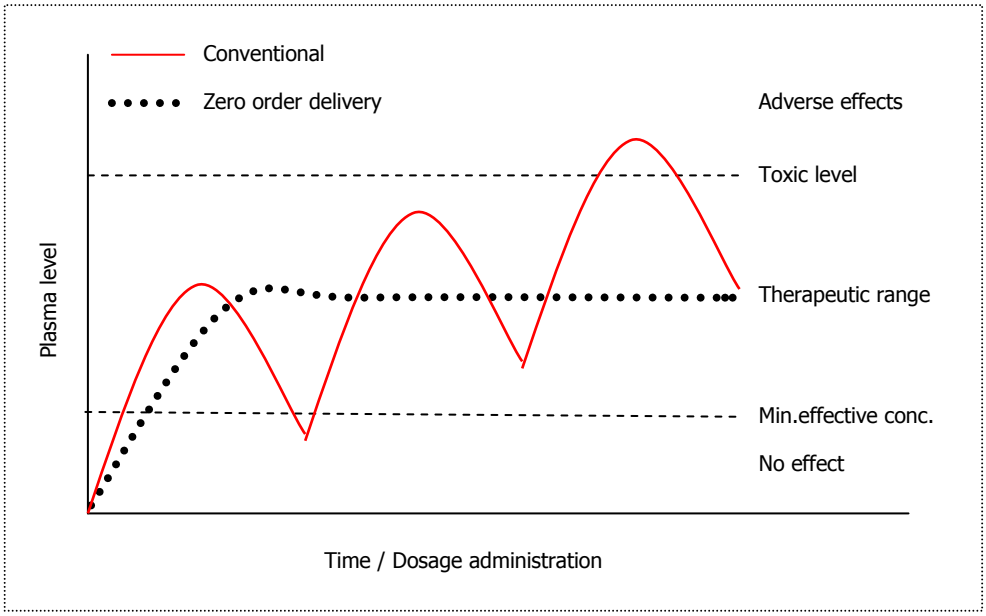


Figure 2. Conventional chemotherapy needs repeating drug administration so that the plasma level of the agent is in therapeutic range [10]

2.2. Controlled Drug Delivery

The term "drug delivery" is defined as placing the drug in a pellet of biodegradable material and sending it where the drug is needed. Actually,

drug delivery is an application of biochemical engineering in which the major goal is having ability to deliver therapeutic agents to a patient in a pulsatile or staggered release profile [11,12].

Controlled drug delivery makes possible the release of an agent in a predesigned manner from the material prepared by the combination of a synthetic or a natural polymer with the active agent [9].

Controlled delivery devices are generally diffusion-based release systems applicable for the release of drugs in systemic circulation or into a localized site. The controlled drug delivery applications include both sustained drug delivery over days, weeks, months, or years and targeted delivery on a one-time or sustained basis. Those applications are required when the continuous drug administration is detrimental and repeated dosing would be difficult or problematic [13,14].

The most important factor in drug delivery system preparation is providing control over the drug delivery. For example, water-soluble drugs should release slowly and low-soluble drugs should release faster due to the aqueous environment in the body. Also, drug delivery to specific sites, delivery of two or more agents with the same formulation, and the biocompatibility, degradability and readily elimination of carrier systems from the body are important parameters for the design and formulation of drug delivery systems [9].

The ideal drug delivery system should have several important properties. The main aim of using the drug delivery systems is to aid optimization of therapy by delivering the appropriate amount of the drug to the site where it is needed the most and at the time when it is most effective. Being inert, biocompatible and mechanically strong are important physical parameters. An

ideal system should also be comfortable for the patient, have high drug loading, be safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize [9,10].

2.2.1. Advantages and Disadvantages of Drug Delivery Systems

Controlled delivery systems offer so many advantages including improved efficacy and reduced toxicity of the drugs by capability of providing a lower and localized dose of drug to the tumor site, and improved patient compliance and convenience [15,16].

Improved efficacy can be shown as therapeutic range stability of the drug in blood as it is shown in Figure 3.

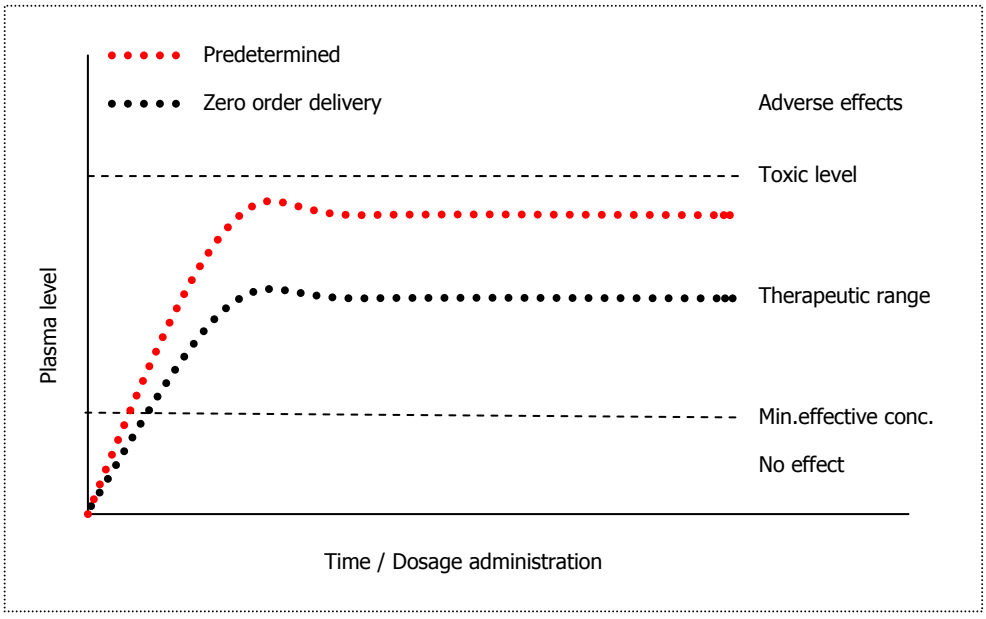


Figure 3. Drug delivery system delivers the active agent in a predetermined way in the therapeutic range and showing zero order release kinetics [10].

The optimum level of drug in blood circulation or in cell plasma should be around the therapeutic range and be stable for continuous effectivity. The lower limit shows the minimum effective drug concentration at which below there is no effect seen and the upper limit demonstrates that the toxic level to the cells. The controlled drug delivery devices can be designed so that they transfer and release the drugs between minimum and toxic level with zero order kinetics. This predetermined manner of drug delivery aids improvement in efficacy of the drug by making the concentration stable in the therapeutic range to obtain the maximum efficacy.

In conventional methods, since therapeutics may have poor solubility, especially the hydrophobic drugs may precipitate in aquous media. However, an effective drug delivery system provides both hydrophobic and hydrophilic environment enhancing the drug solubility [17].

Another problem in conventional methods is the rapid breakdown and elimination of the drug in vivo and loss of activity of the drug following administration. But, DDS protects drug from premature degradation and functions as sustained delivery system.

Moreover, in conventional methods, unfavorable pharmacokinetics may be seen, for instance, drug requiring high doses or continuous infusion is cleared too rapidly by the kidney. In the other side, DDS alter the pharmacokinetic of the drug and reduce clearance. By this way, rapid renal clearance of small molecules is avoided [18].

The poor biodistribution or widespread distribution of drugs in the body can affect normal tissues. However, drug delivery systems lower the volume of distribution and help to reduce side effects in sensitive, non-target tissues [19,20].

There are also potential disadvantages of controlled delivery systems. The possible toxicity or non-biocompatibility of the materials used, undesirable by-products of degradation and the higher cost of controlled-release systems compared with traditional pharmaceutical formulations are some of the points that should be mentioned [9].

The drug carriers in drug delivery systems may be in different forms. All have the same general advantages and disadvantages but also different benefits due to different properties they have. Table 1 summarizes the drug carriers used in drug delivery investigations.

Table 1. Some of the drug delivery systems used in investigations

Form	Formulation	Loading
Polymeric micro/ nanospheres	Different property-spheres can be formulated by using different polymers	Drug is either left inside the polymeric capsule (reservoir devices) or buried into polymeric sphere (matrix devices)
Liposomes	Constituted from phospholipids and cholesterol molecules.	Drug left inside the liposome spheres
Hydrogels	Gels-networks of polymer chains cross-linked together by either chemically or noncovalently	Drug gets trapped in the hydrogel during polymerization
Dendrimers	Created by branching of macromolecular polymers	Drug is arrested between branches or it forms bond with active groups of polymer

Carriers should meet the requirements for effective drug delivery, including water solubility, non-toxicity, non-immunogenicity, in vivo stability and selective delivery to the target site [21].

2.3. Polymers and Polymer Systems for Drug Delivery

Among the various carrier systems, polymeric drug delivery gained popularity in recent years. Polymeric systems help to alter and improve the pharmacokinetic and pharmacodynamic properties of various types of drug molecules in the body while minimizing side effects [22]. The carrier consists of a drug embedded in a polymer matrix or drug encapsulated within a polymeric bead. Polymer is used as a protector of the active agent during the transfer of drug in circulation through the body until it is released. The drug is released either directly into an affected site if the system is targeted or to the blood circulation by way of diffusion through or erosion of the polymer [3].

The polymeric biomaterials are used for targeted drug delivery and controlled release of anticancer drugs in tumor tissues since polymers can meet several requirements for those applications, such as biocompatibility, biodegradability, mechanical strength and ease of processing [23]. The bioavailability is also an important parameter for the practical use of many drugs. An efficient bioavailability of the drug should be obtained by the use of polymeric systems. Polymeric biomaterials help increasing the drug exposure of the tumor cell, keeping the concentration of drug constant in the cell, and also keeping the concentration at a desired level in the blood for solid tumors. Therefore, the slow-release formulations, especially in the form of microspheres have been utilized in place of conventional drug solutions to maintain the intratumoral drug concentration at therapeutic range [24].

As Henry et al. (2002) has pointed out, polymer carriers have several advantages over other delivery methods. They can be prepared as either degradable or non-degradable according to the properties of the polymer used. Since polymeric spheres or shells can be made up of polymers chemically diverse, they are useful for various types of tissue. Additionally, the linkage between monomers to get a polymeric structure can be designed to control where and when the drug is released. However, some polymers have a low drug-carrying capacity due to number of reactive groups on which the drug is attached [1].

Mohanraj and Chen (2006) stated that use of polymeric particles makes targeting easy by manipulating the particle size and surface characteristics. Also, they stated that the release and degradation characteristics can be readily modulated by the choice of matrix constituents and material properties. Moreover, polymeric particles control and sustain release of drug during transportation and alter organ distribution of the drug at the site of localization making subsequent clearance of the drug easier. Thus, polymer systems achieve increase in drug therapeutic efficacy and reduction in side effects [22,25].

2.3.1. Effectivity of Polymeric Systems and Drug Release

The biocompatibility and biodegredability of materials used in polymer synthesis are important characteristics of the biomaterials for their effectivity. Those are depending on some physical and mechanical properties of the polymer. The mechanical and degradation properties of polymer are affected by the combined effects of crystallinity, the molecular weight (M_w), the glass transition temperature (T_g) and the monomer hydrophobicity [13].

Moreover, the factors affecting drug release from polymeric particles are controllable. They are attributed to properties such as polymer molecular weight, as well as microparticle size and size distribution, loaded drug amount and porosity of the microparticles [26].

2.3.2. Poly(D,L-Lactide-co-Glycolide)

The delivery of agents by the use of polymeric materials has attracted considerable attention of researchers. The most widely investigated and advanced polymers having considerable toxicological and clinical data are the aliphatic polyesters based on lactic and glycolic acids.

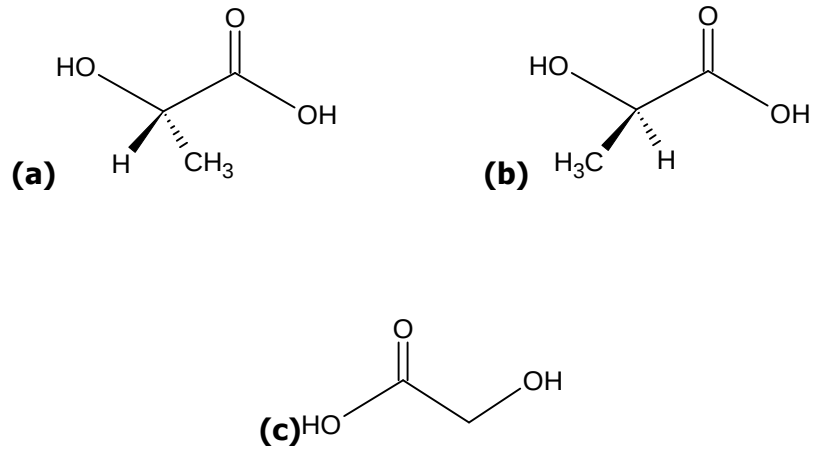


Figure 4. (a) L-lactic acid, (b) D-lactic acid and (c) glycolic acid

Lactic acid exists in two optically active forms. Due to the assymetrical β carbon of lactic acid, D and L stereoisomers exist. The D- and L- forms are optically active, the DL- or meso- form is optically inactive [27,28].

The homo- and copolymers of lactic and glycolic acids are synthesized by the ring-opening melt condensation of the cyclic dimers, lactide and glycolide. Ring opening polymerization of the lactide results in either D-, L- or racemic DL- polymer [27,28].

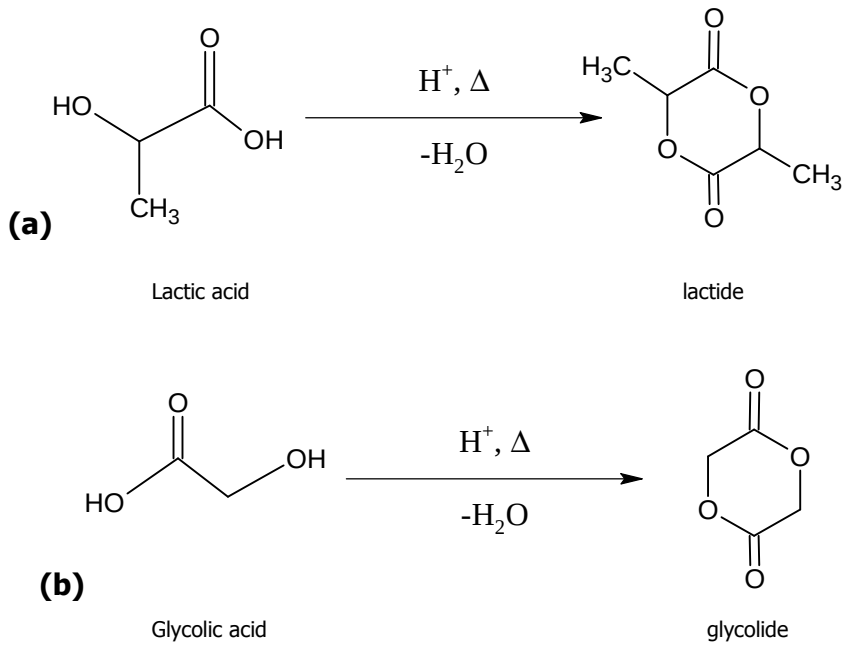
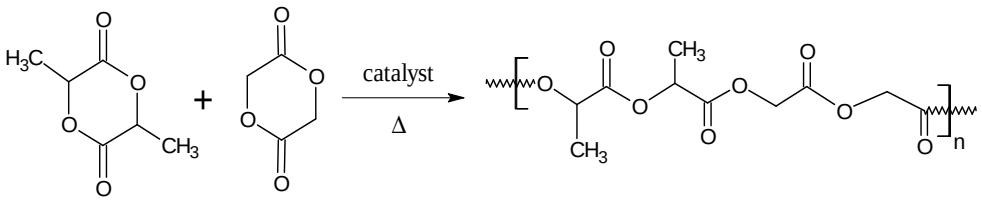


Figure 5. Formation reaction of (a) Lactide and (b) Glycolide

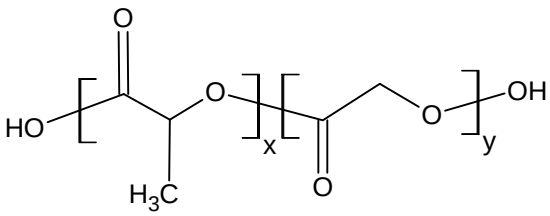
The polymerization is usually conducted over a period of 2-6 hr at about 175^{o}C. During the reaction, catalysts are normally used. The molecular weight of the polymer is controlled by addition of lauryl alcohol during sysnthesis [28].



where; n = number of repeating unit

Figure 6. Condensation reaction of poly(lactide-co-glycolide)

The two high purity L- and D-lactides form stereo regular polymers, D-PLA and L-PLA. These are semi crystalline polymers with a high melting point at about ~180°C and a glass transition temperature in the 55-60°C. However, the racemic poly(DL-lactide) DL-PLA is amorphous and has a lower melting. In addition, the lactic acid polymer is more hydrophobic than the glycolic acid polymer because of the methyl group in its structure [27,28].



where; x = number of units of lactic acid
y = number of units of glycolic acid

Figure 7. General structure of PLGA

2.4. The Role of Vitamin A

Vitamin A is necessary for normal growth, bone development, vision and reproduction and for the maintenance of the integrity of the skin and mucous membranes. A dietary deficiency in vitamin A, may result in night blindness, poor growth, dermatoses, athrophy of epithelial tissues and tissues of the eye and decreased resistance to infection [29,30,31].

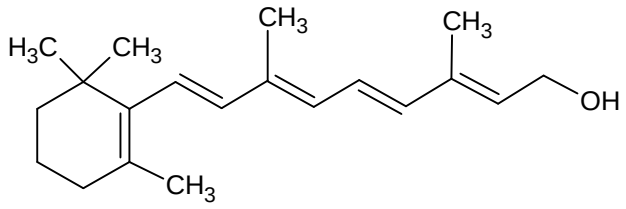
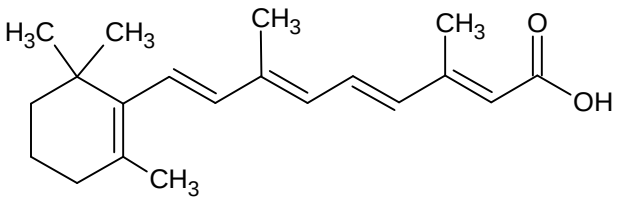
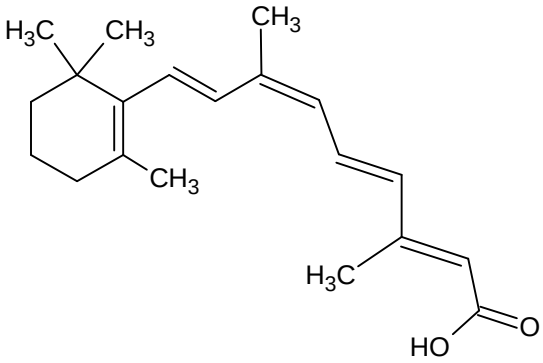
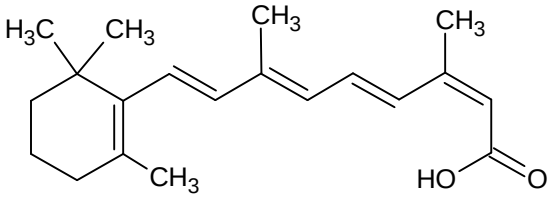


Figure 8. Structure of vitamin A

Vitamin A is one of the group of substances that have similar structures and biological activities of retinoids. Retinoids are natural derivatives of vitamin A and include all-*trans*-, 9-*cis*- and 13-*cis*-retinoic acids (atRA, 9cRA, 13cRA respectively) which are lipophilic. The retinoids regulate cell behaviour during development and play key roles in cell fate determination, cell division, and cell differentiation [32]. The study of Newman et al., 2004 also demonstrated the differentiation capability of retinoic acid [33]. Carotenoids such as β-carotene, sometimes called pro-vitamin A, are water-soluble precursors which are made into vitamin A by the body. Vitamin A and related carotenoid compounds have antioxidant and immune stimulatory properties, thus have a serious role in cancer prevention [34,35,36,37].

Table 2. Retinoic acid analogues

Name	Structure
all-*trans*-retinoic acid	
9-*cis*-retinoic acid	
13-*cis*-retinoic acid	

2.4.1. All-*trans*-Retinoic Acid in Cancer Treatment

Conventional medical cancer treatments, particularly radiotherapy and chemotherapy, are even toxic to normal cells in body because they are designed to kill cells without discrimination. Because the expectations of using DDS in cancer cure, researchers begin to investigate different formulations using various anticancer drugs. The antioxidant and immune stimulatory properties of the retinoids made them an attractive subject in cancer chemotherapy. It was reported after the retinol levels were investigated in the body of cancer patients that cancer incidence and recurrance ratio is higher when the plasma atRA concentration decreased [30,34,38, 37].

Retinoic acid has considerable importance in the prevention and treatment of many cancer types. The mechanisms of this anti-cancer activity, still being researched, and many studies are attributed to the anti-inflammatory and antioxidant properties of retinoic acid.

Especially, retinoids and related compounds such as all-*trans*-Retinoic acid may have therapeutic value in the treatment of human malignant gliomas as it is remarked in the article of Young-II et al. (2003) Because in the study of Rotan, (1991) and Bouterfa et al. (2000) showed that retinoids strongly inhibit proliferation and migration in primary cultures of human glioblastomas multiforme. Those studies suggest that atRA is an adequate chemotherapeutic agent to inhibit local invasion of human malignant gliomas [34].

Moreover, atRA is effective in the treatment of epithelial and hematological malignancies such as breast cancer, head and neck cancer, ovarian adenocarcinoma, and acute promyelocytic leukemia (APL). See-Hee Son et al. (2007) examined the effects of atRA on the proliferation of human ER^- SKBR-3 and ER^+ MCF-7 cell lines. They choose the cell lines having high levels of

retinoic acid receptor α (RARα) since the sensitivity of breast cancer cells to atRA correlates with RARα expression. They infected both SKBR-3 and MCF-7 cells with viruses and then treated those cells with atRA to determine whether atRA induced cell death. They showed that all-*trans*-Retinoic acid caused a significant degree of cell death in human estrogen-receptor negative SKBR-3 (ER^- SKBR-3) and estrogen-receptor positive MCF-7 (ER^+ MCF-7) breast cancer cell lines [34,38].

However, RA has some side effects such as hypertriglyceridemia, mucocutaneous dryness, and headache that limit the clinical applications of atRA. Moreover, prolonged RA treatment reduced plasma RA levels. This situation frequently causes RA resistance in patients with acute promyelocytic leukemia (APL). During carcinogenesis, a resistance to retinoids may develop in solid tumors [34,38].

To overcome the side effects and rapid decrease of atRA due to its short half-life, various kinds of formulation have to be developed. There were several studies done on atRA-loaded microspheres and liposomes, which overcame some problems sourced by atRA. For instance, Young-II Jeong et al. (2003) pointed the study of Giordano et al. (1993), in which they reported that atRA loaded microspheres were effective in reducing the incidence of tractional retinal detachment by a sustained release of atRA. Solid lipid nanoparticles are also reported as a useful formulation to solve the poor aqueous solubility of atRA and able to use it by intravenous injection [34].

2.4.2. Mechanism of Action of All-*trans*-Retinoic Acid

The anticancer effect of retinoids is resulted from the effect of inhibition of cell proliferation and promotion of cell differentiation. Differentiation is a

maturation process during which a determined cell becomes a recognizable, specialized cell. External stimuli, such as growth factors, trigger cells to differentiate. Once differentiated, these specialized cells are usually terminal and nondividing. Many studies have reported the effect of atRA, which primarily involves blocking the transition from the G1 to the S phase, on solid tumors. The presence of retinoid receptors and binding of retinoids to them influence gene expression and cell proliferation. atRA induces cell-cycle arrest in the G1 phase and cyclin D1 decreases by ubiquitination. atRA also influence apoptosis via its effect on nuclear receptors, retinoic acid receptors (RAR) -α, -β and -γ, and retinoid X receptors (RXR) -α, -β and -γ. [36,38,39,40,41].

2.5. Structure and Mechanism of Action of Tamoxifen

Tamoxifen is a synthetic non-steroidal triphenylethylene anti-estrogen compound which is widely used as an effective chemotherapeutic agent for treatment and prevention of breast cancer [42,43].

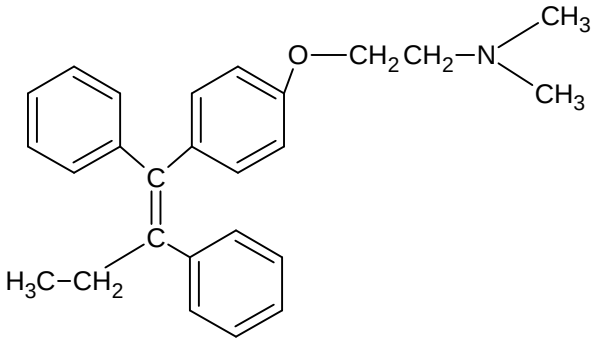


Figure 9. Structure of tamoxifen

Estrogen plays a crucial role in promoting growth of estrogen receptor (ER) positive breast cancer cells because removal of the ovaries in postmenopausal patients causes tumor regression [44]. The estrogen receptor functions to activate or repress the target gene transcription in response to binding of estrogen. The estrogen receptor regulates the proliferation and differentiation of many tissues, including reproductive tissues, and increased exposure to estrogens over time is an established risk factor for the development of breast cancer [42,43].

In most cases, there is no loss of ERα when resistance to endocrine therapy develops. As long as the ERα is present, transcription of estrogen-responsive genes (and tumor growth) may still be stimulated by small amounts of estrogens or anti-estrogens. Increased ER functionality has been found in breast cancer cells which become resistant to manipulations blocking estrogen action [44,45].

Tamoxifen has both estrogen antagonist and agonist activities. It acts as an antagonist in the breast. Binding of tamoxifen to ER blocks the mitogenic effect of estrogen on breast carcinoma through the inhibition of ER activity. However, it behaves as a agonist in the uterus causing endometrial carcinoma depending of the nature of receptors in certain tissues [6,44,46].

2.6. Structure and Mechanism of Action of Tamoxifen Citrate

Tamoxifen citrate is a synthetic non-steroidal triphenylethylene anti-estrogen compound and it has potential applications in breast cancer. It has a complex structure making it slightly soluble in water [47,48].

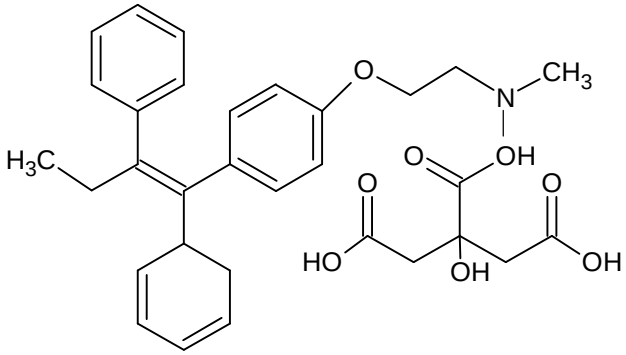


Figure 10. Structure of tamoxifen citrate

Tamoxifen citrate is a selective ER antagonist. It inhibits competitively estradiol binding to the high affinity estrogen receptor, thus inhibits the growth of tumor cells [48,49].

2.7. Structure and Mechanism of Action of Idarubicin

Idarubicin is a member of the class of natural molecules called anthracyclines, which were originally discovered from bacterial sources (*Streptomyces*). It is an analogue of daunorubicin and 5 to 6 times more potent and less cardiotoxic than daunorubicin.

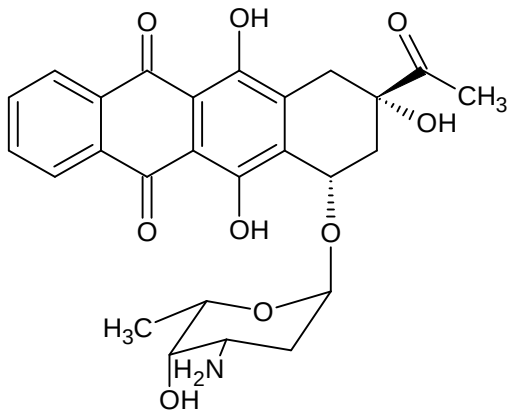


Figure 11. Structure of idarubicin

Cytotoxicity of the agent is generally attributed to intercalation of the drug into DNA or inhibition of DNA topoisomerase II activity resulting in double and single strand DNA breaks.

In addition, idarubicin has mutagenic and carcinogenic properties. The tissue necrosis may occur due to extravasation in days to weeks after the treatment [50].

2.8. Toxicity of Microparticulate Systems in Cell Culture

Different tumor cell cultures are widely used in *in vitro* toxicity studies of controlled drug delivery systems. Researchers try to optimize the formulation parameters of particulate systems according to the nature of different tumor cell types. Moreover, they test the behaviour of tumor cells upon addition of particulate systems including anticancer agents.

In a study of Kang et al. (2004), in vitro anti-tumor activity of paclitaxel, a widely used anticancer drug, released from microemulsions with or without PLGA was investigated on human breast cancer cell line MCF-7. They reported that paclitaxel released from microemulsion containing PLGA continuously and the cytotoxicity against the MCF-7 cell line was affected significantly by the released amount of paclitaxel. Thus, microemulsion containing PLGA enhances its anti-tumor activity as compared to microemulsion without PLGA. They suggested that these result strongly prove that the formulation they applied could be used for clinical trials [51].

Jeong et al. (2003) studied the effect of sterilization process of atRA encapsulated PLGA microspheres on cell growth by using MTT cell proliferation assay. They used U87MG, malignant glioma cell line for the cell viability tests. As a control, they also looked at the cell growth inhibition when free atRA and empty PLGA microparticles were introduced to the cells. They concluded that neither cell growth inhibition properties of atRA nor the drug and microsphere stability was changed by polymer and the sterilization process [34].

Another study by Fonseca et al. (2002) examined the in vitro anti-tumoral activity of paclitaxel incorporated in the PLGA nanoparticles using a human small cell lung cancer cell line (NCI-H69). The results demonstrated that both

the incubation time and and concentration play a major role in the *in vitro* cytotoxicity of paclitaxel. Cell toxicity was higher for longer periods of incubation with the drug since larger number of cells enter the G2 and M cell cycle phases during which paclitaxel is more active. Incubation of the cells with only paclitaxel contributed to 70% reduction in cell viability whereas paclitaxel-loaded nanoparticles allowed a cytotoxic effect of almost 100%. Therefore, they explained the enhancement of paclitaxel activity mediated by its incorporation into nanoparticles by those systems acting as a reservoir for drug protects the drug from epimerization and hydrolysis. Thus, they provided not only a sustained release of drug but also contributed to the maintenance of its activity [52].

Gupte and Çiftçi (2004) studied the cytotoxic activity of PLGA microspheres containing paclitaxel, 5-FU + paclitaxel or 5-FU alone. They used metastatic breast cancer cell line MDA-MB435 S. They concluded that cell viability depends on the concentration of drug. They observed a rapid reduction of cell viability when incubated with a high concentration of free paclitaxel and free 5-FU whereas a lesser reduction in cell viability was observed with a lower concentration of free drugs. On the other hand, microspheres containing paclitaxel or 5-FU alone resulted in the reduction of cell viability less than it was in the case of free drugs. While the combination microspheres resulted in the least reduction in cell viability compared to others because paclitaxel and 5-FU have antagonictic relationship due to the G1-S arresting agent such as 5-FU prevents the majority of cells from progressing to the G2-M phase of the cell cycle where the anti-mitotic agents such as paclitaxel exerts their greatest cytotoxic effect. Therefore, Gupte and Çiftçi (2004) concluded that it is important to consider carefully when combining anti-neoplastic drugs that exert their cytotoxic action at different phases of cell cycle [53].

2.9. Objectives of the Study

In this study it was aimed to prepare and evaluate the anticancer drug carrying system using biodegradable copolymer poly(D,L-lactide-co-glycolide). For this approach, followings were explored;

1. To prepare polymer microparticles as drug carrying matrices;
    a. Chemotherapeutic hydrophobic drugs, atRA, tamoxifen, tamoxifen citrate and idarubicin were loaded to the polymeric system.

    b. Poly(D,L-lactide-co-glycolide) microparticles containing drug were studied with various formulation parameters such as drug amount and stirring rate. Prepared microparticles were characterized in terms of morphology and encapsulation efficiency.

2. Release studies were done for the evaluation of drug release rates and to achieve the sustained release of hydrophobic drugs incorporated to the biodegradable copolymer.

3. Cytotoxicity tests were done on MCF-7 Human breast cancer cell line for the investigation of drug and polymer toxicity by using sterilized empty and all-*trans*-Retinoic acid-loaded microparticles.

Anticancer drug encapsulated biodegradable microparticles are considered to be a useful approach in increasing the drug efficacy and reduce the side effects. Thus, more effective therapies can be achieved in further *in vivo* studies [3,34].

CHAPTER III

EXPERIMENTAL

3.1. Materials

3.1.1. Materials Used for Drug Carrying PLGA Microparticle System

Important chemicals used for the preparation and characterization of microparticles and drug release are listed in Table 3.

Table 3. List of chemicals for microparticle preparation and release studies

Chemicals	Properties
Poly(D,L-Lactide-co-Glycolide) (PLGA)	(75:25, MW: 66,000-107,000), Sigma Chemicals
All-*trans*-Retinoic acid (atRA)	min 98% HPLC, Sigma Chemicals
Tamoxifen	Sigma Chemicals
Tamoxifen citrate	TEVA 10 mg, film kaplı tablet
Idarubicin.hydrochloride	Pharmacia (Zavedos® 10 mg)
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich
Polyvinyl alcohol (PVA)	MW: 30.000-70.000 Sigma-Aldrich
Dichloromethane (DCM)	Riedel-de Haën
Chloroform	Lab-Scan Analytical Sciences
Methanol	Merck

3.1.2. Materials Used for Cell Culture Studies

XTT based colorimetric assay kit, 0,25% Trypsin EDTA and Tryphan blue were purchased from Biological Industries, Israel.

RPMI 1640 medium (1X), 2 g/l $NaHCO_3$ w stable glutamine was purchased from Biochrom AG, Deutchland.

MCF-7 Human breast carcinoma cell line was obtained from Şap Enstitüsü, Ankara.

Phosphate buffered saline tablets (PBS) were purchased from Sigma-Aldrich, USA.

3.2. Experimental Methods

3.2.1. Spontaneous Emulsification Solvent Evaporation (ESE) Method

In the course of preparation of microparticles, the spontaneous emulsification solvent evaporation (ESE) method was used. ESE is mainly a two-step process: the emulsification of a polymer solution containing the encapsulated substance, followed by particle hardening through solvent evaporation and polymer precipitation [54,55,56].

The polymer is dissolved in a suitable water-immiscible, volatile solvent, and the bioactive molecule is dispersed in or dissolved in this polymeric solution. The resultant solution or dispersion is then emulsified in an aqueous continuous phase that contains a surface-active agent such as poly(vinyl alcohol) (PVA) to give an oil in water (o/w) emulsion and form discrete droplets [57,58,59,60]. During this emulsification process, polymer solution is broken up in microdroplets by the shear stress produced either by homogenizer, sonicator or whirl mixer in the presence of a surface active agent. This first step mainly determines the microparticle size distribution [54].

In order for the microparticles to form, the organic solvent must first diffuse into the aquous phase and then evaporate at the water/air interface so that the polymer forms hardened microparticles. As solvent evaporation occurs, the microparticles harden and free flowing microparticles can be obtained after suitable filtration and drying [57,59,61].

Particle formation mechanism is crucial for size distribution and morphology, which in turn determines the delivery system behaviour during encapsulation and release.

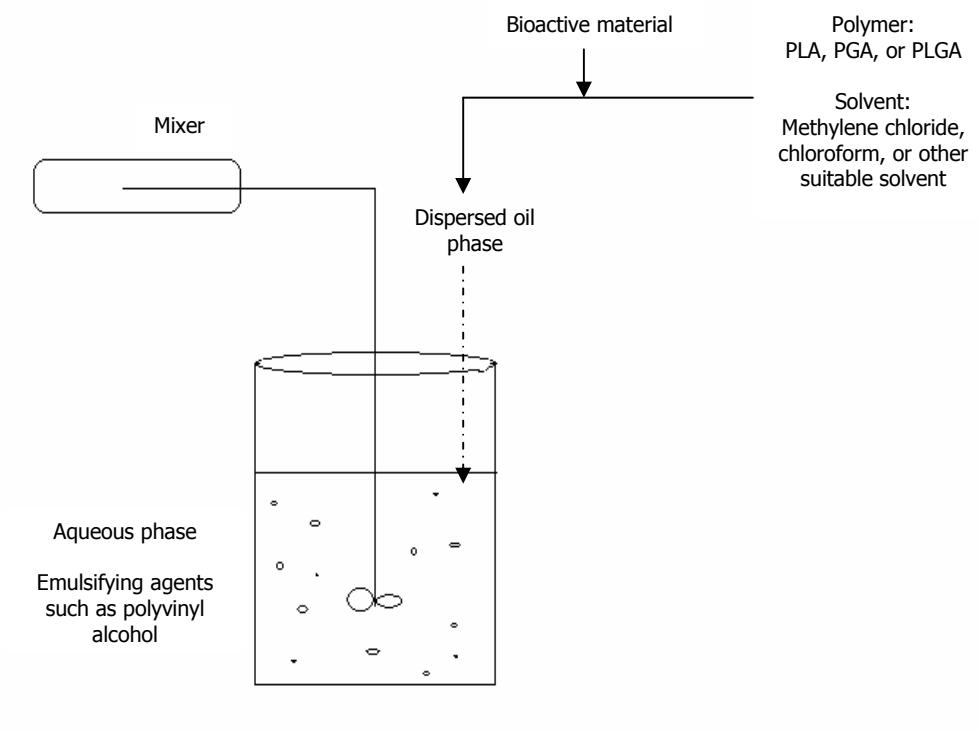


Figure 12. Schematic diagram of the ESE method [57]

3.2.2. Preparation of PLGA Microparticles

3.2.2.1. Preparation of Empty PLGA Microparticles

Empty PLGA microparticles were prepared by using spontaneous emulsification solvent evaporation method. This method was used with single emulsion (oil in water, o/w) at room temperature, and microparticles were prepared non-aseptically.

Firstly, certain amount of PLGA (50 mg) was dissolved in dichloromethane (4 ml), organic solvent. This solution was added into 50 ml of freshly prepared polyvinyl alcohol (PVA) solution (2% w/v) with a dropwise fashion by the help of a syringe while mechanically stirring at 800 rpm. Then, the mixture was mechanically stirred at 800 rpm for 2 hours for homogenous distribution of microparticles. The resulted oil in water emulsion was further stirred overnight at 400 rpm with a magnetic stirrer (Velp Scientifica, Italy) to completely evaporate the organic solvent and hardening of microparticles. Microparticles were obtained by centrifugation at 9000 rpm (Sigma Centrifuge 3K30, Germany) for 15 min at 10^{o}C. Then, they were washed with 10 ml distilled water twice and freeze dried (HETO Maxi Dry Lyo).

3.2.2.2. Preparation of Drug Loaded PLGA Microparticles

Drug loaded poly(D,L-lactide-co-glycolide) microparticles were prepared for controlled release of one of these hydrophobic anticancer drugs: all-*trans*-Retinoic acid, tamoxifen, tamoxifen citrate and idarubicin. Drug loaded PLGA microparticles were prepared by using spontaneous emulsification solvent evaporation method. Single emulsification process was progressed (oil in water, o/w) at room temperature, and microparticles were prepared non-aseptically.

3.2.2.2.1. Preparation of All-*trans*-Retinoic Acid-Loaded Microparticles

PLGA (25 mg) was dissolved in 2 ml dichloromethane. All-*trans*-Retinoic acid was added in changing amounts (1 mg / 1,5 mg / 2 mg / 3 mg) for different batches. The solution was vortexed (Nüve-NM 1101) until it became

homogenous. This solution was added into 25 ml of freshly prepared polyvinyl alcohol (PVA) solution (2% w/v) with a dropwise fashion by the help of a syringe while mechanically stirring at 800 rpm. Then, the mixture was mechanically stirred at 800 rpm for 2 hours for homogenous distribution of microparticles. The resulted oil in water emulsion was further stirred overnight at 400 rpm with a magnetic stirrer to completely evaporate the organic solvent and hardening of microparticles. Microparticles were obtained by centrifugation at 9000 rpm for 15 min at 10^{o}C. Then, they were washed with 10 ml distilled water twice and freeze dried.

3.2.2.2.2. Preparation of Tamoxifen-Loaded and Idarubicin-Loaded Microparticles

PLGA (25 mg) was dissolved in 2 ml dichloromethane. Tamoxifien/idarubicin was added (1 mg) to the solution. The solution was vortexed until it became homogenous. This solution was added into 25 ml of freshly prepared polyvinyl alcohol (PVA) solution (2% w/v) with a dropwise fashion by the help of a syringe while mechanically stirring at 800 rpm. Then, the mixture was mechanically stirred at 800 rpm for 2 hours for homogenous distribution of microparticles. The resulted oil in water emulsion was further stirred overnight at 400 rpm with a magnetic stirrer to completely evaporate the organic solvent and hardening of microparticles. Microparticles were obtained by centrifugation at 9000 rpm for 15 min at 10^{o}C. Then, they were washed with 10 ml distilled water twice and freeze dried.

3.2.2.2.3. Preparation of Tamoxifen Citrate-Loaded Microparticles

PLGA (25 mg) was dissolved in 5 ml dichloromethane:methanol (1:1) mixture. Tamoxifen citrate was added in changing amounts (1 mg or 2 mg) for different batches. The solution was vortexed until it became homogenous. It was added into 50 ml of freshly prepared polyvinyl alcohol (PVA) solution (2% w/v) with a dropwise fashion by the help of a syringe while mechanically stirring at 800 rpm. Then, the mixture was mechanically stirred at 800 rpm for 2 hours for homogenous distribution of microparticles. The resulted oil in water emulsion was further stirred overnight at 400 rpm with a magnetic stirrer to completely evaporate the organic solvent and hardening of microparticles. Microparticles were obtained by centrifugation at 9000 rpm for 15 min at 10^{o}C. Then, they were washed with 10 ml distilled water twice and freeze dried.

Table 4. PLGA microparticles prepared by changing parameters

Sample PLGA microparticle	Concentration of Polymer (w/v)	Stabilizator amount (%)	Drug used	Drug / Polymer ratio	Stirring rate (rpm)
MP0	12.5	2	-	-	400
MP1	12.5	2	-	-	800
MP2	12.5	2	atRA	0.04	800
MP3	12.5	2	atRA	0.06	800
MP4	12.5	2	atRA	0.08	800
MP5	12.5	2	atRA	0.12	800
MP6	12.5	2	TMX	0.04	800
MP7	12.5	2	TMX-cit	0.04	800
MP8	12.5	2	TMX-cit	0.08	800
MP9	12.5	2	IDA	0.04	800

3.2.4. Characterization of Microparticles

3.2.4.1. Morphological Analysis of Microparticles

The morphological investigation of microparticles were done by inverted light microscopy (Olympus CX41RF) and scanning electron microscopy (LEO 438 Vp).

The morphology analysis shows the size, shape and surface properties of microparticles.

Scanning electron microscopy (SEM) micrographs were obtained after coating with gold under vacuum (Au-Pd coating Emitor K550X).

3.2.4.2. Particle Size Analysis of Microparticles

The average size and size distribution curves of empty and all-*trans*-Retinoic acid-loaded microparticles were determined by using a particle size analyzer (MALVERN Mastersizer 2000).

3.2.5. Drug Encapsulation Efficiency

Drug encapsulation efficiencies of the prepared microparticles was measured by UV spectrophotometer (Shimadzu, UV-1208) at λ = 365 nm for all-*trans*-Retinoic acid, at λ = 274 nm for tamoxifen, at λ = 254 nm for tamoxifen citrate and at λ = 486 nm for idarubicin.

Certain amount of freeze-dried sample was dissolved in dichloromethane (DCM) and vortexed until the solution become clear. The solution was then analyzed by spectrophotometer at specified wavelength to see the amount of drug loaded in the microparticles. Empty PLGA microparticles were used as blank.

Drug encapsulation efficiencies were calculated by using the following formula:

$$D.E.E = \frac{amount\ of\ drug\ loaded}{amount\ of\ drug\ used\ initially} x100 \qquad \text{(Eqn.1)}$$

where; D.E.E. = Drug Encapsulation Efficiency

Moreover, the supernatant and wash solutions were run at spectrophotometer to confirm the drug encapsulation efficiencies calculated by using loaded drug amount.

The measurements were done after extraction with dichloromethane (DCM). All measurements were performed in duplicate.

This time;

$$D.E.E = \frac{amount\ of\ drug\ used\ initially - amount\ of\ drug\ measured}{amount\ of\ drug\ used\ initially} x100 \quad \text{(Eqn2)}$$

A calibration curve of standard retinoic acid solution was used to obtain retinoic acid concentration, which was linear over studied range with a correlation coefficient of $R^2 = 0.9906$.

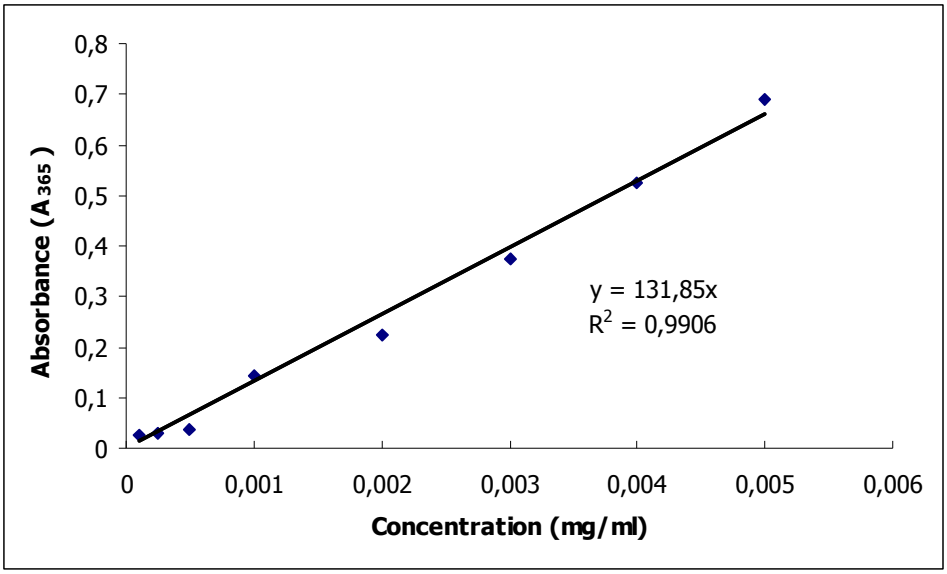


Figure 13. Absorbance readings in a spectrophotometer (Shimadzu, UV-1208) at different concentrations of atRA. atRA was dissolved in dichloromethane.

A calibration curve of standard tamoxifen solution was used to obtain tamoxifen concentration, which was linear over studied range with a correlation coefficient of $R^2 = 0.9769$.

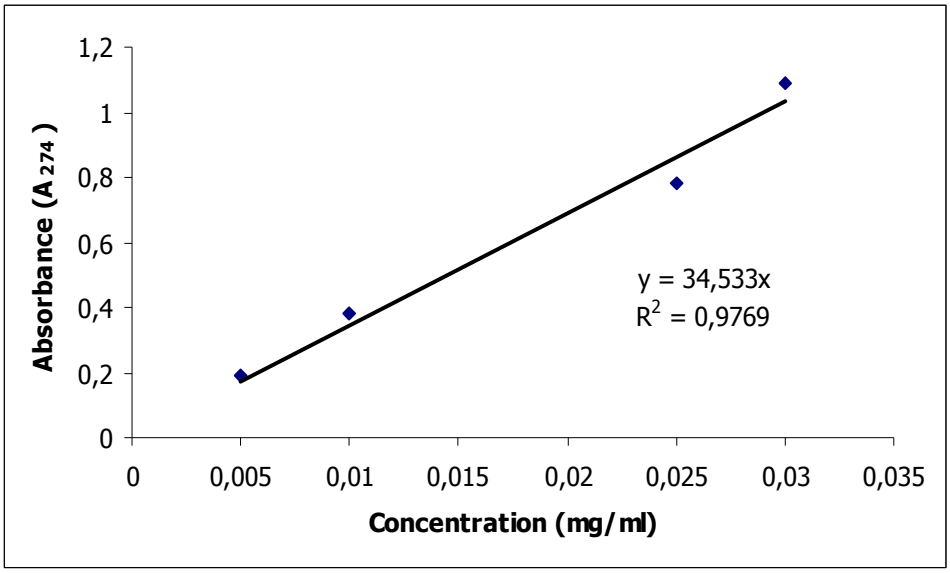


Figure 14. Absorbance (λ = 274) versus concentration curve for tamoxifen. Tamoxifen was dissolved in dichloromethane and analyzed by Shimadzu, UV-1208 spectrophotometer.

A calibration curve of standard tamoxifen citrate solution was used to obtain tamoxifen citrate concentration, which was linear over studied range with a correlation coefficient of $R^2 = 0.9929$.

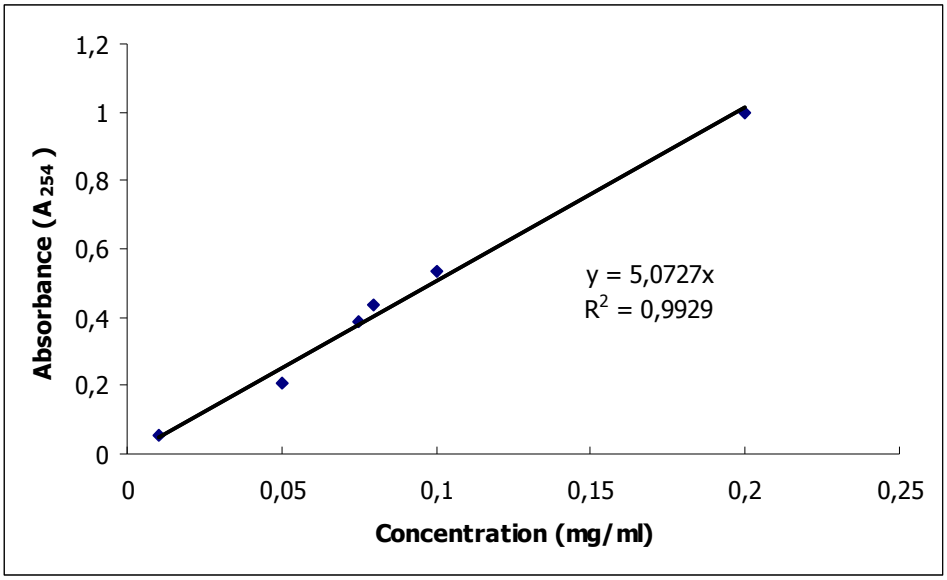


Figure 15. Absorbance (λ = 254) versus concentration curve for tamoxifen citrate. Tamoxifen citrate was dissolved in dichloromethane and analyzed by Shimadzu, UV-1208 spectrophotometer.

A calibration curve of standard idarubicin solution was used to obtain idarubicin concentration, which was linear over studied range with a correlation coefficient of $R^2 = 0.994$.

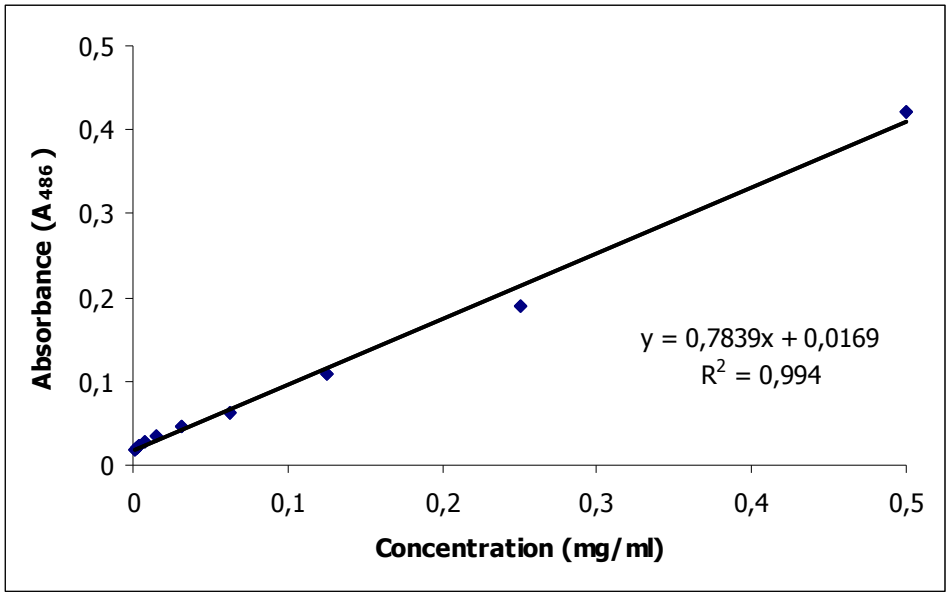


Figure 16. Absorbance (λ = 486) versus concentration curve for idarubicin. Idarubicin was dissolved in dichloromethane and analyzed by Shimadzu, UV-1208 spectrophotometer.

3.2.6. Drug Release

In vitro release of drugs, all-*trans*-Retinoic acid, tamoxifen and tamoxifen citrate, were performed in phosphate buffered saline (PBS, 0.01 M, pH 7.4) solution at 37°C.

In drug release studies, MP2, MP4, MP5, MP6, MP7 and MP8 batches (Table 4) were studied to investigate the release profiles of microparticles prepared by using different drug concentrations.

Certain amount of drug-loaded poly(D,L-lactide-co-glycolide) microparticles were dispersed in 10 ml of freshly prepared phosphate buffered saline solution. They were placed in a shaker (New Brunswick Scientific-G24) at 100 rpm and 37 ^{o}C for horizontal shaking. At specific time points, the samples were taken and centrifuged at 2500 rpm for 10 minutes. Supernatant was collected and was extracted with same amount of DCM. Analysis were done at λ = 365 nm for atRA, at λ = 274 nm for tamoxifen and at λ = 254 nm for tamoxifen citrate by UV spectrophotometer (Shimadzu, UV-1208). DCM was used as blank. The experiments were performed in duplicate.

Microparticles in pellet were resuspended in 10 ml freshly prepared PBS solution and placed in shaker at 100 rpm and 37 ^{o}C.

Calibration curves given in Section 3.2.5 at pages 37-40 were used to calculate the amount of drugs released from the microparticles.

The extraction efficiency was calculated to find the recovery factor as the drug was extracted from aquous phase to organic phase. Certain amount of standard drug was dispersed in PBS and extracted with same amount of DCM. The resultant value was used as a correction factor in calculations.

3.2.7. *In Vitro* Cytotoxicity Tests

Cytotoxicity of empty and all-*trans*-Retinoic acid-loaded microparticles were determined by inhibition of cell growth. MCF-7 Human breast carcinoma cell line was obtained from Şap Enstitüsü, Ankara

Firstly, flat-bottomed 96 well plates were loaded with 100 µl of RPMI 1640 medium except the control wells (Each control well was loaded with 150 µl of medium). Aseptically prepared microparticles were dispersed in PBS solution at maximum concentration of 200 µM. 100 µl of microparticle solution was placed into the first well and serially diluted to obtain different concentrations of microparticle solutions.

Following these, the experimental cells were counted by light microscope using trypan blue exclusion method [62]. Then, 50 µl of cells were seeded at a concentration of 0.1 x 10^6 cells/ml in 96-well plate. They were incubated with twelve different concentrations of empty and all-*trans*-Retinoic acid-loaded microparticles in triplicate for 96 hours in a CO_2 incubator (Heraeus Hera Cell) at 37^{o}C. The cell viability was determined by XTT cytotoxicity assay.

XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, is a yellow tetrazolium salt which active cells reduce to orange colored compounds of formazan. The dye formed is water soluble and the dye intensity, from which viable cell number can be determined, can be read at a given wavelength with a spectrophotometer.

XTT reagent was added to each well after incubation of cells with empty and drug-loaded microparticles at 37^{o}C for 96 hours. The plates were incubated further 3 hours in CO_2 incubator. Following, the absorbance of the samples

were measured with ELISA reader (SPECTRAmax 340PC) at a wavelength of 500 nm. All experiments were performed in triplicates.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Preparation and Characterization of PLGA Microparticles

Polymer microparticles were prepared by applying o/w single emulsion technique as described in Section 3.2.2. As stabilizator, PVA was used. Various formulation parameters (Table 4) were examined. Loaded microparticles were prepared at various drug concentrations.

4.1.1. Morphological Analysis of Microparticles

4.1.1.2. Inverted Light Microscopy Analysis

The prepared microparticles were examined by inverted light microscopy. Observation of the samples revealed the shape and gave information about the size of microparticles. Moreover, microscopy made possible to observe the effect of stirring rate.

4.1.1.2.1. Empty Microparticles

In the microscopic observation, it was seen that empty microparticles were spherical in shape. The ones prepared at a stirring rate of 800 rpm were

smaller than the microparticles prepared at a stirring rate of 400 rpm as shown in Figure 17 and Figure 18.

The stirring rate is an important parameter for the size of prepared microparticles. By changing the stirring rate during preparation it was possible to prepare big or small-sized particles. The effect of stirring rate on microparticles was examined on empty PLGA microparticles and observed by inverted light microscopy (Figure 17 and 18).

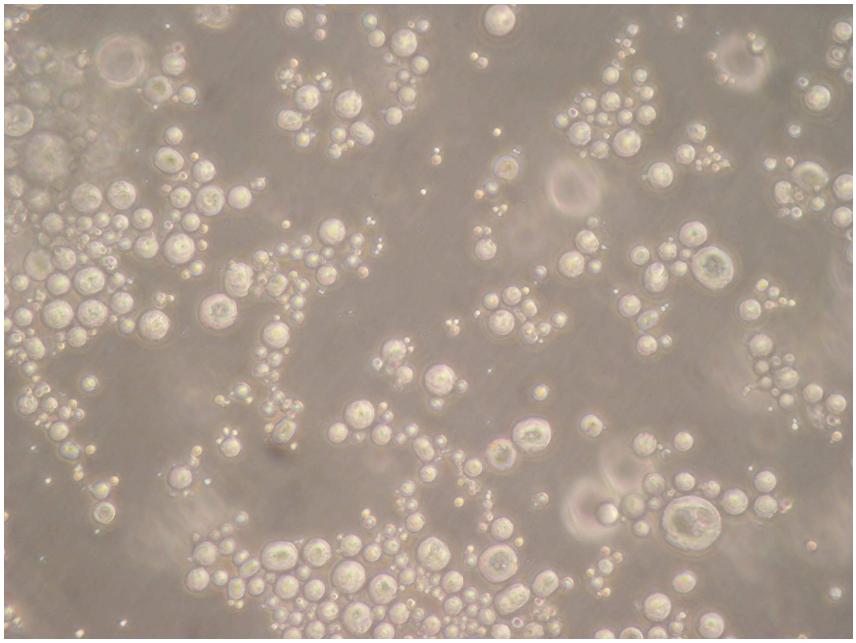


Figure 17. Empty MPs prepared at 400 rpm – MP0 (Table 4) (400X)

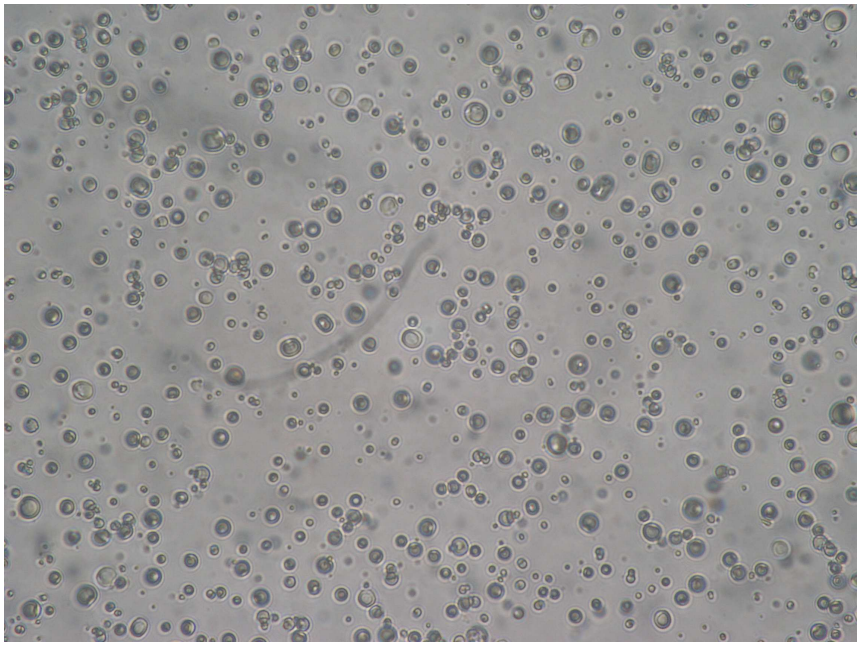


Figure 18. Empty MPs prepared at 800 rpm – MP1 (Table 4) (400X)

When two results were compared (Figure 17 and Figure 18), it was concluded that the microparticles prepared at a stirring rate of 800 rpm was smaller and the size distribution of the microparticles was more uniform. Therefore, the stirring rate was optimized at 800 rpm so that smaller and more uniform microparticles can be obtained. Therefore the following studies were proceeded with microparticles prepared at 800 rpm.

4.1.1.2.2. Drug-Loaded Microparticles

The samples loaded with all-*trans*-Retinoic acid, tamoxifen, tamoxifen citrate and idarubicin were also examined with inverted light microscopy. Figure 19, 20, 21, Figure 22, Figure 23, 24 and Figure 25 which belong to mentioned drugs above respectively confirmed the microparticle formation by spontaneous emulsification solvent evaporation (ESE) method.

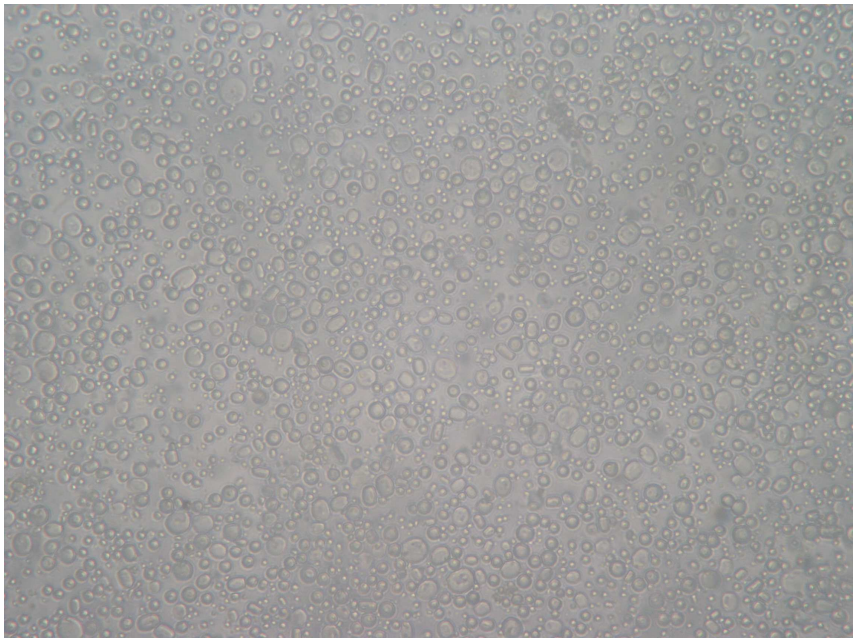


Figure 19. atRA-loaded MPs with a drug/polymer ratio 0.04 prepared at room temperature – MP2 (Table 4) (400X)

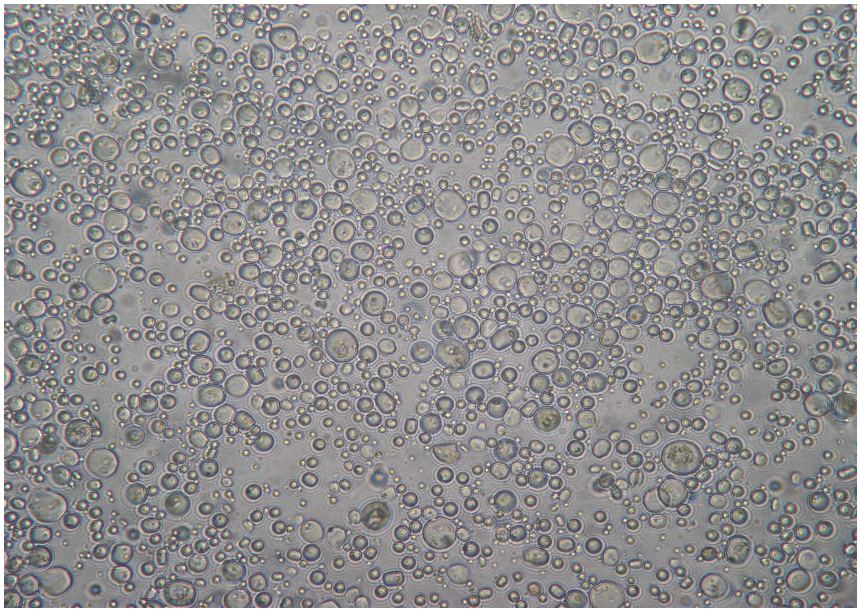


Figure 20. atRA-loaded MPs with a drug/polymer ratio 0.08 prepared at room temperature – MP4 (Table 4) (400X)

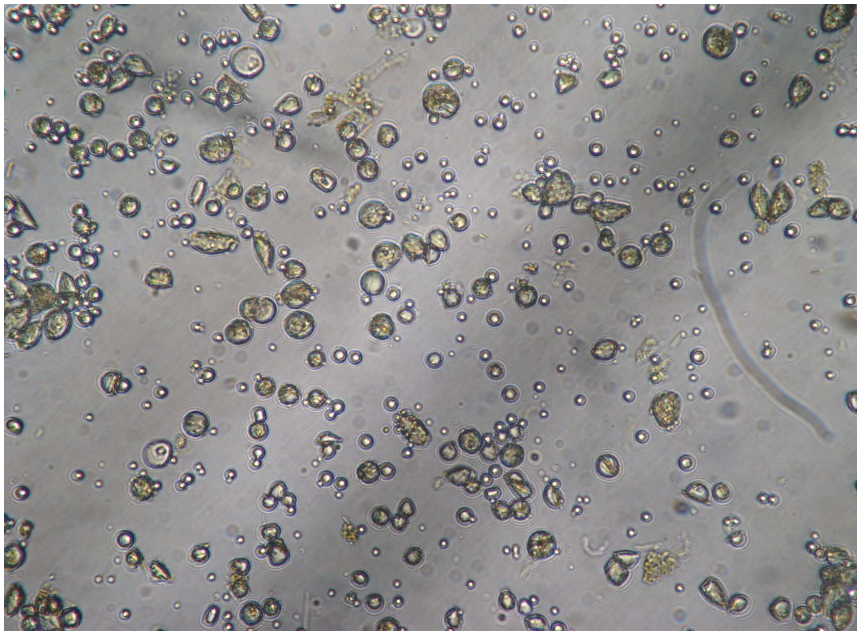


Figure 21. atRA-loaded MPs with a drug/polymer ratio 0.12 prepared at room temperature – MP5 (Table 4) (400X)

Figure 19, 20 and 21 show all-*trans*-Retinoic acid-loaded microspheres. All images confirmed the formation of microparticles with ESE method.

There were considerable difference in sizes of atRA-loaded microparticles. Figure 19, Figure 20 and Figure 21 show microparticles prepared by using 1 mg, 2 mg and 3 mg atRA respectively. According to those figures, increase in drug amount in preparation caused increase in microparticle size. Size distribution became less uniform as more drug was used in preparation. In addition, in batch MP5, the shapes of the microparticles were disrupted and there was no homogeneity. Therefore, increase in drug concentration used in preparation step causes formation of less uniform microparticles.

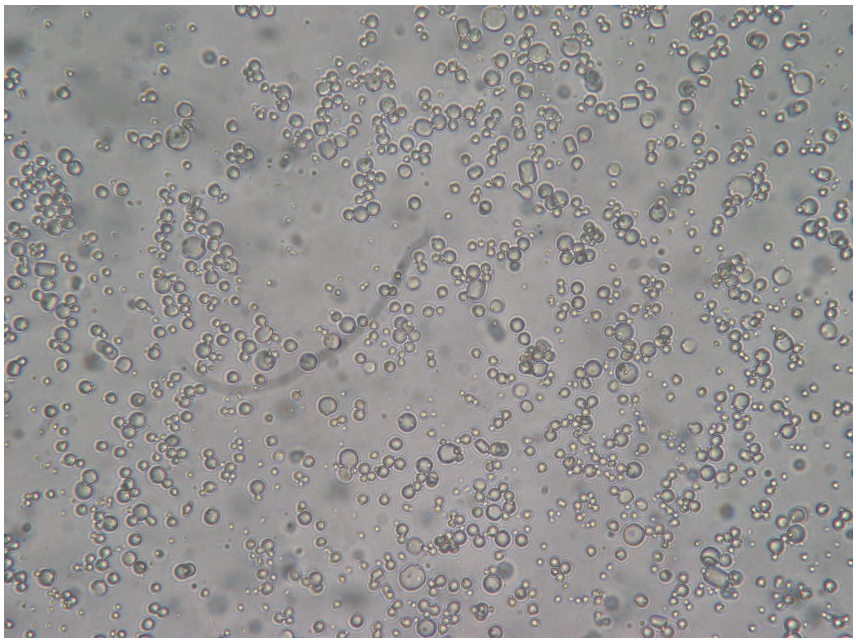


Figure 22. TMX-loaded MPs with a drug/polymer ratio 0.04 prepared at room temperature – MP6 (Table 4) (400X)

Tamoxifen-loaded microparticles (batch MP6) were shown at Figure 22. They were spherical and uniform in size as it was seen from micrographs.

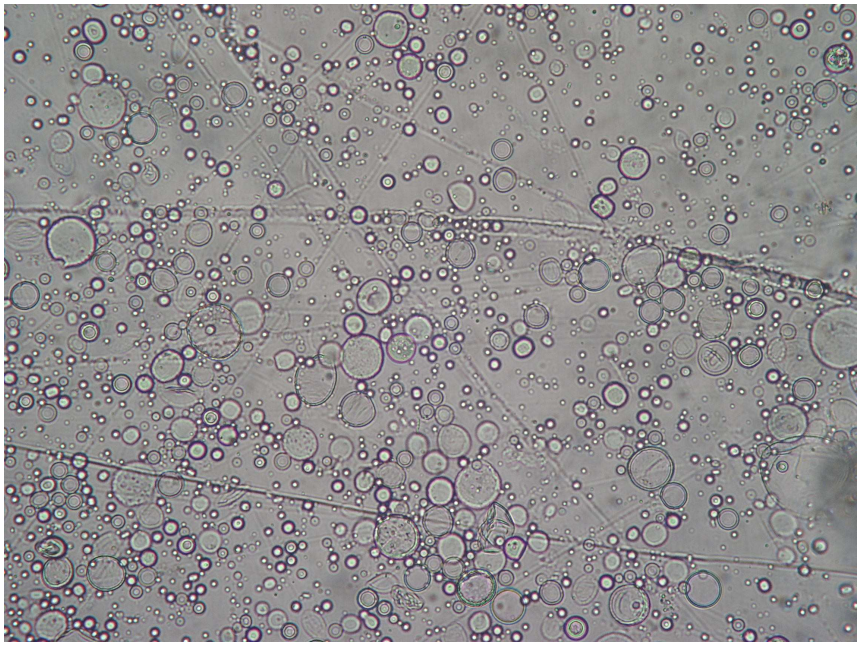


Figure 23. TMX-Cit-loaded MPs with a drug/polymer ratio 0.04 prepared at room temperature – MP7 (Table 4) (400X)

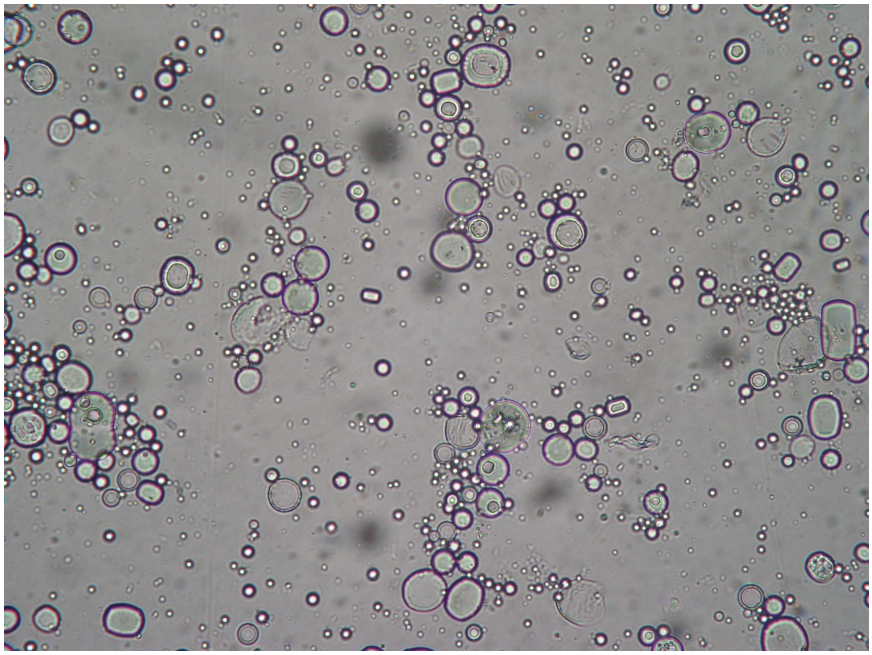


Figure 24. TMX-Cit-loaded MPs with a drug/polymer ratio 0.08 prepared at room temperature – MP8 (Table 4) (400X)

Tamoxifen citrate-loaded microparticles (batch MP7 and MP8) were spherical in shape in both batches. There was no considerable difference between MP7 and MP8 micrographs seen at Figure 23 and Figure 24. They were similar in their sizes and shapes. However, tamoxifen citrate-loaded PLGA microparticles were not uniform in size. The uniformity of those microparticles might be affected from the organic phase used during preparation.

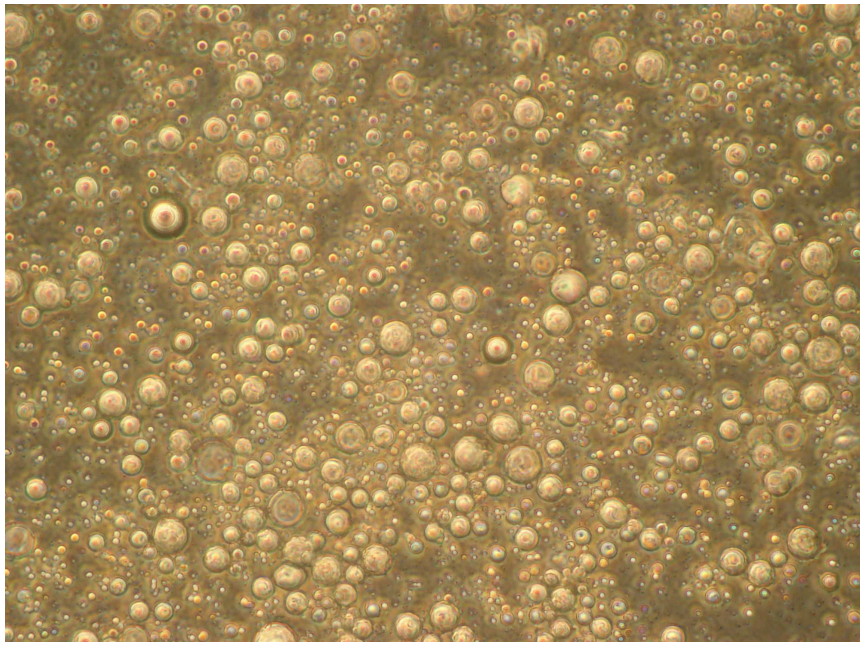


Figure 25. IDA-loaded MPs with a drug/polymer ratio 0.04 prepared at room temperature – MP9 (Table 4) (400X)

Idarubicin-loaded microparticles (batch MP9) were shown at Figure 25. They were spherical and almost uniform in size.

According to the micrographs, it was observed that microparticles at MP2 and MP6 were similar in size. However, microparticles at MP7 and MP9 were bigger than MP2 and MP6, although same amount of drug was used during preparation. There was increase in size depending on increase in drug concentration used as microparticles were prepared in atRA microparticles whereas no difference in tamoxifen citrate microparticles. On the other hand, all of the microparticles were spherical in shape as observed in figures above.

4.1.1.3. Scanning Electron Microscopy (SEM) Analysis

Size, shape and surface characteristics of microparticles were determined closely by SEM analysis.

As can be seen from the following micrographs given at Figure 26, 27, 28, 29, 30, 31, 32 and 33, the prepared microparticles were generally spherical in shape, almost smooth and no pores on them. Nevertheless, some microparticles had distorted shapes and aggregation of microparticles was observed as the drug amount used during preparation increased.

Figure 26 showed PLGA microparticles prepared without loading any drug inside. The particles had completely smooth surfaces and were spherical in shape. They were about 5-10 μm in size and almost homogenous in size distribution. When drug was loaded, the size of microparticles increased and become more diverse, thus size distribution became less uniform.

Figure 27, Figure 28 and Figure 29 showed all-*trans*-Retinoic acid-loaded PLGA microparticles with changing atRA concentrations. Microparticles were about 2-5 μm, 2-6 μm and 5-20 μm in size in Figure 27, Figure 28 and Figure 29 respectively. The microparticle size increased with increasing atRA concentration. Moreover, the uniformity of microparticles was decreased with increasing atRA concentration. When Figure 27 and Figure 29 were compared, it could be also seen that the shapes of microparticles became more distorted for the latter one due to higher amount of drug loading.

Figure 30 showed tamoxifen-loaded PLGA microparticles. They were spherical and 3-8 μm in size. They had smooth surfaces, their sizes diversed slightly and distribution of microparticles were almost uniform.

Figure 31 and Figure 32 showed tamoxifen citrate-loaded PLGA microparticles. Their sizes were almost 1-10 μm, and 5-20 μm in size in Figure 31 and Figure 32 respectively. The microparticles were spherical, however they were not uniform in size. This difference seen at size distribution might result from organic phase used during preparation of microparticles. Moreover, it could be seen from the Figure 32 that some microparticles had pores on them which could result in higher release rates.

Figure 33 showed SEM micrograph of idarubicin-loaded PLGA microparticles. All of the microparticles were not spherical but there were some egg-shaped ones. This might result from the nature of drug. Their sizes were almost 2-6 μm in size.

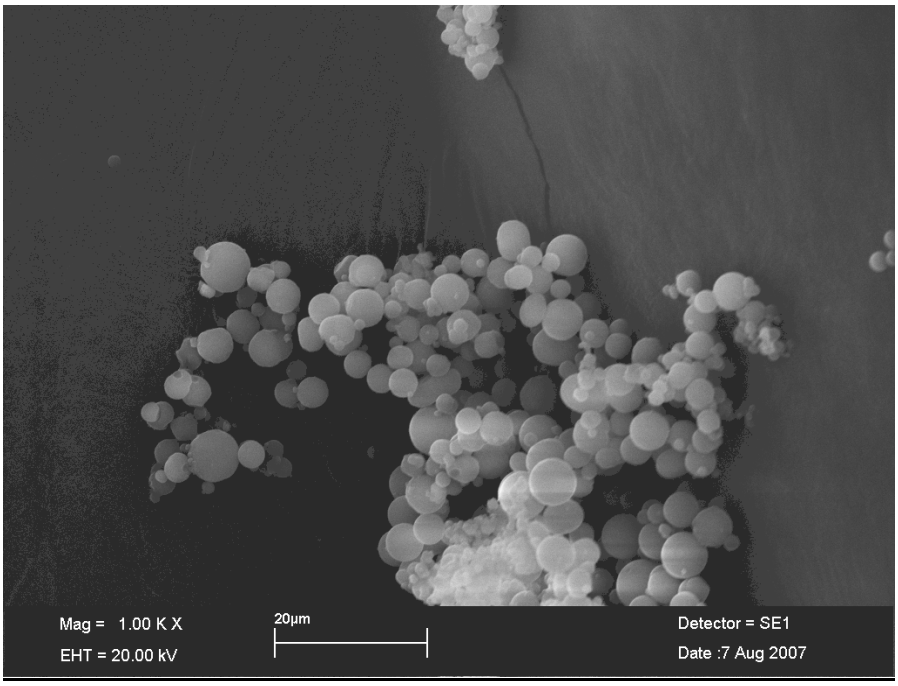


Figure 26. SEM micrograph of empty PLGA microparticles (MP1) after Au-Pd coating (1.00 K X)

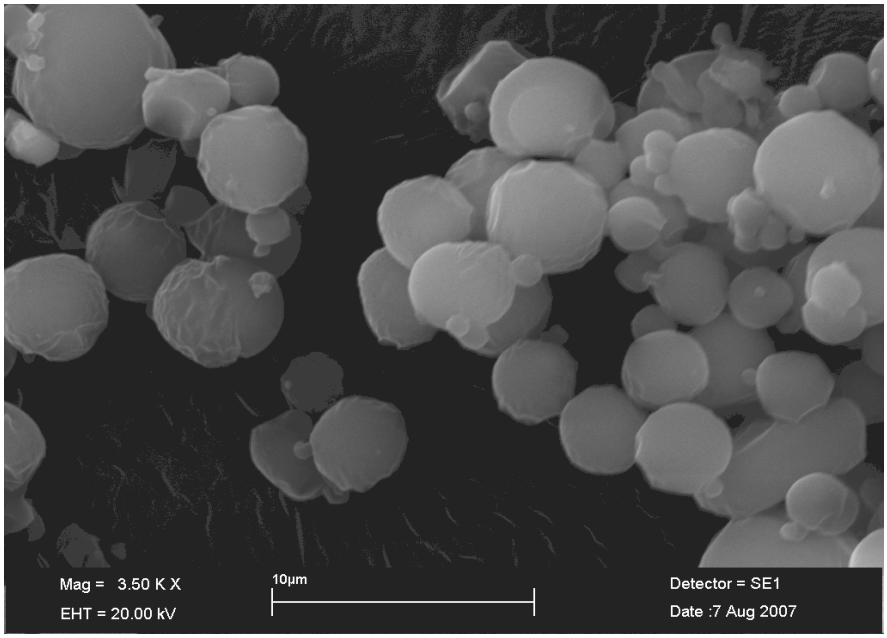


Figure 27. SEM micrograph of atRA-loaded PLGA microparticles with drug/polymer ratio 0.04 prepared at room temperature (MP2) after Au-Pd coating (3.50 K X)

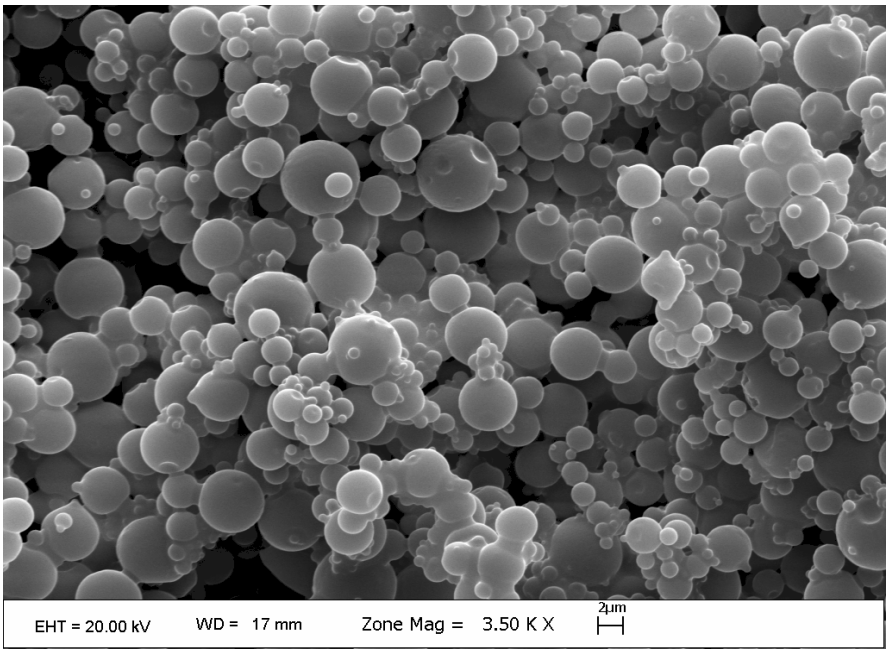


Figure 28. SEM micrograph of atRA-loaded PLGA microparticles with drug/polymer ratio 0.08 prepared at room temperature (MP4) after Au-Pd coating (3.50 K X)

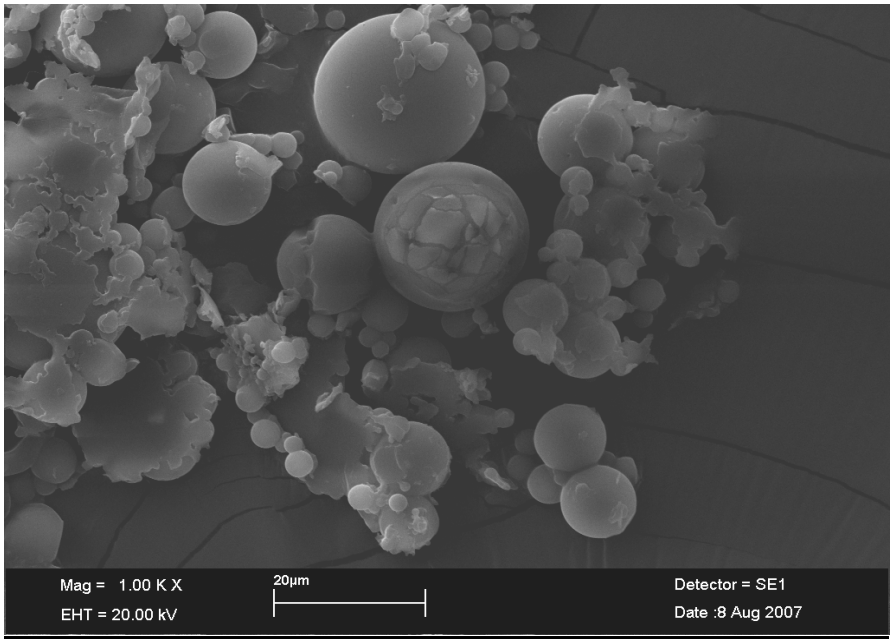


Figure 29. SEM micrograph of atRA-loaded PLGA microparticles with drug/polymer ratio 0.12 prepared at room temperature (MP5) after Au-Pd coating (1.00 K X)

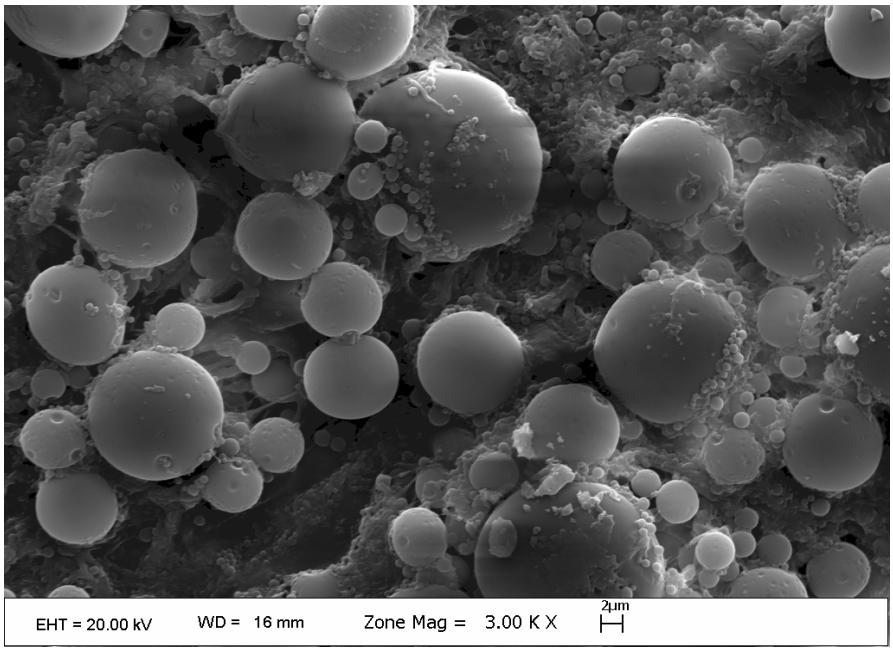


Figure 30. SEM micrograph of TMX-loaded PLGA microparticles with drug/polymer ratio 0.04 prepared at room temperature (MP6) after Au-Pd coating (3.00 K X)

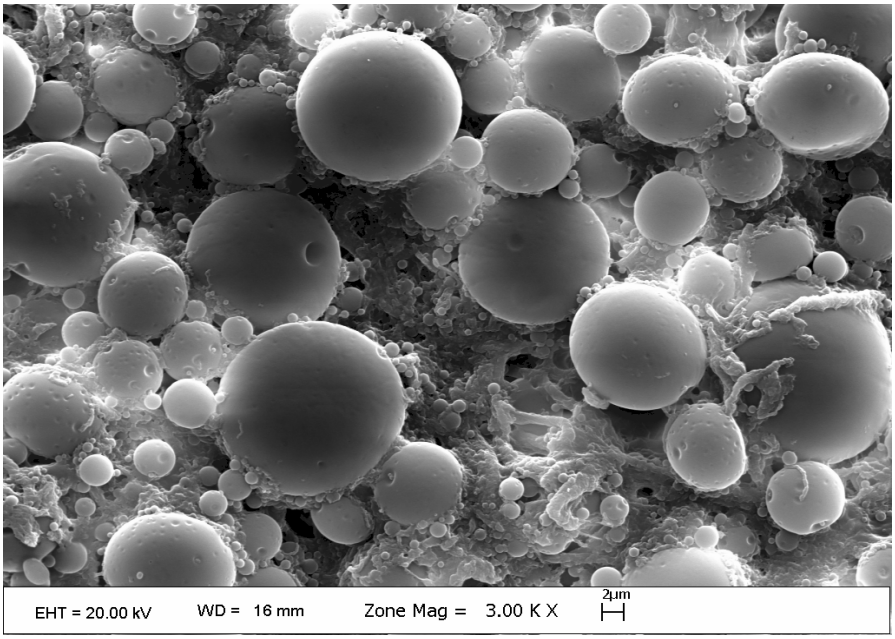


Figure 31. SEM micrograph of TMX-Cit-loaded PLGA microparticles with drug/polymer ratio 0.04 prepared at room temperature (MP7) after Au-Pd coating (3.00 K X)

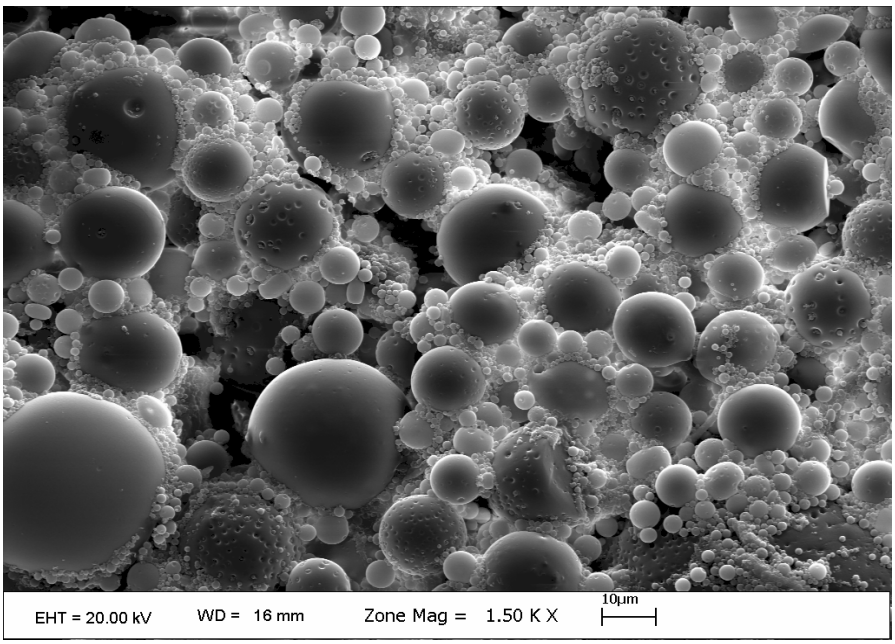


Figure 32. SEM micrograph of TMX-Cit-loaded PLGA microparticles with drug/polymer ratio 0.08 prepared at room temperature (MP8) after Au-Pd coating (1.50 K X)

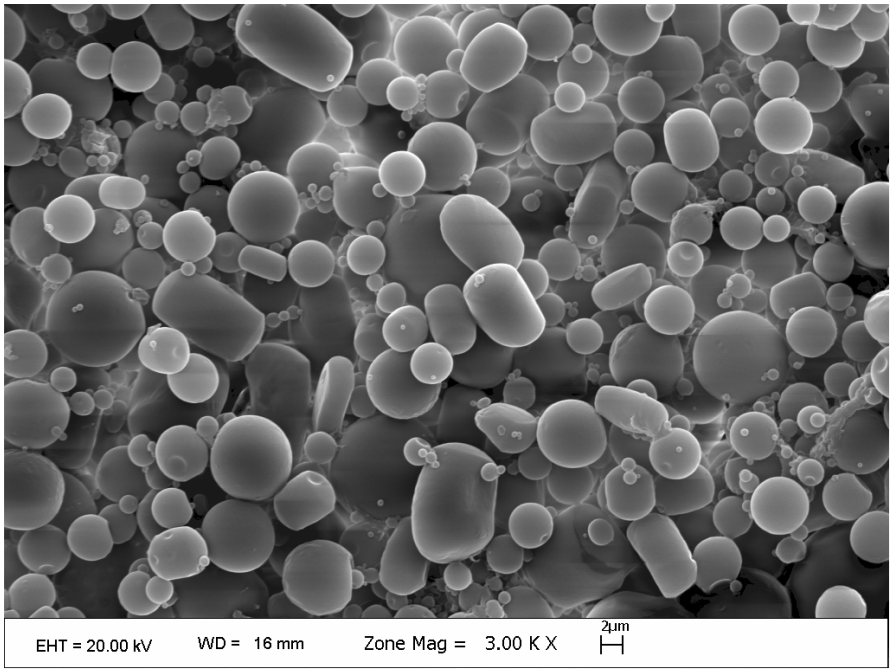


Figure 33. SEM micrograph of IDA-loaded PLGA microparticles with drug/polymer ratio 0.04 prepared at room temperature (MP9) after Au-Pd coating (3.00 K X)

4.1.1.4. Size Distribution Analysis

The average sizes of empty and all-*trans*-Retinoic acid-loaded microparticles were determined and size distribution curves were obtained. The particle size distribution curves of empty and all-*trans*-Retinoic acid-loaded microparticles were given at Figure A1, and Figure A2 in Appendix A.

Sizes of empty microparticles ranged approximately between 1-10 μm and mostly their sizes were around 4-6 μm. Moreover, sizes of all-*trans*-Retinoic acid-loaded microparticles also ranged between 1-10 μm and average sizes of microparticles were similar to empty microparticles. The size distribution of both microparticles were rather uniform according to the particle size distribution curves given in Appendix A.

4.2. Drug Encapsulation Efficiency

Drug encapsulation efficiency is the ratio of the amount of drug loaded to microparticles to that of the amount of the drug used initially. The encapsulation efficiencies of microparticles were calculated by using Equation 1 in Section 3.2.5 at page 36.

According to the formula mentioned at related section, the result of encapsulation efficiencies were given below:

Table 5. Encapsulation efficiencies of drug-loaded microparticles

Sample **PLGA microparticles**	**Drug/Polymer** **ratio**	**Encapsulation efficiency** **(%)**
MP2	0.04	8.43 ± 0.08
MP3	0.06	42.58 ± 0.4
MP4	0.08	49.65 ± 0.8
MP5	0.12	88.8 ± 0.7
MP6	0.04	8.50 ± 0.4
MP7	0.04	11.90 ± 0.3
MP8	0.08	23.95 ± 0.2
MP9	0.04	17.43 ± 0.2

It was observed from the Table 5 that changing in drug/polymer ratio affected the extend of drug content. The encapsulation efficiencies of all-*trans*-Retinoic acid-loaded microparticles (MP2, MP3, MP4 and MP5) and tamoxifen citrate-

loaded microparticles (MP7 and MP8) revealed that the encapsulation efficiency increased when the drug concentration used in preparation increased. The only variable parameter was the drug concentration in encapsulation efficiency studies.

The percent encapsulation efficiencies of atRA-loaded PLGA microparticles (MP2, MP3, MP4, MP5) increased as the drug concentration used initially increased. Figure 34 demonstrated the effect of drug concentration on percent encapsulation efficiency.

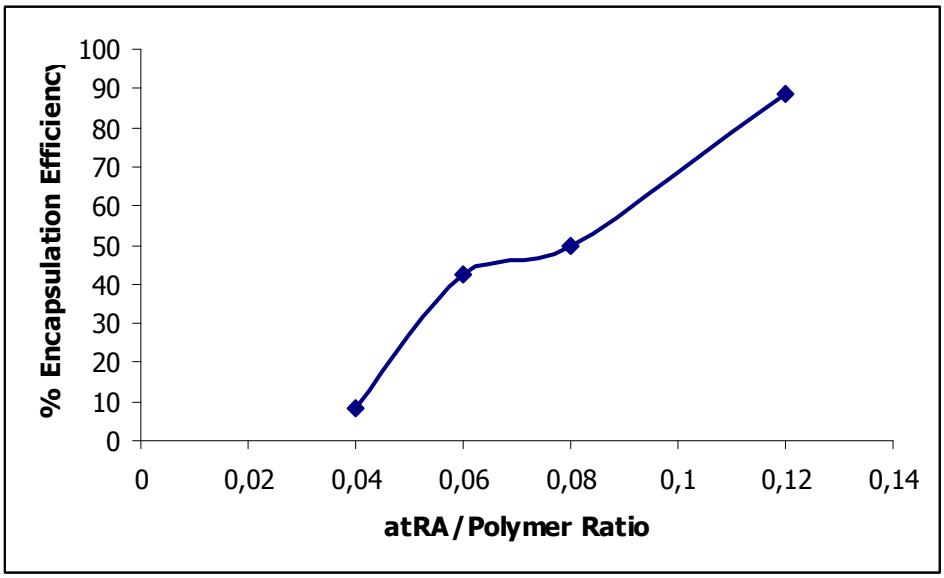


Figure 34. Effect of atRA concentration on percent encapsulation efficiency.

The study of Jeong et al. (2003), investigated the effect of changing parameters in preparation, such as PVA content and molecular weight, polymer molecular weight and atRA weight. According to the results of their study, the loading efficiency of retinoic acid increased with increasing atRA weight. The study of Jeong et al. (2003) supported the increasing effect of

atRA concentration on encapsulation efficiency as it was demonstrated in Figure 34 [34].

When the percent encapsulation efficiencies of different drugs having same drug/polymer ratio (MP2, MP6, MP7 and MP9) were compared, it was observed that MP2 and MP6 had nearly the same percent encapsulation efficiencies (8.43 ± 0.08 and 8.50 ± 0.4 respectively) whereas MP7 and MP9 (11.90 ± 0.3 and 17.43 ± 0.2 respectively) had higher efficiencies.

In the study of Sehra and Dhake (2005), tamoxifen citrate-loaded PLGA microspheres were prepared and the drug content of microspheres prepared with variable parameters was determined. According to this study, the encapsulation efficiency of different batches was found in the range of 75-90%. According to Table 5, the encapsulation efficiency of tamoxifen citrate-loaded PLGA microparticles (MP7 and MP8) was found 11.90% and 23.95% respectively. The difference between results might be due to preparation conditions and variable parameters which they investigated, such as polymer and stabilizator concentration, solvent used and other experimental conditions [48].

4.3. *In vitro* Drug Release Studies

The release profiles of all-*trans*-Retinoic acid, tamoxifen and tamoxifen citrate from PLGA microparticles were determined by spectrophotometric measurement of released drug in phosphate buffer (0.01 M, pH 7.4, 10 ml, at 37^{o}C). The experimental was described in Section 3.2.6 at page 40. The absorbance at λ = 365 nm, λ = 274 nm and λ = 254 nm were measured for atRA, tamoxifen and tamoxifen citrate respectively. The release medium was replaced with same amount of fresh buffer after each mesurement. The results of duplicate tests were used in order to calculate the average amount of released drug.

A cumulative percent average drug release versus time graph was obtained for each sample. The release trends of all-*trans*-Retinoic acid, tamoxifen and tamoxifen citrate from PLGA microparticles are given in Figure 35, Figure 36 and Figure 37, respectively. The raw data of cumulative percent average drug release amounts of each studied drug were also given at Table B1, Table B2 and Table B3 in Appendix B.

4.3.1. *In vitro* Release of All-*trans*-Retinoic Acid from PLGA Microparticles

UV absorption spectrum of all-*trans*-Retinoic acid was obtained and spectrophotometrical measurements were done at λ = 365 nm.

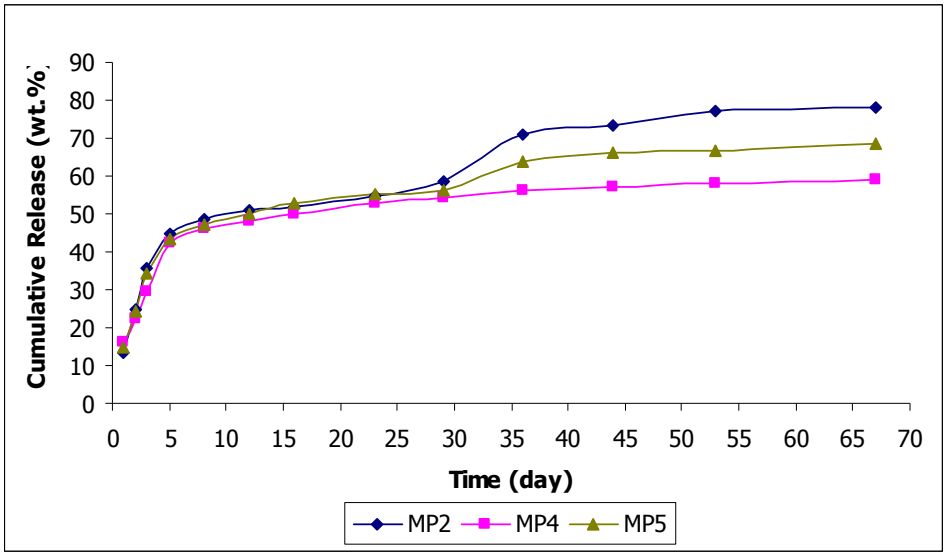


Figure 35. Release profile of all-*trans*-Retinoic acid from PLGA microparticles (Drug was released in PBS medium, 0.01 M, pH 7.4, with horizontal shaking at 100 rpm at 37°C.)

MP2: drug/polymer ratio is 0.04, MP4: drug/polymer ratio is 0.08, MP5: drug/polymer ratio is 0.12. All samples were prepared at room temperature.

In release profile of atRA, it was observed that an early rapid release was followed by a slower release period. This initial burst might caused from the release of drugs bound onto the microparticles or drugs embedded close to the surface.

By the end of fifth day, it was observed that 40-50% of atRA was released from all of atRA-loaded microparticles. At 53. day 50-70% of atRA was released and the release rate became nearly stable.

The release trend of atRA from PLGA microparticles was found to be changed slightly depending on the concentration of atRA. For lower drug containing sample (MP2), more rapid drug release profiles were observed and high drug containing sample (MP5), drug release was slower as it can be observed from Figure 35.

In the study of Young-II Jeong et al. (2003), atRA-loaded PLGA microspheres were prepared and the drug release rate was investigated. They investigated microspheres prepared by changing parameters and their release trends. They also looked for the effect of atRA weight on atRA release characteristics from PLGA microspheres. The result of their release study demonstrated that there was a slight initial burst of atRA during first 5-7 days followed by a slower release. At about 55. days, release of atRA reached approximately 95% for microparticles containing low amount of drug (0.2%, w/v) and 30% for microparticles containing higher amount of drug (0.8%, w/v). They concluded that the higher the drug weights the lower the release rate [34]. Young-II Jeong et al. (2002) and Young-II Jeong et al. (2003) pointed out the reason which Gref et al. (1994) described clearly that hydrophobic drug was crystallized inside the microparticles at higher drug contents and phase seperation occurs. However, at lower drug contents, molecular dispersion occured inside the microspheres. Therefore, crystallized hydrophobic drug dissolved and released slower than dispersed drug [34,63]. According to this conclusion, the reason of the difference on release patterns of MP2, MP4 and MP5 might be either being crystallization in or dispersed throughout the PLGA microparticles.

4.3.2. *In vitro* Release of Tamoxifen from PLGA Microparticles

UV absorption spectrum of tamoxifen was obtained and spectrophotometrical measurements were done at λ = 274 nm.

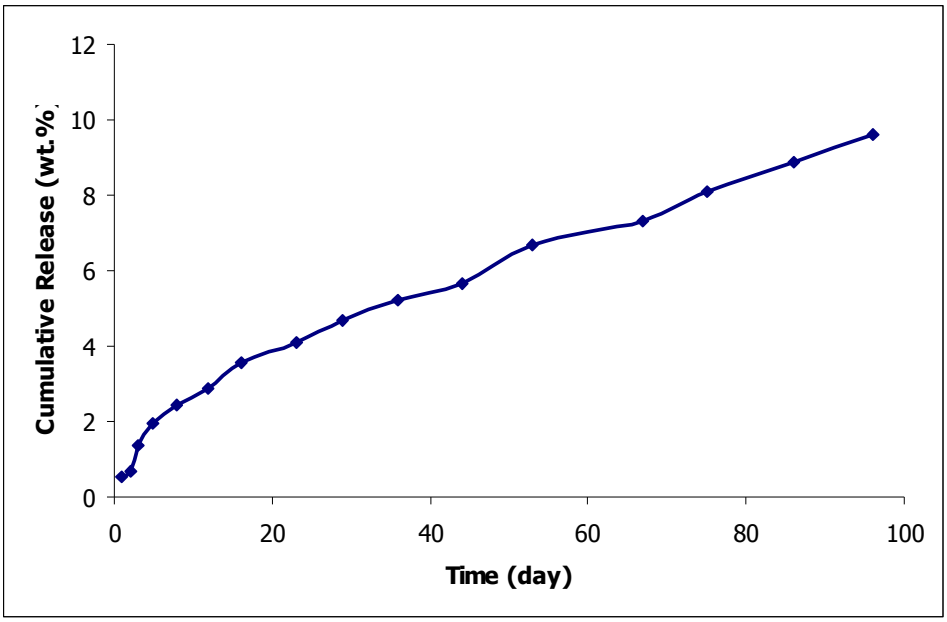


Figure 36. Release profile of tamoxifen from PLGA microparticles (Drug was released in PBS medium, 0.01 M, pH 7.4, with horizontal shaking at 100 rpm at 37°C.) The drug/polymer ratio is 0.04 and sample was prepared at room temperature.

In release profile of tamoxifen, an early rapid release was observed, however, it was followed by a slower and controlled release period. By the end of fourth day, it was observed that 2% of loaded tamoxifen was released from microparticles. Towards 90th day, 10% of loaded drug released.

The study of Eyövge (2005), demonstrated a similar release pattern for tamoxifen release from PLGA microparticles with an initial burst release.

However, initial burst release was observed ~5% in 1-2 days and cumulative release reached ~30% at about 15th day [6]. The microparticle preparation conditions was probably the reason of this difference. Although the encapsulation efficiency of tamoxifen was very low in this study, the release pattern is acceptably good. The release of drug from microparticles was slow and controlable as it was desired.

4.3.3. *In vitro* Release of Tamoxifen Citrate from PLGA Microparticles

UV absorption spectrum of tamoxifen citrate was obtained and spectrophotometrical measurements were done at λ = 254 nm.

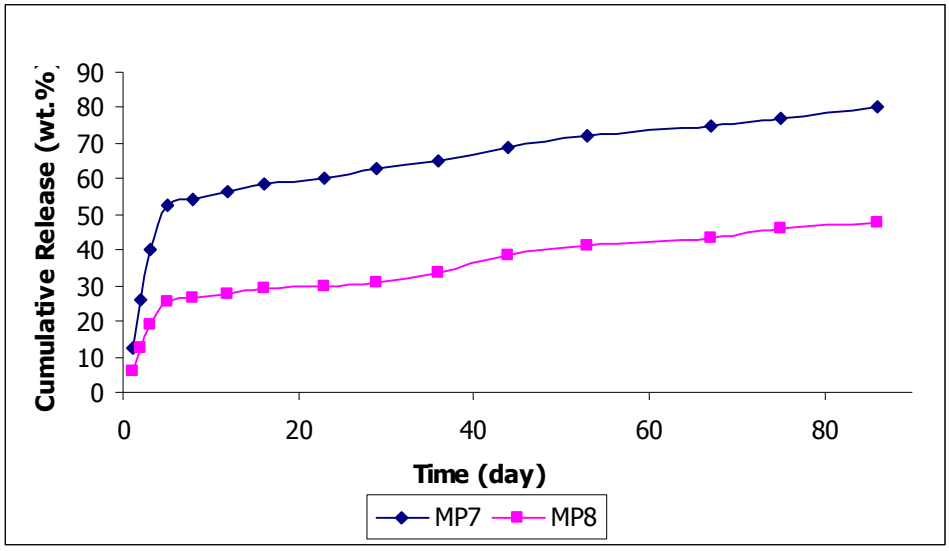


Figure 37. Release profile of tamoxifen citrate from PLGA microparticles (Drug was released in PBS medium, 0.01 M, pH 7.4, with horizontal shaking at 100 rpm at 37^{o}C.) —◆—MP7: drug/polymer ratio is 0.04, —■—MP8: drug/polymer ratio is 0.08. All samples were prepared at room temperature.

In release profile of tamoxifen citrate, it was observed that an early rapid release was followed by a slower release period as in the case of atRA.

The initial release rate of tamoxifen citrate from microparticles was changing depending on the concentration of drug. Low drug contained sample (MP7) released encapsulated drug more rapidly whereas higher drug contained sample (MP8) released the drug more slower as it can be observed from Figure 37. However, the release trends were similar. By the fifth day, it was observed that approximately 60% of drug was released from MP7 and 30% from MP8 microparticles. Further release patterns were similar for MP7 and MP8, and the drug release rate became nearly equal. Nevertheless, after 80 days MP7 microparticles released ~80% of loaded drug whereas MP8 microparticles released only half of it, ~40% of loaded drug.

In the study of Sehra and Dhake (2005), the release rate of tamoxifen citrate-loaded PLGA microspheres was also investigated and release pattern of microparticles prepared with variable parameters was determined. According to this study, cumulative percent release of tamoxifen citrate of different batches were found similar. They showed a very slight initial burst release in an hour and ~30% of drug was released in every batches. After 12 hours, 85-95% of drug was released [48]. Contrary to study of Sehra and Dhake, it was clear that a more controlled and prolonged release profile was obtained in this study over 80 days.

4.4. *In Vitro* Cytotoxicity Tests

Cytotoxicity of empty and all-*trans*-Retinoic acid-loaded microparticles was determined by measuring the inhibition of growth of MCF-7 human breast cancer cells. Following the incubation of cells with empty and atRA-loaded microparticles, XTT based cytotoxicity assay was used to determine the cell viability.

The cytotoxic effect of empty PLGA microparticles were determined by XTT test using MCF-7 human breast cancer cell line. Figure 38 demonstrates the results:

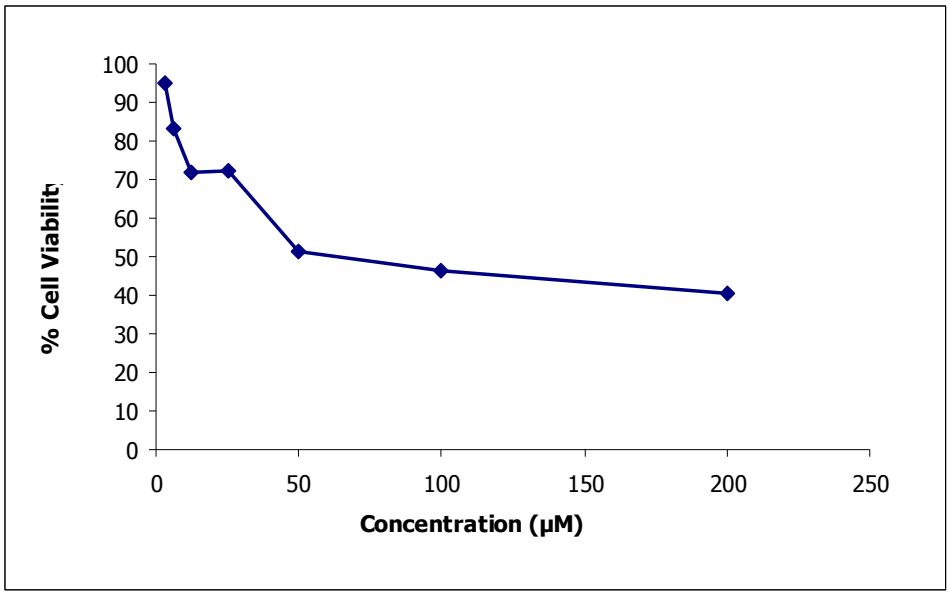


Figure 38. Cell viability after 4 days of incubation with sterile empty PLGA microparticles (MP1) (Table 4).

According to the graph, empty PLGA microparticles had a toxic effect on breast cancer cells. After 4 days of incubation of cells with empty PLGA

microparticles at different concentrations, cell growth inhibition was observed. The IC_{50} value of empty microparticles was found to be 91.77 μM at which 50% of cells was dead.

In the study of Jeong et al. (2003), the cell growth inhibition by PLGA microspheres containing atRA and empty PLGA microspheres were compared. The cell viability tests were performed on U87MG, malignant glioma cell line. They concluded that the empty microspheres do not affect the cells [34]. However, Figure 38 showed some toxic effect of empty PLGA microparticles on MCF-7 cells. This may originate mainly from the preparation of microparticles or types of cells. The organic phase, DCM, used during microparticle preparation was toxic; the microparticles may exert toxic effect if the organic phase could not be removed properly.

The cytotoxic effect of atRA-loaded PLGA microparticles were determined by XTT test using MCF-7 human breast cancer cell line. Figure 39 demonstrates the results:

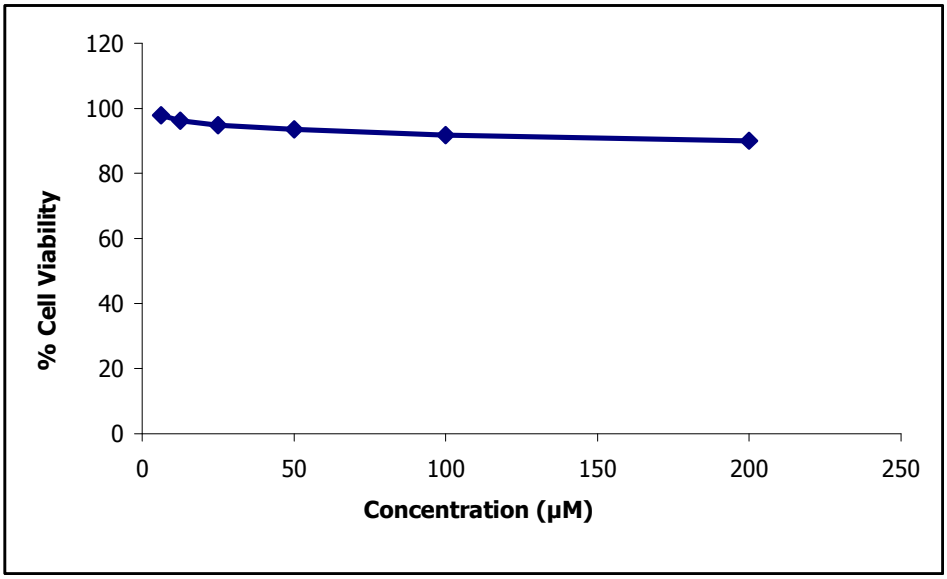


Figure 39. Cell viability after 4 days of incubation with sterile atRA-loaded PLGA microparticles (MP4) (Table 4).

According to the result, all-*trans*-Retinoic acid-loaded PLGA microparticles had a slightly toxic effect on breast cancer cells. After 4 days of incubation of cells with atRA-loaded PLGA microparticles at different concentrations, it was observed that the cell viability was decreased. However, cell growth inhibition was very slight. The initial burst of atRA does not seem to be effective for toxicity, since initial burst release of drug at first 3-4 days was only 20-30% of loaded drug and the release of atRA was very slow (Figure 35).

Although free atRA had toxic effects on different kinds of cells [40,64], and combination of atRA with docetaxel [65] or zoledronic acid [66] had strong

synergistic cytotoxic effect on prostate carcinoma cell lines, it exerted its toxicity slightly on human breast cancer cells when encapsulated within PLGA. According to Jeong et al. (2003), empty microparticles did not affect cell cytotoxicity whereas atRA-loaded PLGA microparticles had significant toxicity on cells [34]. In the study of Jeong et al., atRA-loaded PLGA microparticles were dissolved in dimethyl sulfoxide (DMSO) which dissolved PLGA. The cytotoxicity experiment was then progressed in the presence of free atRA and polymer. However, in this study, atRA-loaded PLGA microparticles were dispersed in aquous solution so that cells were affected only released atRA from microparticles during cytotoxicity study. Since the release of atRA was slow and the cytotoxicity experiment was progressed over 96 hours, released atRA was totally from initial burst. Probably, the initial burst concentration of atRA (20-30%) from microparticles in 4 days of incubation was not so effective to the cell toxicity. Nevertheless, the released amount of atRA might have some proliferative effect on cells so that it could interfere with and neutralize toxic effect of organic phase used during preparation of microparticles.

CHAPTER V

CONCLUSIONS

Polymeric controlled release systems of anticancer drugs have many advantages over conventional chemotherapy. For instance, reduction in side effects of toxic anticancer drugs, prevention of drug resistance in continuous exposure, increment in efficacy of drug therapies and application of drugs in wide range, especially the toxic ones.

In this study, poly(D,L-lactide-co-glycolide) microparticles containing different hydrophobic drugs, namely all-*trans*-Retinoic acid, tamoxifen, tamoxifen citrate and idarubicin, were prepared and chacterized as drug carrier matrices for controlled release of anticancer drugs. In the preparation of microparticles loaded with all-*trans*-Retinoic acid, tamoxifen and idarubicin, dichloromethane was used as organic phase in which those hyrophobic drugs were dissolved. However, tamoxifen citrate was dissolved in dichloromethane:methanol (1:1) mixture because it was highly hydrophobic due to its complex structure. Drug release from microparticles were also studied. The results can be summarized as:

Morphological analysis makes possible to investigate the size, shape and surface properties of microparticles.

- Inverted light microscopy confirmed the formation of both empty and drug-loaded microparticles.

- Scanning electron micrographs revealed that the microparticles were smooth and spherical in shape. Their sizes differed in the range of 2-20 μm and almost homogenous in size distribution. However, as the loaded drug amount increased, the sizes of microparticles increased and the size distribution became less uniform. Moreover, aggregation and distortion increased as loaded drug amount increased.
- Microparticles were spherical in shape. However, when the drug amount was high, the shape of atRA-loaded microparticles lost its homogeneity.
- According to size distribution curves, the sizes of empty and all-*trans*-Retinoic acid-loaded microparticles ranged approximately between 1-10 μm and mostly their sizes were around 4-6 μm. The size distribution of both microparticles were rather uniform.

Encapsulation efficiencies were investigated to reveal the efficiency of drug-loading inside the microparticles.

- The drug encapsulation efficiencies of four different drugs, atRA, TMX, TMX-Cit and IDA, were found as 8.43 ± 0.08%, 8.50 ± 0.4%, 11.90 ± 0.3% and 17.43 ± 0.2% respectively when the drug/polymer ratio was 0.04. According to these results, idarubicin had higher efficiency than other drugs at the same concentration.
- atRA-loaded PLGA microparticles having a drug/polymer ratio of 0.04 (MP2) had low encapsulation efficiency (8.43%) since the drug concentration was low during preparation. It was also revealed that encapsulation efficiency increased when the drug concentration used in preparation increased. When MP2, MP3, MP4 and MP5 batches were compared it was clear that the

encapsulation efficiency increased (8.43%, 42.58%, 49.65%, and 88.8% respectively) with increasing drug amount during preparation. It was similar for TMX-Cit-loaded microparticles (batches MP7 and MP8, 11.90% and 23.95% respectively). Therefore, it was concluded that changing in drug/polymer ratio affected the extend of drug content.

- atRA-loaded PLGA microparticles having a drug/polymer ratio of 0.04 (MP2) was found to have the lowest encapsulation efficiency. Contrary, atRA-loaded PLGA microparticles having a drug/polymer ratio of 0.12 (MP5) was found to have the highest encapsulation efficiency. However, their shapes were irregular and the size distribution was not uniform. Therefore, atRA-loaded PLGA microparticles having a drug/polymer ratio of 0.08 (MP4) was found as the most optimized microparticles having spherical shape, small and uniform in size and rather high encapsulation efficiency.

The drug release studies were done to observe the release trends of the drugs. Generally, all of atRA, TMX and TMX-Cit, released from microparticles with biphasic kinetics, an initial burst was followed by a slower release.

- The release trend of atRA was found to be changed slightly depending on the concentration of atRA. Increase in drug content caused a slower release pattern as in the case of drug release from TMX-Cit-loaded microparticles.
- By the end of 4-5 days, ~45% of atRA was released from atRA-loaded microparticles, whereas ~2% of tamoxifen was released from TMX-loaded microparticles and ~60% of drug was released from TMX-Cit-loaded microparticles.

- At around the steady state release period, 70-80% of atRA was released at about 50 days, whereas ~80% of TMX-Cit was released by 80. day from microparticles prepared with using 1 mg drug.
- The release rate was very slow for TMX microparticles. Towards 90. day, 10% of TMX released. However, in controlled release systems, this type of release pattern was generally desired.

In vitro cytotoxicity test reveal that the cytotoxicities of empty and all-*trans*-Retinoic acid-loaded microparticles. The inhibition of cell growth in MCF-7 human breast cancer cell line was determined by XTT cytotoxicity assay.

- After 4 days of incubation of cells with empty and atRA-loaded PLGA microparticles at different concentrations, cell growth inhibition was observed.
- The empty microparticles demonstrate some toxicity and affect the cell viability. The toxic effect of empty microparticles may be due to the organic phase remained in microparticle solution during preparation.
- The cell growth inhibition was slight for atRA-loaded PLGA microparticles. Probably, the initial burst concentration of atRA (20-30%) from microparticles in 4 days of incubation was not so effective to the cell toxicity.

As a final conclusion, the biocompatible and biodegradable drug-loaded poly(D,L-lactide-co-glycolide) microparticles are promising devices for controlled drug delivery. However, this study demonstrated a preliminary information about the drug-loaded PLGA microparticles, especially with atRA, further *in vitro* and *in vivo* studies were required.

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APPENDIX A

PARTICLE SIZE DISTRIBUTIONS OF MICROPARTICLES

Particle Name: Default	**Accessory Name:** Hydro 2000S (A)	**Analysis model:** General purpose	**Sensitivityl:** Normal
Particle RI: 1.520	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.89 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 2.222 %	**Result Emulation:** Off
Concentration: 0.0078 %Vol	**Span :** 1.828	**Uniformity:** 0.694	**Result units:** Volume
Specific Surface Area: 2.03 m^2/g	**Surface Weighted Mean D[3,2]:** 2.954 um	**Vol. Weighted Mean D[4,3]:** 5.312 um	

d(0.1): 1.379 um d(0.5): 4.183 um d(0.9): 9.028 um

Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %
0.020	0.00	0.142	0.00	1.002	4.28	7.096	80.16	50.238	99.73	355.656	100.00
0.022	0.00	0.159	0.00	1.125	6.07	7.962	85.40	56.368	99.89	399.052	100.00
0.025	0.00	0.178	0.00	1.262	8.17	8.934	89.66	63.246	99.98	447.744	100.00
0.028	0.00	0.200	0.00	1.416	10.57	10.024	92.90	70.963	100.00	502.377	100.00
0.032	0.00	0.224	0.00	1.589	13.25	11.247	95.17	79.621	100.00	563.677	100.00
0.036	0.00	0.252	0.00	1.783	16.19	12.619	96.61	89.337	100.00	632.456	100.00
0.040	0.00	0.283	0.00	2.000	19.40	14.159	97.42	100.237	100.00	709.627	100.00
0.045	0.00	0.317	0.00	2.244	22.91	15.887	97.79	112.468	100.00	796.214	100.00
0.050	0.00	0.356	0.00	2.518	26.78	17.825	97.94	126.191	100.00	893.367	100.00
0.056	0.00	0.399	0.00	2.825	31.09	20.000	97.98	141.589	100.00	1002.374	100.00
0.063	0.00	0.448	0.00	3.170	35.95	22.440	98.02	158.866	100.00	1124.683	100.00
0.071	0.00	0.502	0.00	3.557	41.38	25.179	98.10	178.250	100.00	1261.915	100.00
0.080	0.00	0.564	0.04	3.991	47.39	28.251	98.26	200.000	100.00	1415.892	100.00
0.089	0.00	0.632	0.30	4.477	53.88	31.698	98.51	224.404	100.00	1588.656	100.00
0.100	0.00	0.710	0.84	5.024	60.69	35.566	98.82	251.785	100.00	1782.502	100.00
0.112	0.00	0.796	1.67	5.637	67.53	39.905	99.15	282.508	100.00	2000.000	100.00
0.126	0.00	0.893	2.81	6.325	74.13	44.774	99.47	316.979	100.00		

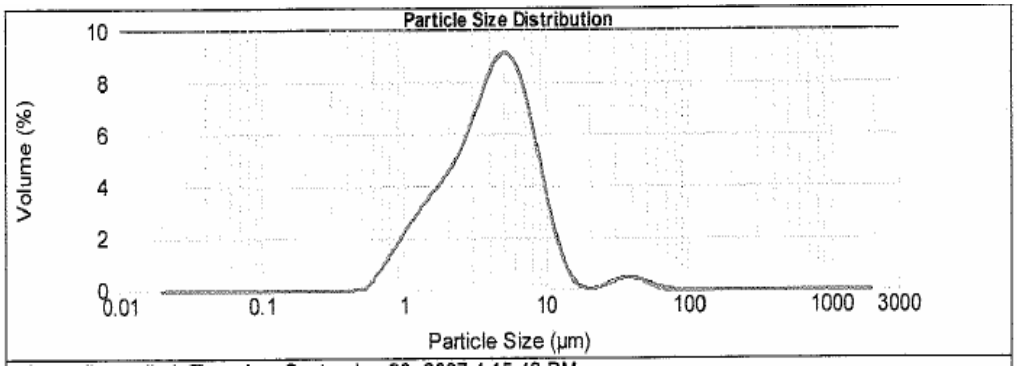

Figure A1. The histogram table and average particle size distribution curve of empty PLGA microparticles prepared at room temperature (MP1).

Particle Name: Default	**Accessory Name:** Hydro 2000S (A)	**Analysis model:** General purpose	**Sensitivity!:** Normal
Particle RI: 1.520	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.99 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 2.222 %	**Result Emulation:** Off
Concentration: 0.0072 %Vol	**Span :** 1.294	**Uniformity:** 0.404	**Result units:** Volume
Specific Surface Area: 2.23 m²/g	**Surface Weighted Mean D[3,2]:** 2.689 um	**Vol. Weighted Mean D[4,3]:** 3.395 um	

d(0.1): 1.611 um d(0.5): 3.085 um d(0.9): 5.604 um

Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %
0.020	0.00	0.142	0.00	1.002	1.53	7.096	96.87	50.238	100.00	355.656	100.00
0.022	0.00	0.159	0.00	1.125	2.57	7.962	98.54	56.368	100.00	399.052	100.00
0.025	0.00	0.178	0.00	1.262	4.11	8.934	99.46	63.246	100.00	447.744	100.00
0.028	0.00	0.200	0.00	1.416	6.37	10.024	99.86	70.963	100.00	502.377	100.00
0.032	0.00	0.224	0.00	1.589	9.53	11.247	99.98	79.621	100.00	563.677	100.00
0.036	0.00	0.252	0.00	1.783	13.81	12.619	100.00	89.337	100.00	632.456	100.00
0.040	0.00	0.283	0.00	2.000	19.33	14.159	100.00	100.237	100.00	709.627	100.00
0.045	0.00	0.317	0.00	2.244	26.13	15.887	100.00	112.468	100.00	796.214	100.00
0.050	0.00	0.356	0.00	2.518	34.07	17.825	100.00	126.191	100.00	893.357	100.00
0.056	0.00	0.399	0.00	2.825	42.89	20.000	100.00	141.589	100.00	1002.374	100.00
0.063	0.00	0.448	0.00	3.170	52.20	22.440	100.00	158.865	100.00	1124.683	100.00
0.071	0.00	0.502	0.00	3.557	61.50	25.179	100.00	178.250	100.00	1261.915	100.00
0.080	0.00	0.564	0.00	3.991	70.32	28.251	100.00	200.000	100.00	1415.892	100.00
0.089	0.00	0.632	0.05	4.477	78.23	31.698	100.00	224.404	100.00	1588.656	100.00
0.100	0.00	0.710	0.17	5.024	84.92	35.566	100.00	251.785	100.00	1782.502	100.00
0.112	0.00	0.796	0.43	5.637	90.23	39.905	100.00	282.508	100.00	2000.000	100.00
0.126	0.00	0.893	0.85	6.325	94.18	44.774	100.00	316.979	100.00		

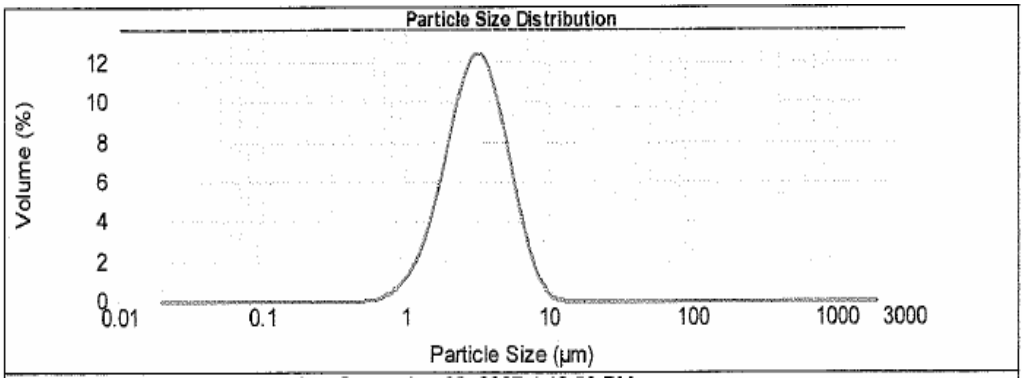

Figure A2. The histogram table and average particle size distribution curve of all-*trans*-Retinoic acid-loaded PLGA microparticles with a drug/polymer ratio is 0.08 prepared at room temperature (MP4).

APPENDIX B

IN VITRO DRUG RELEASE VALUES FOR PLGA MICROPARTICLES

Table B1. Cumulative average % release of all-*trans*-Retinoic acid from PLGA microparticles with a drug/polymer ratio is 0.04 (MP2), 0.08 (MP4), 0.12 (MP5) prepared at RT

	Cumulative Average % Release		
Time (day)	**MP2**	**MP4**	**MP5**
1	13.37	16.13	14.67
2	24.81	22.46	24.095
3	35.52	29.68	34.345
5	44.94	42.32	43.24
8	48.80	46.08	47.175
12	50.75	47.95	50.13
16	52.09	49.92	53.075
23	54.73	52.74	55.225
29	58.45	54.33	56.035
36	70.99	56.19	63.76
44	73.11	56.99	66.14
53	77.2	58.31	66.885
67	77.93	59.27	68.345

Table B2. Cumulative average % release of tamoxifen from PLGA microparticles with a drug/polymer ratio is 0.04 prepared at RT

	Cumulative Average % Release
Time (day)	**MP6**
1	0.56
2	0.69
3	1.35
5	1.95
8	2.45
12	2.86
16	3.55
23	4.08
29	4.68
36	5.23
44	5.64
53	6.66
67	7.30
75	8.08
86	8.89
96	9.63

Table B3. Cumulative average % release of tamoxifen citrate from atRA-loaded PLGA microparticles with a drug/polymer ratio is 0.04 (MP7), 0.08 (MP8) prepared at RT

	Cumulative Average % Release	
Time (day)	**MP7**	**MP8**
1	12.54	6.13
2	25.95	12.44
3	39.94	19.10
5	52.47	25.54
8	54.12	26.40
12	56.38	27.87
16	58.65	29.26
23	60.31	29.82
29	62.67	30.85
36	64.97	33.59
44	68.87	38.27
53	72.25	41.41
67	74.99	43.37
75	76.85	45.90
86	80.23	47.68