PREPARATION AND CHARACTERIZATION OF POLY(D,L-LACTIDE-CO-GLYCOLIDE) MICROSPHERES FOR CONTROLLED RELEASE OF ANTICANCER DRUGS

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF POLY(D,L-LACTIDE-CO-GLYCOLIDE) MICROSPHERES FOR CONTROLLED RELEASE OF ANTICANCER DRUGS

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Breast cancer is the most frequent type of cancer seen in woman. Chemotherapy is one of the most important treatments for breast 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 anticancer 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, it was aimed to prepare such a controlled release system for anticancer drugs which are used in breast cancer treatment by using biodegradable copolymer poly(D,L-lactide-co-glycolide) and to characterize in terms of morphology, size, drug content and drug release rate.

In the first part of this study; empty and drug loaded poly (D,L-lactide-co-glycolide) microspheres were prepared. Two sets of empty poly(D,L-lactide-co-glycolide) microspheres were prepared by solvent evaporation technique with single emulsion (oil/water) to determine the effect of stirring rate on size of microspheres. Increase in stirring rate caused decrease in size of microspheres. Drug loaded poly(D,L-lactide-co-glycolide) microspheres were prepared for controlled release of anticancer drugs which are used in breast cancer treatment namely; 5-fluorouracil, methotrexate and tamoxifen by using solvent evaporation technique either with double emulsion (water/oil/water) or single emulsion (oil/water).

In the second part of this study; empty and drug loaded microspheres were characterized. Inverted light microscopy and scanning electron microscopy were used to examine morphology and size of microspheres. Drug content of microspheres and amount of released drug were determined and drug release profile was obtained for each anticancer drug separetely.

Key words : Controlled drug release, anticancer drugs, biodegradable polymers, poly(D,L-lactide-co-glycolide)

ÖZ

ANTİKANSER İLAÇLARININ KONTROLLÜ SALIMI İÇİN POLİ (D,L-LAKTİD-KO-GLİKOLİD) MİKROKÜRELERİN HAZIRLANMASI VE KARAKTERİZASYONU

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Meme kanseri kadınlarda en sık görülen kanser türüdür. Kemoterapi, meme kanseri için en önemli tedavilerden biridir. Fakat, sistemik toksisite, ilaç dirençliliği ve kandaki değişken ilaç kinetiği kemoterapinin ciddi problemleridir. Biyoyıkımlı polimerlerin antikanser ilaçlarının kontrollü salımı için kullanılması son yıllarda popülarite kazanmıştır. Antikanser ilaçlarının polimer taşıyıcılardan salımının tedavinin etkisini artırması, sistemik toksisiteyi azaltması ve kanser hücreleri tarafından geliştirilen ilaç dirençliğini önlemesi gibi faydaları vardır.

Bu çalışmada, biyoyıkımlı kopolimer poli(D,L-laktid-ko-glikolid) kullanılarak, meme kanseri tedavisinde kullanılan antikanser ilaçları için kontrollü salım sistemi hazırlanması ve morfoloji, büyüklük, ilaç miktarı ve ilaç salım hızları yönünden karakterize edilmesi planlanmıştır.

Bu çalışmanın ilk kısmında, boş ve ilaç yüklenmiş poli(D,L-laktid-ko-glikolid) mikroküreler hazırlanmıştır. Karıştırma hızının mikroküre büyüklüğüne etkisini belirlemek için, boş poli(D,L-laktid-ko-glikolid) mikroküreler tek emülsiyonlu (yağ/su) çözücü buharlaştırma tekniği kullanılarak iki grup olarak hazırlanmıştır. Karıştırma hızının artması, mikroküre büyüklüğünün azalmasına sebep olmuştur. İlaç yüklenmiş poli(D,L-laktid-ko-glikolid) mikroküreler meme kanseri tedavisinde kullanılan 5-fluorourasil, methotreksat ve tamoksifen isimli antikanser ilaçlarının kontrollü salımı için ikili (su/yağ/su) yada tek emülsiyonlu (yağ/su) çözücü buharlaştırma tekniği kullanılarak hazırlanmıştır.

Bu çalışmanın ikinci kısmında; boş ve ilaç yüklenmiş mikroküreler karakterize edilmiştir. Mikroküre morfoloji ve büyüklüğünü incelemek için inverted ışık mikroskobu ve taramalı elektron mikroskobu kullanılmıştır. Mikrokürelerdeki ilaç miktarı ve salınan ilaç miktarı belirlenmiş ve her antikanser ilacı için ayrı salım profili elde edilmiştir.

Anahtar Sözcükler : Kontrollü ilaç salımı, antikanser ilaçları, biyoyıkımlı polimerler, poli(D,L-laktid-ko-glikolid)

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ABBREVIATIONS

PLGA	POLY(LACTIDE-CO-GLYCOLIDE)
PLA	POLY(LACTIDE)
PGA	POLY(GLYCOLIDE)
PVA	POLYVINL ALCOHOL
PEG	POLYETHLENE GLYCOL
5-FU	5-FLUOROURACIL
SEM	SCANNING ELECTRON MICROSCOPY
W/O/W	WATER IN OIL IN WATER
O/W	OIL IN WATER

CHAPTER I

INTRODUCTION

1.1 Origin and Development of Cancer

Cancer is a genetic disease that seriously threats human health. Genetic alterations due to mutations of DNA cause uncontrolled growth of cells in any part of the body, which results in the formation of tumors that have the ability to invade tissues and metastasize to distant organs.

DNA alterations take place during DNA replication. However, these alterations are usually fixed due to the presence of DNA replication fidelity. Mutations that damage DNA can also result due to exposure to some chemical compounds (Bertram, 2001). These chemicals can be found either in the environment or in the foods such as aflatoxin that is found in the improperly stored foods, benzopyrene, dimethylnitrosamine and nickel compounds that are the major constituents of tobacco smoke. Also, radiation such as ultraviolet radiation and ionizing radiation causes DNA mutations, which can lead to cancer such as skin cancer. A small percentage of human cancers are believed to be due to oncogenic viruses.

Carcinogenesis is a multistep process. Interaction of carcinogen with DNA that results in uncontrolled cell division can be considered as tumor initiation, because some of these mutations cause one of these dividing cells to grow rapidly into tumor. Accumulations of additional mutations, activation of oncogenes, inactivation of tumor suppressor genes, defective DNA repair mechanisms are the main factors which cause tumor progression.

1.2 Biology of Cancer

Cancer cells show different properties that distinguish them from normal cells (Rieger, 2004). Their two main characteristics are uncontrolled growth and metastasis. Uncontrolled proliferation is due to damages on the genome of these cells. Metastasis is the ability of cancer cells to move from one part of the body to another part. It occurs in several steps. First, cancer cells secrete digestive enzymes that digest extracellular matrix and cause the invasion of surrouding tissue toward the blood vessels. Then some of the cancer cells enter either into the circulatory system or into the lymphatic system. After that, they adhere to any part of the body and continue to divide.

Formation of new blood vessels that is known as angiogenesis by cancer cells in the tumor favors metastasis. Due to high permeability of these new blood vessels, cancer cells penetrate easily into these vessels and enter into circulatory system. Extensive angiogenesis and so hypervasculature, enhanced vascular permeability and impaired lymphatic drainage are characteristics of tumor cells that is known as enhanced permeability and retention (EPR) effect (Maeda *et al*., 2000) which is schematically shown in Figure 1.1.

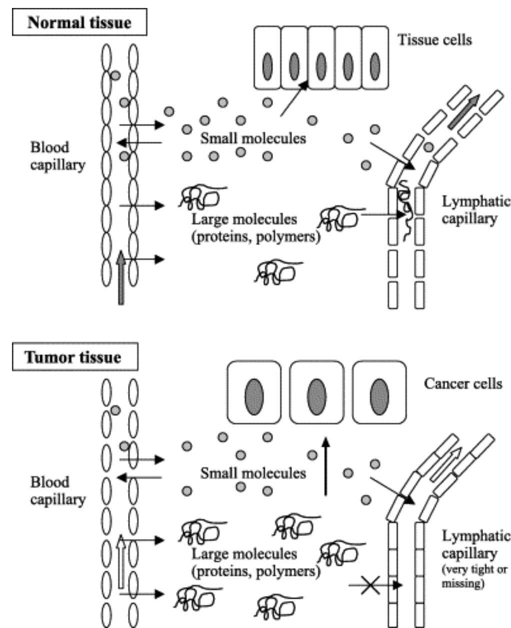

Figure 1.1 Enhanced permeability and retention (EPR) effect (Ulbrich, 2004)

Enhanced vascular permeability of tumor capillaries and impaired or missing lymphatic clearance of macromolecules, which is not observed in normal cells result in accumulation of macromolecules in tumor tissue. This property of cancer cells can be used as a strategy for the delivery of polymer-drug conjugates to the tumor cells (Ulbrich, 2004).

Also cancer cells are different from normal cells in their differentiation. Instead of undergoing differentiation, they continue to abnormal proliferation. For example as in the case of leukemia; differention of leukemic cells is blocked and they continue to proliferate. Some changes that result in the distinctive properties of cancer cells can be observed at the cell surface of cancer cells. They are less adhesive to other cells and to the extracellular matrix components due to reduced expression of cell surface adhesion molecules, which leads to reduction in the interaction with other cells.

Reduced adhesivenes favors the spread of cancer cells to the other sides of the body. Also, reduced adhesivenes causes cytoskeletal and morphological changes. Cancer cells have disorganized cytoskeleton elements and irregular shapes. In addition to these, appearance of some new cell surface proteins that are tumor associated antigens can be seen on some cancer cells.

In normal cells, telomeres at the end of linear chromosomes are shortened after each DNA replication. Telomerase prevents the shortening of chromosomes. Since telomerase is synthesized from telomeres, telomere shortening causes the aging of cells and after many division telomere is lost and cells die. However, maintenance of telomerase activity in some type of cancer cells makes them immortal (Masutomi *et al.*, 2003; Bayne *et al.*, 2005).

1.3 Cancer Chemotherapy

Cancer chemotherapy is the one of the most important ways for the treatment of cancer. While surgery and radiotherapy provide elimination of localized cancer cells, chemotherapy causes the elimination of both localized and metastatic cancer cells.

Cancer chemotherapy is the use of any anticancer drug either to kill the cancer cells or to control their proliferation. Various types of anticancer drugs with different modes of action are available for cancer therapy. Some of the anticancer drugs are antimetabolites of purines, pyrimidines and folic acid. Mercaptopurine, 5-fluorouracil and methotrexate are some of the examples of these kind of agents that interfere with the synthesis of nucleic acids and thereby inhibit DNA synthesis. Another group of anticancer drugs are DNA interacting agents that are known as tumor antibiotics that directly damage the DNA via the noncovalently binding to DNA such as doxorubicin (Di Marco, 1975).

On the other hand, the other group of DNA interacting agents that are known as alkylating agents such as cyclophosphamide damage DNA through covalent linkages. In addition to these, some of the anticancer drugs prevent mitosis due to either inhibition of mitotic spindles from functioning or inhibition of microtubule assembly. Paclitaxel (Schiff *et al.,* 1979) and vincristine are in this group. Tamoxifen is another anticancer drug that has a different mechanism of action than drugs which are mentioned above. It acts as an estrogen antagonist for the estrogen receptors in the breast.

Structure and mechanism of action of 5-Fluorouracil, methotrexate and tamoxifen are described in the following part since these drugs will be used as model drugs in this study.

1.3.1 Chemical Structure and Mechanism of Action of 5-Fluorouracil

5-Fluorouracil that is a pyrimidine antimetabolite has the activity against various types of cancers such as colon, breast and pancreatic cancers (Calabresi *et al*., 1985). It is a fluorinated analog of pyrimidine base uracil as shown in Figure 1.2

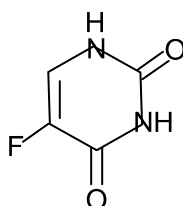

Figure 1.2 Chemical structure of 5-FU

Its active form that is fluorodeoxyuridine monophosphate is the inhibitor of thymidylate synthase that is the key enzyme in thymidylate synthesis. It inhibits DNA synthesis through the inhibition of the production of thymidine base. Its another active form that is fluorouridine triphosphate is incorporated into RNA.

1.3.2 Chemical Structure and Mechanism of Action of Methotrexate

Methotrexate is an antimetabolite of folic acid that is used for the treatment of some types of cancer such as breast cancer, head and neck cancer. It is also used as an anti-inflammatory and an immunosuppressive drug in some non-neoplastic diseases. (Genestier et al., 2000)

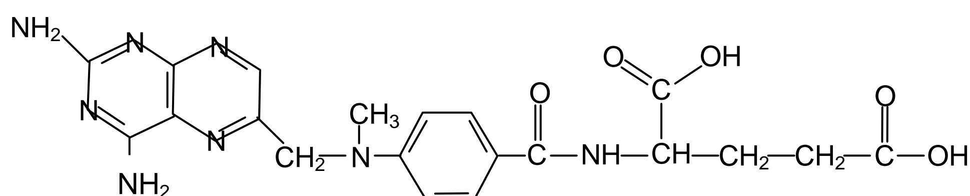

Figure 1.3 Chemical structure of methotrexate

Methotrexate is an inhibitor of enzyme dihydrofolate reductase (DHFR). Enzyme dihydrofolate reductase (DHFR) causes the folic acid functions as cofactor by converting folic acid to reduced folate cofactors (tetrahydrofolate) in the synthesis of DNA and RNA precursors such as thymidylate and purines. Inhibition of this enzyme leads to depletion of cofactors required for the synthesis of thymidylate and purines.

1.3.3 Chemical Structure and Mechanism of Action of Tamoxifen

Tamoxifen that is a nonsteroidal antiestrogen is used for the estrogen dependent breast cancers. It is a triphenylethylene anticancer drug as shown in Figure1.4.

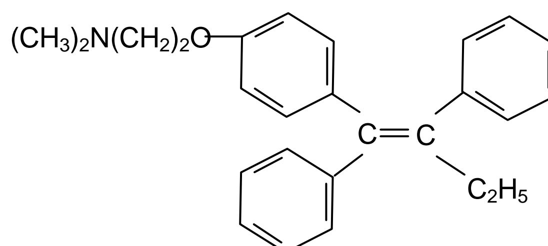

Figure 1.4 Chemical structure of tamoxifen

Although estrogen has beneficial functions such as in female reproduction and in the regulation of bone mineral composition, it is also involved in the development of cancer (Weisz and Bresciani, 1993).

Estrogen exerts its mitogenic affect by binding to two estrogen receptors; Erα and Erβ that are the nuclear receptors of the superfamily of transcription factors. Binding of estrogen to estrogen receptors activates these receptors and they further act on certain DNA sequences and cause the transcription of growth factors and other proteins (Tsai and O'Malley, 1994).

Tamoxifen acts as an estrogen antagonist for the estrogen receptors in the breast. Binding of tamoxifen to the estrogen receptor inhibits the estrogen mediated mitogenic activity in the breast through the suppression of the biological activity of estrogen receptors.

Tamoxifen binds to estrogen receptors in its 4-hydroxytamoxifen form. Tamoxifen is considered as selective tissue estrogen receptor modulater (SERM). Because while acting as an estrogen antagonist in the breast, it acts as an estrogen agonist in the uterus so it can cause the development of endometrium cancer, which is due to the nature of the receptors in certain tissues.

1.4 Problems of Cancer Chemotherapy

Chemotherapy is one of the currently available treatment options for cancer. However, chemotherapy has some drawbacks which effect the success of treatment. One of the most important drawbacks is associated with the toxicity and nonspecificity of the anticancer drugs. Anticancer drugs are highly toxic compounds. They show their effects both on cancer cells and normal cells without discrimination, which leads to side effects. Bone marrow cells, intestinal epithelium cells can be affected adversely. Patients are open to infections due to their low leucocyte counts. Also liver and kidney can be damaged by the toxicity of anticancer drugs that are metabolized through these organs.

Another important problem of the chemotherapy is development of drug resistance by cancer cells. Development of resistance against multiple anticancer drugs is known as multiple drug resistance (MDR). Overexpression of transport proteins that are p-glycoproteins in the membrane of cancer cells causes the efflux of anticancer drugs (Filipits, 2004), which reduces the effectivenes of chemotherapeutic agent and success of treatment. Use of drug combinations in cancer chemotherapy is a way to enhance the efficiency of the treatment.

Combination chemotherapy has important advantages when drugs that have different mechanism of action and minimally overlapping toxicities are selected. However, there are complex interactions between anticancer drugs either synergistic or antagonistic (Smorenburg *et al*., 2001).

In addition, some problems are related to the unstable kinetics of the drug in the blood between dosing periods. In traditional cancer chemotherapy, anticancer drug is administrated at regular time intervals. Drug concentration in the blood fluctuates between dosing periods as shown in Figure 1.5.

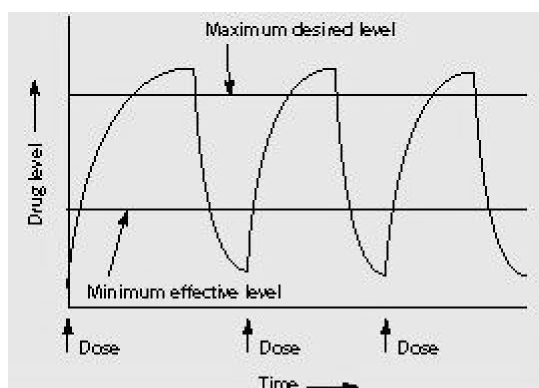

Figure 1.5 Traditional drug delivery dosing
(Brannon-Peppas, 1997)

After the first dose of a drug is administrated into the blood, drug concentration increases rapidly and it can exceed the maximum tolerable concentration above which the drug is highly toxic. After that, drug concentration decreases and it can drop under the minimum effective concentration until the next dose of a drug is given.

Below the minimum effective concentration, drug is not effective, which reduces the success of treatment. Also presence of low concentration of drug in the tumor can contribute to the development of drug resistance.

1.5 Drug Delivery Systems

Presence of drawbacks that include systemic toxicity, nonselectivity for tumor cells over normal cells, insufficient drug concentration in the tumor and development of drug resistance by cancer cells signal the need for different drug delivery strategies. Number of drug delivery systems have been developed to compensate for the problems that arise in chemotherapy. The common objectives of these drug delivery systems are to adjust kinetics of the drug in the blood and to alter tissue distribution of drug in the body in such a way that to enhance efficiency of treatment and to eliminate side effects.

Drug delivery systems aim controlled release of drug from a carrier and/or drug targetting. In controlled drug release systems, drug is combined with a carrier material from which the drug is released. Maintenance of therapeutic drug levels in the blood for a determined time is the main advantage of controlled release systems over traditional chemotherapy as shown in Figure 1.6.

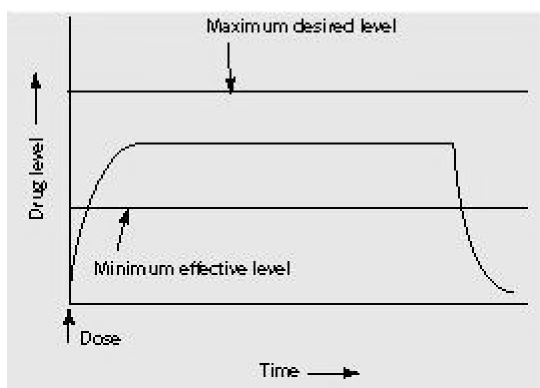

Figure 1.6 Controlled drug delivery dosing (Brannon-Peppas, 1997)

Peaks above the maximum tolerable concentration and below the minimum effective concentration are not observed, which increases the efficiency of treatment and eliminate problems of chemotherapy that are described in Section 1.4.

In addition to controlled drug release, targetting of the drug increases the specificity of the drug for tumor cells over normal cells.

Most widely used drug delivery systems for cancer therapy are liposomes, monoclonal antibodies (drug targetting) and polymeric drug delivery systems.

1.5.1 Liposomes

Liposomes were discovered in 1960s (Bangham *et al.*,1965). In 1970s they were suggested as drug carriers (Gregoriadis, 1976). They have been under extensive study for delivery of a variety of agents such as DNA, antigen, blood components and anticancer drugs since that time.

Liposomes are closed lipid bilayers. Self assembly of phospholipid molecules in an aqueous environment results in the formation of liposomes. Phospholipid molecules form a lipid bilayer in such a way that hydrophilic phosphate heads face to outside and hydrophobic tails are faced to the inside of the bilayer. Both hydrophobic and hydrophilic drugs can be carried by liposomes. While aqueous soluble drugs are being encapsulated into aqueous part, hydophobic drugs are placed within the lipid bilayer.

Use of liposomes for the delivery of anticancer drugs has an advantage of reduced systemic toxicity. They provide a means of passive targetting due to their increased uptake by higher permeable blood vessels in tumors and due to differences in their uptake by several types of cells, tissues and organs (Maruyama, 2000).

However, main drawback of liposomes in drug delivery applications is their instability and short circulation time in the blood. They are uptaken by liver macrophages and eliminated from the circulation by liver and spleen (the reticuloendothelial system) (Gregoriadis *et al*., 1972). One of the mostly studied anticancer drugs for liposomal drug delivery applications is doxorubicin which is an anticancer drug with high systemic toxicity. One of such liposome encapsulated doxorubicin is Myocet TM. Although liposomal formulation of doxorubicin caused reduction in systemic toxicity, Myocet TM showed limited degree of prolonged circulation.(Park, 2002a)

Discovery of liposome surface ligands in late 1980s such as polyoxyethylene (Blume *et al*.,1990) and monosialoganglioside (Gabizon *et al*., 1992) partially increased the circulation time of liposomes in the blood. Preparation of liposomes that contain compounds like cholesterol and high phase transition temperature lipids can improve the stability of liposomes in the blood. Daunoxome that is liposomal formulation of anticancer drug is prepared from high phase transition lipids (Forssen *et al*., 1997). Daunoxome has long circulation time due to its relatively small liposome size and rigid bilayer composition.

In addition, incorporation of hydrophilic polymer polyethylene glycol (PEG) chains to the outer surface of liposomes is another way to increase the blood circulation time of liposomes. These type of liposomes are called stealth liposomes, meaning sterically stabilized liposomes (Storm and Crommelin, 1998).

PEG groups reduced coating (opsonisation) of these liposomes by serving as a barrier against plasma proteins.

Doxil that is pegylated liposomal doxorubicin, is approved for human use. It has a long circulation time in the blood even the most prolonged circulation to date with a half life of 55 hours. However, development of some skin toxicity problems can be seen in people who are treated with Doxil (Park,2002a).

In addition to these**,** heat (Grebe *et al*., 1997), pH and light (Gerasimov *et al*., 1997) sensitive liposomes can be developed for specific functional performance.

Liposomal formulation of other anticancer drugs such as paclitaxel (Crosasso, Ceruti and Brusa *et al*., 2000), 5-fluorouracil lipid analogue (Doi et *al*.,1994), vincristine (Tokudome *et al*., 1996) are in preclinical trials.

To achieve active tumor targetting, liposomes can be covered by specific antibody fragments or specific antibodies that are called monoclonal antibodies for tumor specific membrane proteins, which results in the formation of immunoliposomes.

Monoclonal antibodies can be attached to either surface of the shealth liposomes or to the distal ends of PEG chains. Anti-HER 2 immunoliposomes that were loaded with doxorubicin showed efficient and selective anticancer activity against HER2-overexpressing tumors. Its efficiency was superior to other treatments such as free doxorubicin, liposomal doxorubicin, free monoclonal antibody trastuzumab and combination of trastuzumab with doxorubicin (Park, 2002b).

1.5.2 Monoclonal Antibodies

Use of monoclonal antibodies (mAb) that recognize only cancer cell specific antigens for drug delivery applications provides specific targetting of the drug to cancer cells, which enhances the effect of chemotherapeutic agent and reduces systemic toxicity.

Monoclonal antibodies that were developed by Kohler and Milstein in 1975 (Kohler *et al*.,1975) were produced from a single clone. Monoclonal antibodies have been used for therapy of cancer. Binding of mAb to cancer cell specific antigen causes immunological response on the target cell through antibody dependent cell mediated cytotoxicity and complement dependent cytotoxicity (Waldmann, 2003). However, to increase the efficiency of treatment, mAbs are used with anticancer drugs.

For delivery of anticancer drugs, monoclonal antibodies are used in different ways. One of the ways is the combination of monoclonal antibodies with cytotoxic drugs, which results in the formation of antibody drug immunoconjugates. Trastusumab is a recombinant humanized mAb against the product of HER2 (erbB2, neu) protooncogenes. Although combination of trastuzumab with doxorubicin was highly effective, it resulted in cardiotoxicity (Slamon *et al*., 2001). Therefore in clinical applications combination of trastuzumab with liposomal doxorubicin is prefered .

Antibody Directed Prodrug Therapy (ADEPT) is another way of using monoclonal antibodies for cancer therapy (Senter *et al*.,2001; Bagshawe *et al*., 1999) It is a two step approach to decrease systemic toxicity. Binding of specific antibody in an antibody-enzyme conjugate to the specific antigen on tumor cells is the first step. In the second step, non toxic prodrug which is matched with the pre–targeted enzyme is systemically administrated. Then nontoxic prodrug is converted into toxic drug by the pre-targeted enzyme only in tumor cells.

In addition to these, radiolabeled monoclonal antibodies are used for both diagnosis and treatment of cancer (Serengulam *et al*.,2000).

However use of specific antibodies (monoclonal antibodies) for drug targetting can create difficulty because it is difficult to prepare a specific antibody for a specific antigen and most of the tumor antigens are not known.

1.5.3 Polymeric Drug Delivery Systems

Use of polymeric materials has gained popularity in many areas such as in tissue engineering, in drug delivery applications, implantation of medical devices and bone repair (Drury and Money, 2003; Griffith, 2000; Jagur-Gradzinski, 1999; Hubbel, 1998, Lee and Mooney, 2001; Hoffman, 2002).

Polymers can be used in different ways to deliver drugs. Drug can be contained either by the polymer (reservoir devices) or within the polymer network (matrix devices). Also drug carrying polymer can contain an attached additive such as additional polymer, hydrophilic component to improve the release of drug from the polymeric carrier.

Another application of polymers is the use of polymers in polymer-drug conjugates for the delivery of anticancer drugs into tumor tissues. Conjugation of drugs with soluble biocompatible carriers may cause the accumulation of drug mostly into cancer cells. This passive targetting of drugs depends on the physiological features of the tumor cells that are not seen in normal cells. Presence of enhanced vascular permeability and poor lymphatic clearance in tumor cells cause the accumulation of large molecules in tumors as described in Section 1.2. Different combinations of polymer, drugs and linkers have been developed (Ulbrich, 2004) so that they can show reduced toxicity towards normal tissues and more cytotoxicity to cancer cells than the free drug. .

Polymer microspheres are the one of the largest growing applications of polymers in controlled drug release systems. Polymer microspheres are carrier systems in which drug can be either encapsulated by a polymer coat or entrapped within the polymer network. An ideal controlled release system for a device should show zero order drug release that means release of drug is independent of time.

1.5.3.1 Biodegradable Polymers in Controlled Drug Release

In controlled drug release systems biodegradable polymers are increasingly utilized for controlled release of variety of drugs. One of the main advantages of using biodegradable polymers is that surgical removal of the drug carrier is not required after the drug release is completed. They degrade into smaller fragments which can be excreted from the body.

Biodegradable polymers for controlled drug release systems are either natural or synthetic in origin (Chandra and Rustgi, 1998). Natural biodegradable polymers are found in the cells of organisms (animals and bacteria). They can be polysaccharides (alginic acid, chitosan), peptides of natural origin (gelatin) and bacterial polyesters.

Biodegradable polymers for controlled drug release systems must have biocompatibility, required mechanical properties, required biodegradation kinetics and drug compatibility.

Biocompatibility means, both the polymeric material and its degradation products should be nontoxic and should not harm to body. Also they should not create any inflammatory response, immunogenic reactions, allergic reactions and cancer. Natural polymers have the advantage of biocompatibility. However, some drawbacks associate with natural biodegradable polymers. One of the drawback is related to their mechanical properties. They have shorter or longer degradation time than the desired one. Another disadvantage is that they are obtained in limited amounts. Also some purity and batch to batch variation problems can be observed with natural biodegradable polymers.

Various synthetic biodegradable polymers have been increasingly used and studied in controlled drug delivery systems due to lack of most of the drawbacks associated with the natural biodegradable polymers. Synthetic biodegradable polymers have advantages over natural ones in controlled drug release systems.

One of the most important advantage is adjustment of drug release rate and time in accordance to the desired way by altering the properties of polymer. Some of these properties include polymer molecular weight, crystallinity, hydrophobic/hydrophilic properties that effect the degradation rate of polymers. Biodegradation of these polymers take place through the hydrolysis of unstable linkages in their backbone.

Another advantage of synthetic biodegradable polymers is their batch-to-batch uniformity. Also, more reliable sources of synthetic biodegradable polymers are present.

Some of the studied synthetic biodegradable polymers in controlled drug release systems are polyesters, polyorthoesters (Deshpande *et al*., 1998), polyanhydrides (Kipper *et al*., 2002).

The most widely used and studied type of synthetic biodegradable polymers are polyesters of poly(lactide), poly(glycolide) and especially their copolymers poly(lactide-co-glycolide) due to their excellent biocompatibility and controllable biodegradability.

1.5.3.2 Poly(lactide), poly(glycolide) and poly(lactide-co-glycolide)

Use of these polymers for biomedical applications has a long history. Polymers of glycolic acid and lactic acid were firstly used as biodegradable sutures (Gilding *et al* ., 1979). In 1970s Yolles and coworkers reported the use of lactic acid based polymers for drug delivery (Conti *et al*., 1991), since then these polymers have been used for controlled release of variety of agents and also as medical devices.

Ring opening polymerization of the monomers of these polymers results in the high molecular weight polymers. Each of them have different degradation rate due to their different properties.

Poly(glycolide) (PGA) is the simplest aliphatic polyester. Monomer of PGA is glycolide, which is synthesized from the dimerization of glycolic acid. Ring opening polymerization of glycolide results in the formation of poly(glycolide) as shown in Figure 1.7.

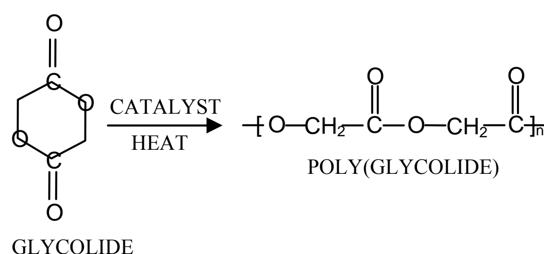

Figure 1.7 Synthesis and structure of poly(glycolide)

Lactide monomer is the cyclic dimer of lactic acid. Ring opening polymerization of lactide results in the formation of poly(lactide) as shown in Figure 1.8.

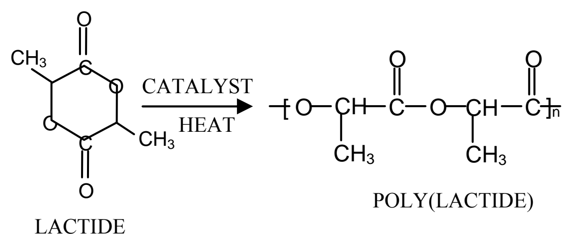

Figure 1.8 Synthesis and structure of poly(lactide)

There are two optical isomers of lactic acid, L-lactic acid and D-lactic acid. Polylactide polymers are group of polymers; poly(L-lactide) and poly(D,L-lactide). High regularity of Poly(L-lactide) chain structure leads to semi crystalline polymer. On the other hand, poly(D,L-lactide) that consist of irregular distribution of both L-lactide and D-lactide is an amorphous polymer (Wu, 1995). Semicrystalline poly(L-lactide) is more resistant to hydrolytic degradation than amorphous poly(D,L-lactide) since cristallinity hinders biodegradation, which causes longer degradation time for poly(L-lactide) than poly(D,L-lactide).

PGA and poly(L-lactide) (PLLA) show high tensile strenght (Middeleton and Tipton, 2000) which makes them more suitable for orthopedic applications such as load bearing applications in which degradation of polymeric implant allows the slowly transfer of load to healing bone. (Athanasiou *et al*., 1998) Barber reported the list of commercially available orthopedic devices, most of which were made of PLLA (Barber, 1988).

To improve and extend the properties of these polymers, their copolymers are designed for both device and drug delivery applications. Poly(lactide-co-glycolide) (PLGA) is a copolymer of lactide and glycolide monomers as shown in Figure 1.9.

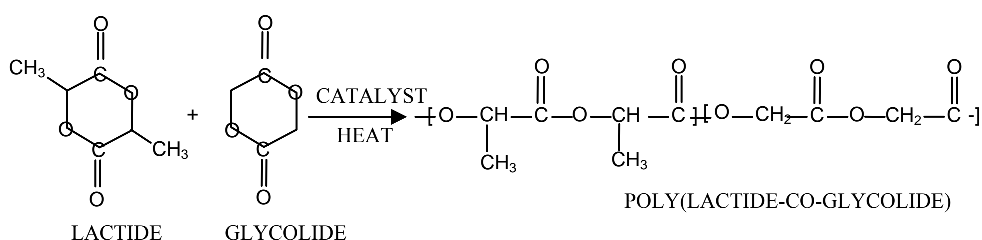

Figure 1.9 Structure and synthesis of poly(lactide-co-glycolide)

PLGAs prepared from PLLA and PGA are crystalline copolymers, on the other hand PLGAs that consist of D,L-lactide and glycolide are amorphous. (Lewis., 1990; Wu., 1995). Poly(D,L-lactide-co-glycolide) is more amorphous than poly (D,L-lactide), which makes Poly(D,L-lactide-co-glycolide) more suitable for drug delivery applications.

Biodegradable poly(D,L-lactide-co-glycolide) copolymers with different lactide/glycolide ratios have been increasingly utilized for controlled release of anticancer drugs due to their excellent biocompatibility (Athanasiou *et al*., 1996) and controllable biodegradability (Lu *et al*., 2000).

Among poly(D,L-lactide-co-glycolide) copolymers; lactide:glycolide 50:50 has the fastest degradation rate. Copolymers with lactide:glycolide 65:35, 75:25 and 85:15 have progressively longer in vivo half lives. Copolymers with lower lactide/glycolide ratio degrade faster than copolymers with higher lactide/glycolide ratio. Since lactic acid is more hydrophobic than glycolic acid, copolymers with higher lactide/glycolide ratio are more hydrophobic, so absorb less water and degrade more slowly.

Hydrolysis of ester bonds in aqueous environments such as body fluids results in the degradation of polymer PLGA into its monomers that are lactide and glycolide, which do not cause harm to body. These degraded products are eliminated by the Krebs cycle as carbondioxide and water and excreted from the kidney in the urine**.**

One of the most widely used and studied controlled release system is PLGA microsphere system. PLGA microspheres were prepared for controlled release of variety of agents; for small molecule drugs (Boisdron-Celle *et al*., 1995; Yeh, *et al*., 2001; Birnbaum *et al*., 2000; Reza and Whateley, 1998; Giordano *et al*., 1993; Feng *et al*., 2000), for protein delivery (Cao and Schoichet, 1999), for viral delivery (Beer *et al*., 1998).

They can provide controlled release of drugs at a desired rate and in a desired time. Rate of drug release from microspheres depends on polymer type and properties, molecular weight, monomer composition, processing conditions and properties of drug.

Polymer microspheres can be prepared by using different methods such as solvent evaporation, spray drying, solvent extraction with polymer precipitation and multiple emulsion. Solvent evaporation based on emulsion is the common method for the preparation of drug loaded PLGA microparticles.

Paclitaxel is the one of the mostly studied anticancer drugs in controlled release systems with these type of polymers under different experimental conditions. A group of scientist prepared controlled release systems for paclitaxel by changing the experimental conditions (Feng and Huang., 2001; Mu and Feng., 2002; Feng *et al*., 2002; Mu and Feng., 2001). Recent study of Mu and Feng (2003) showed release profile of paclitaxel from nanoparticles that were prepared from poly(D,L-lactide) and poly (D,L-lactide-co-glycolide) with two different monomeric ratios (75:25 and 50:50) by using vitamin E TPGS as the emulsifier. While PLGA (75:25) and PLGA(50:50) were being showed similar release properties, PLLA gave the slowest release (Mu and Feng ., 2003).

In another study paclitaxel release from microspheres of new kind of polymer PLA-PEG-PLA triblock copolymer was performed (Ruan and Feng., 2003). In corporation of PEG that is hydrophilic polymer into hydrophobic PLA chain, which results in the formation of triblock copolymer PLA-PEG-PLA enhanced the drug release.

Some of the studies prepared PLGA microspheres that contain combination of chemotherapeutic agents and also their separete formulations, such as combination of paclitaxel with 5-FU (Gupte *et al*., 2004) and combination of antisense oligonucleotides against oncogenes with 5-FU (Hussain *et al*.,2002). Also they were performed the cell culture cytotoxicity studies.

In addition, more recent study of Lin *et al*. (2005) performed in-vitro release of doxorubicin from PLGA microparticles that were prepared by spray drying technique.

1.6 Aim of The Study

Use of biodegradable polymer microspheres for controlled release of anticancer drugs has advantages of improvement in the efficiency of the treatment, reduction in systemic toxicity and prevention of drug resistance developed by the cancer cells. Aim of this study is to prepare controlled release systems for anticancer drugs, 5-fluorouracil, methotrexate and tamoxifen which are commonly used in breast cancer treatment. Biodegradable copolymer poly(D,L-lactide-co-glycolide) was used in the preparation of microparticle carriers and they are characterized in terms of morphology, encapsulation efficiency and drug release rates.

In this study, prepared controlled release systems for anticancer drugs was aimed to be used in further studies of cell culture experiments. The main aim of the cell culture experiments will be the testing of the development of drug resistance in breast cancer cell lines MCF7 to compare direct drug application and drug release from poly(D,L-lactide-co-glycolide) microspheres.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Poly(D,L-Lactide-co-Glycolide) (75:15, MW: 90,000-126,000) was purchased from Sigma Chemicals.

Phosphate buffered saline tablets and polyvinyl alcohol were purchased from Sigma Chemicals and chloroform was purchased from Sure Chem. Products LTS.

2.2 Methods

2.2.1 Preparation of PLGA Microspheres

2.2.1.1 Preparation of Empty PLGA Microspheres

Empty PLGA microspheres were prepared by using solvent evaporation technique with single emulsion (oil in water o/w) at room temperature.

Two sets of empty PLGA microspheres were prepared. For each, certain amount of PLGA (50mg) was dissolved in organic solvent chloroform (2ml), which was then added into 50ml of aqueous polyvinyl alcohol (PVA) solution (2% w/v) with a dropwise fashion. The mixtures were then mechanically stirred with a magnetic stirrer at 400 rpm and at 800 rpm for 4 hours. The resulted oil in water emulsion was further stirred overnight at 200 rpm and at 400 rpm to completely evaporate the organic solvent. Afterwards, microspheres were obtained by centrifugation at 9000 rpm (Sigma Centrifuge 3K30) for 10 min. Then they were washed with distilled water and dried.

2.2.1.2 Preparation of Drug Loaded PLGA Microspheres

Drug loaded poly(D,L-lactide-co-glycolide) microspheres were prepared for controlled release of anticancer drugs namely 5-FU, methotrexate and tamoxifen. Drug loaded PLGA microspheres were prepared by using solvent evaporation technique either with double emulsion (water in oil in water) or single emulsion (oil in water) at room temperature.

For the preparation of 5-FU loaded poly(D,L-lactide-co-glycolide) microspheres and methotrexate loaded poly(D,L-lactide-co-glycolide) microspheres the same procedure was followed. Aqueous solution of each drug (5mg 5-FU in 0.1 ml aqueous solution and 5mg methotrexate in 0.2 ml aqueous solution) that is called initial (internal) aqueous phase was added into organic phase that contains dissolved polymer PLGA (50mg) in solvent chloroform (2ml, organic phase) and mixed. The mixture was rapidly added into 50 ml of aqueous PVA solution (2% w/v) with a dropwise fashion. Then the mixture was stirred at 800 rpm for 4 hours. The resulted water in oil in water emulsion (double emulsion w/o/w) was further stirred overnight at 400 rpm to completely evaporate the organic solvent. Afterwards microparticles were obtained by centrifugation at 9000 rpm (Sigma Centrifuge 3K30) for 10 min. Then they were washed with distilled water and dried.

For the preparation of tamoxifen loaded poly(D,L-lactide-co-glycolide) microspheres; certain amount of drug (1mg) was added to organic solvent chloroform (2 ml) with the polymer PLGA (50mg). The organic phase was added into 50 ml of aqueous PVA solution (2% w/v) with a dropwise fashion. The formed mixture was mechanically stirred with magnetic stirrer at 800 rpm for 4 hours. The resulted oil in water emulsion (single emulsion o/w) was further stirred overnight at 400 rpm to completely evaporate the organic solvent. Afterwards microspheres were obtained by centrifugation at 9000 rpm (Sigma Centrifuge 3K30)) for 10 min. Then they were washed with distilled water and dried.

2.2.2 Characterization of Microspheres

2.2.2.1 Morphological Analysis of Microspheres

Morphological characteristics of microspheres were examined by both inverted light microscopy (Olympus CKX41) and scanning electron microscopy (SEM, Noran, JSM-6400).

Size and shape of microspheres were analyzed firstly by inverted light microscopy and micrographs were obtained.

Scanning electron microscopy (SEM, Noran, JSM-6400) was used to determine shape, size and surface morphology of the microspheres. Before analysis by SEM, samples were coated with gold by sputter coating technique. According to the SEM micrographs of the microspheres, size of microspheres were determined and size distribution profiles were obtained by counting the number of microspheres from SEM micrographs of different areas.

2.2.2.2 Determination of Drug Content of Microspheres

Drug content of microspheres were determined by evaluating the unloaded drug amount both in the initial supernatant obtained after centrifugation and in the washing solution by spectrophotometer (Shimadzu, UV-1208) at 266 nm for 5-FU, at 258 nm for methotrexate and at 274 nm for tamoxifen. Encapsulation efficiency is the ratio of the amount of encapsulated drug to the amount of drug used in the preparation of drug loaded microspheres. Encapsulation efficiencies were calculated according to the formula:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Weight of drug loaded}}{\text{Weight of drug input}} \cdot 100$$

2.2.2.3 Drug Release from Microspheres

In-vitro drug release from microspheres was performed by incubation in phosphate buffered saline (PBS, 0.01M, pH 7.4) at 37° C.

Microspheres (4mg) were placed in release medium phosphate buffered saline (PBS, 2ml, 0,01M, pH 7.4) and shaken with orbital shaker at 37° C. At various time points by the end of 1. 4. 6. 10. and 14. days the samples were removed and centrifuged at 9000 rpm for 15min (micromax RF) and the supernatant was removed and replaced with an equivalent volume of PBS. The supernatant was analyzed by spectrophotometer at 266 nm for 5-FU, at 258 nm for methotrexate and at 274 nm for tamoxifen. For each drug, calibration curve was prepared with known amounts of drug, one of them is shown below in Figure 2.1 as an example and amount of released drugs were determined.

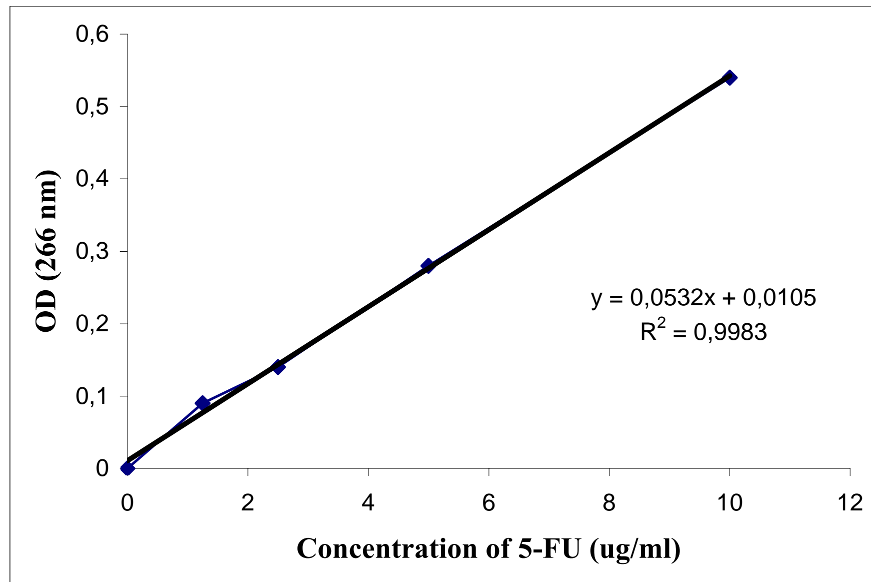

Figure 2.1 Calibration curve for 5-FU

5-FU concentration is 0-10 μg/ml in phosphate buffer (pH 7.4)

Results of duplicate tests were used to calculate the percentage of average amount of released drug. Drug release profiles were obtained from the cumulative values of released drug percentages.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Morphological Analysis of Microspheres

3.1.1 Inverted Light Microscopy Analysis

All the prepared samples were analyzed by the inverted light microscopy to visualize the microspheres and to observe their shapes.

Observation of the samples by the inverted light microscopy confirmed the formation of microspheres.

Empty PLGA microspheres were examined to observe the effect of stirring rate on microsphere size. Microspheres that were prepared at stirring rate of 800 rpm as shown in Figure 3.2 were smaller than the microspheres that were prepared at stirring rate of 400 rpm as shown in Figure 3.1. As observed from the figures the size distribution of the microspheres was more uniform in Figure 3.2 than that of Figure 3.1. Therefore, drug loaded PLGA microspheres were prepared at 800 rpm in order to obtain small and more uniform microspheres.

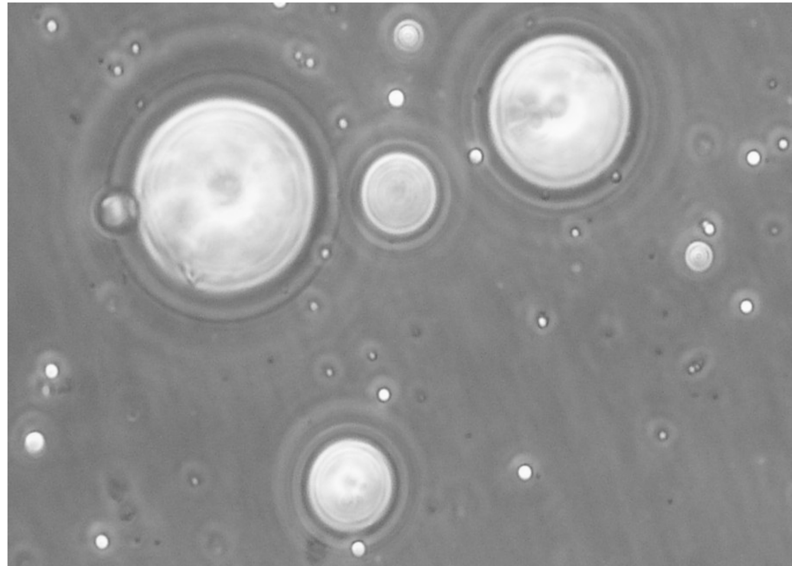

Figure 3.1 Inverted light microscopy micrograph (40x) of empty PLGA microspheres prepared at stirring rate of 400rpm.

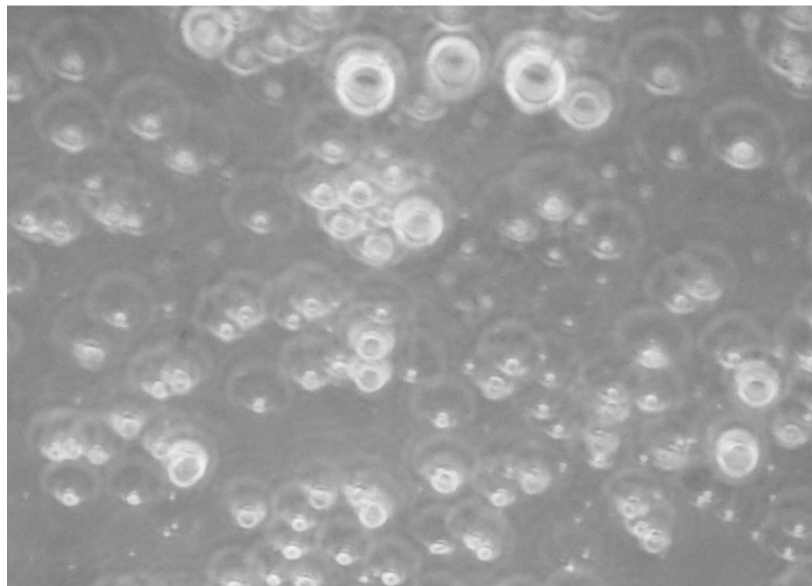

Figure 3.2 Inverted light microscopy micrograph (40x) of empty PLGA microspheres prepared at stirring rate of 800rpm.

The analysis of 5-FU, methotrexate and tamoxifen loaded PLGA samples by inverted light microscopy also confirmed the formation of microspheres as shown in Figure 3.3, 3.4 and 3.5.

From the figures it was observed that; there were differences in size and shape of 5-FU, methotrexate and tamoxifen loaded PLGA microspheres. Observation of the 5-FU loaded PLGA microspheres from Figure 3.3 revealed that they were greater in size compared to others. As observed from Figure 3.4, size of the methotrexate loaded microspheres were close to 5-FU loaded microspheres and greater than the tamoxifen loaded microspheres. On the other hand, size of tamoxifen loaded microspheres were smaller compared to others and all of the microspheres were spherical in shape as observed in Figure 3.5.

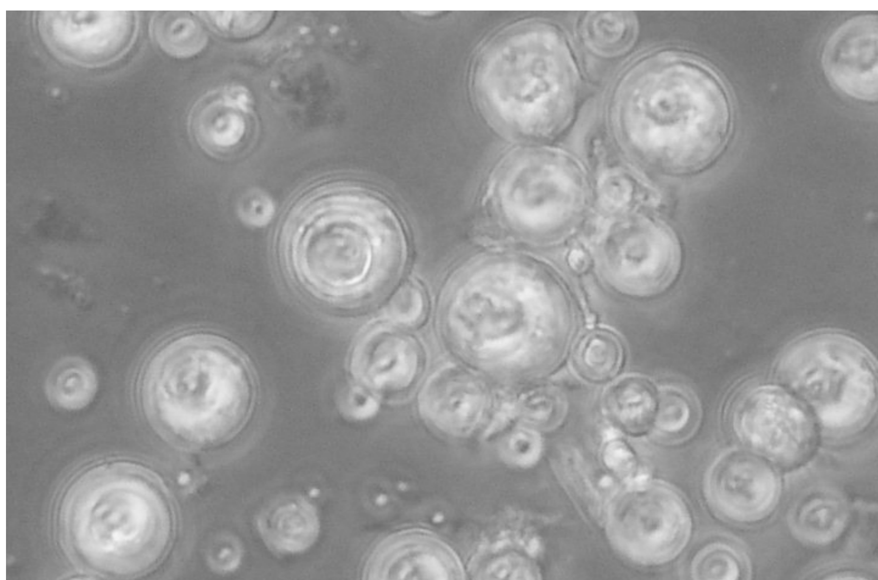

Figure 3.3 Inverted light micrograph (40x) of 5-FU loaded PLGA microspheres

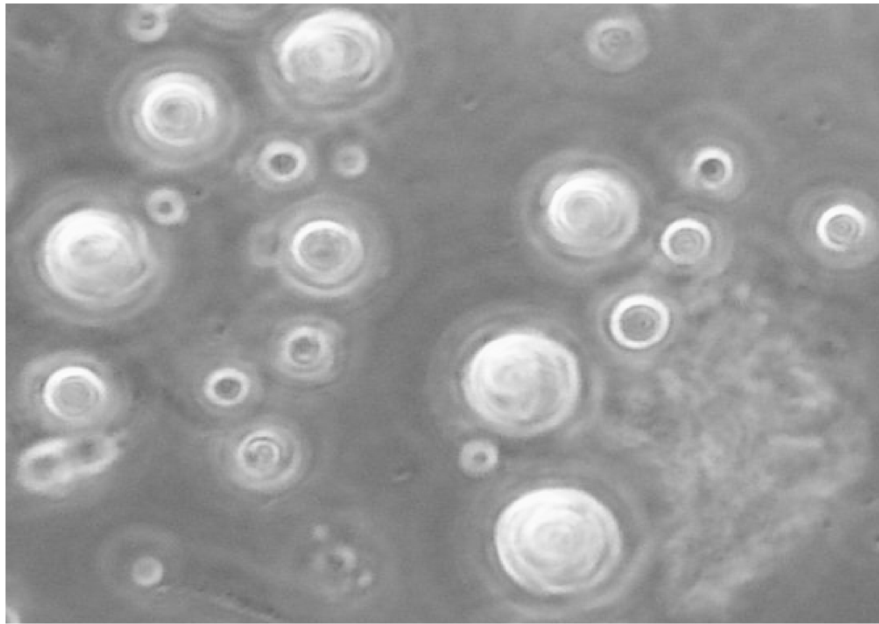

Figure 3.4 Inverted light micrograph (40x) of methotrexate loaded PLGA microspheres

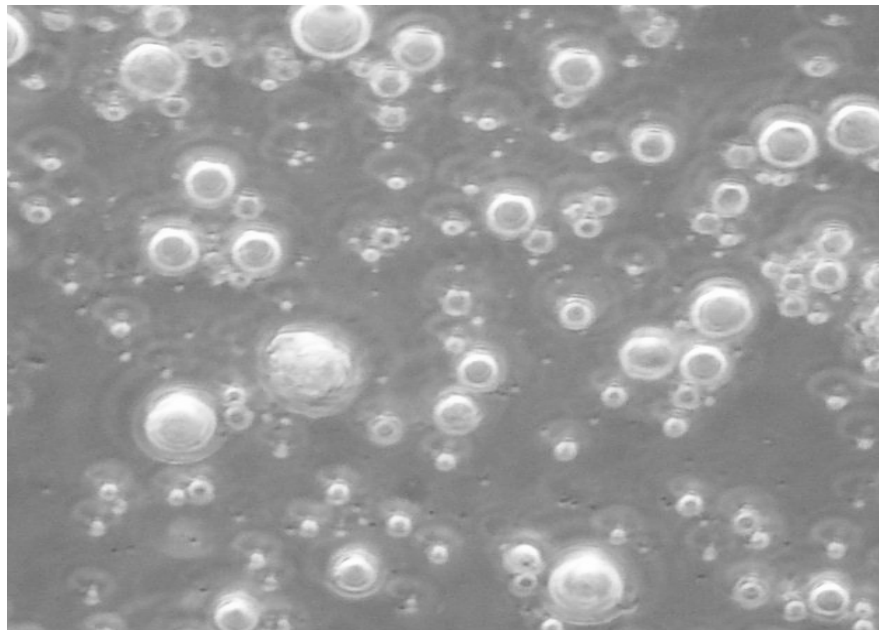

Figure 3.5 Inverted light micrograph (40x) of tamoxifen loaded PLGA microspheres

3.1.2 Scanning Electron Microscopy Analysis

Morphological characteristics; size, shape and surface properties of 5-FU, methotrexate and tamoxifen loaded microspheres were analyzed by scanning electron microscopy (SEM) after the samples were coated with gold. Size of the microspheres were determined from SEM micrographs and size distribution profiles were obtained by counting the number of microspheres from SEM micrographs of different regions.

Scanning electron microscopy observations showed that 5-FU loaded microspheres that were prepared by solvent evaporation technique with double emulsion were mostly spherical in shape, although there were some rare nonspherical ones. Some microspheres had smooth surfaces and some had rough surface. There were pores and deformations on the surface of some of the microspheres as seen in Figure 3.6.

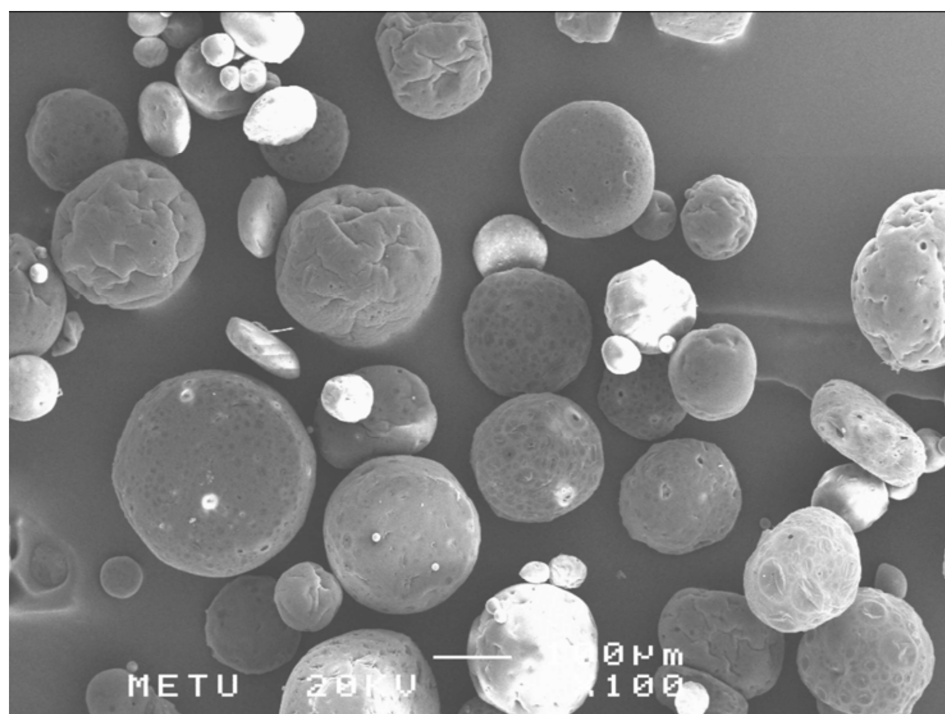

Figure 3.6 Scanning electron micrograph of 5-fluorouracil loaded PLGA microspheres after coating the sample with gold.

Analysis of methotrexate loaded microspheres revealed that they were mostly spherical in shape. Most of the microspheres had smooth surface and also pores were observed on some of the microspheres as shown in Figure 3.7.

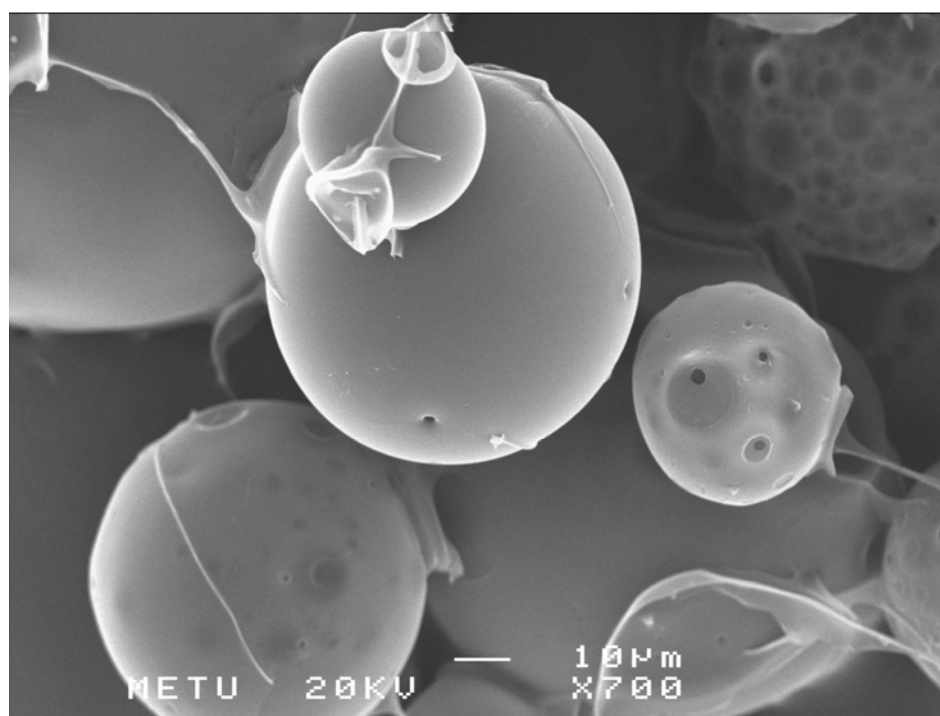

Figure 3.7 Scanning electron micrograph of methotrexate loaded PLGA microspheres after coating the sample with gold.

On the other hand, SEM analysis of tamoxifen loaded microspheres that were prepared by solvent evaporation technique with single emulsion showed that they were spherical in shape and they had smooth surfaces as shown in Figure 3.8 and in Figure 3.9. As observed from these micrographs, there were no nonspherical microparticles and size distribution was more uniform compared to 5-FU and methotrexate loaded PLGA microspheres.

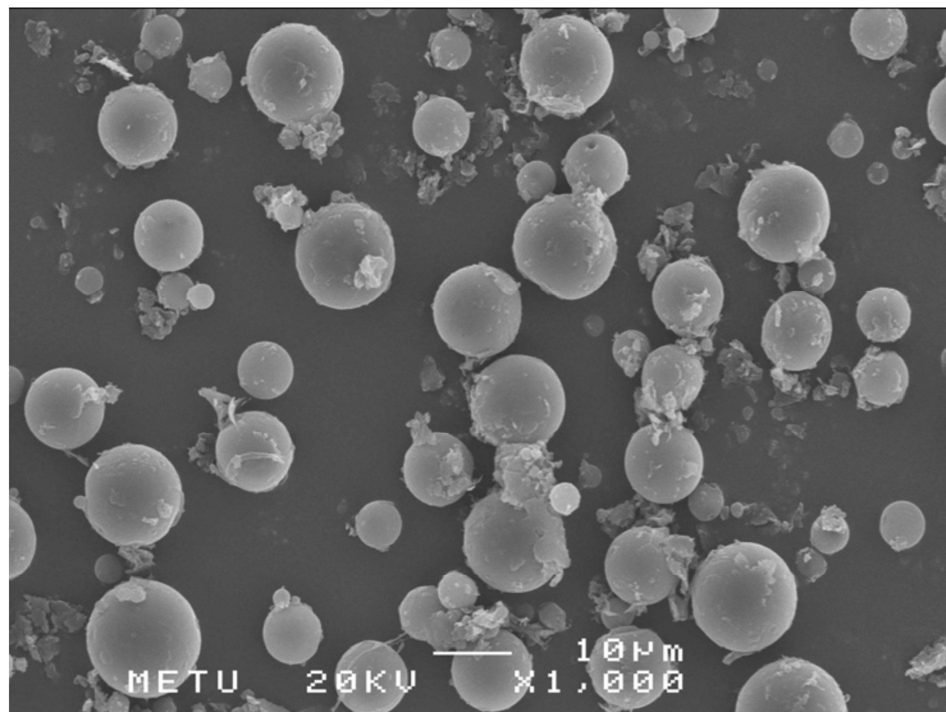

Figure 3.8 Scanning electron micrograph of tamoxifen loaded PLGA microspheres after coating the sample with gold.

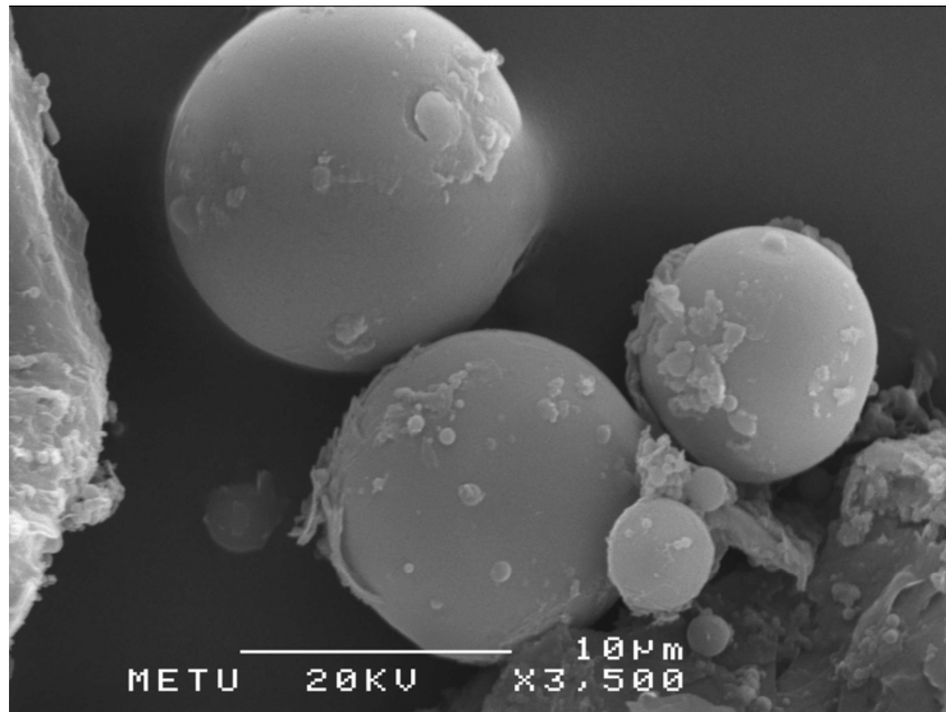

Figure 3.9 Scanning electron micrograph of tamoxifen loaded PLGA microspheres after coating the sample with gold.

Observations above were consistent with the report of the Rosca *et al.* (2004). They have reported that while spherical microparticles are formed in single emulsion formulations, microparticles with different morphologies were produced in double emulsion formulations. Nonspherical, deformed microspheres that were prepared by double emulsion procedure could be generated due to the coalescence of the inner aqueous microdroplets in the emulsion droplet into one final microdroplet that had nonspherical structure during the solvent evaporation. Also breaking of the polymer wall causes the formation of holes on the surface of microspheres that were prepared by double emulsion.

According to Rosca *et al.* (2004); the reason for the generation of microparticles with different morphologies was due to different drug content of microparticles in double emulsion formulations. They have reported that presence of either one or more than one inner aqueous microdroplets in the emulsion droplet causes the generation of microparticles with different morphologies.

Presence of one inner aqueous microdroplet in the emulsion droplet causes the formation of either close or open microcapsules. Closer diameter of inner aqueous microdroplet to the diameter of emulsion microdroplet causes the breaking of the polymer wall during the solvent evaporation and causes the formation of open microcapsules. Whereas, presence of one inner microdroplet with much smaller diameter than the diameter of emulsion microdroplet causes the generation of close microcapsules.

On the other hand, it has been also reported in the study of Rosca *et al.* (2004) that; more than one inner aqueous microdroplets in the emulsion microdroplet with diameter few times smaller than the diameter of emulsion microdroplet causes the formation of microcapsules. Inner microdroplets can coalesce into one final microdroplet and change the spherical shape. Whereas presence of more than one inner aqueous microdroplets in the emulsion microdroplet with diameter much smaller than the diameter of emulsion microdroplet generates honeycomb structure. However, in single emulsion formulations coalescence and breaking of the polymer do not occur, therefore microspheres with smooth surfaces can be obtained.

The size of the microspheres could be determined by SEM micrographs. Size of 5-FU loaded microspheres ranged between 14-314 µm (Figure 3.10) and most of them were smaller than 100µm. Size of the methotrexate loaded microspheres were in a range of 15-185 µm (Figure 3.11) On the other hand, there were significant difference in the size range of tamoxifen loaded microspheres. Their size ranged between 1-17 µm (Figure 3.12) which is much smaller than the microspheres loaded with either methotrexate or 5-FU. Size distribution of tamoxifen loaded microspheres were also more uniform compared to that of 5-FU or methotrexate loaded microspheres.

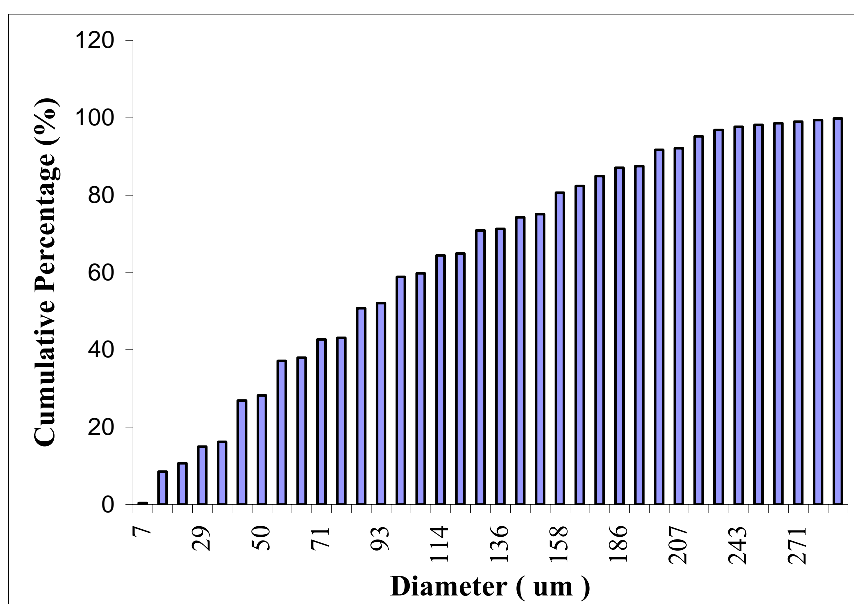

Figure 3.10 Size distribution of 5-FU loaded microspheres (ranged between 14-314 µm) obtained from SEM micrographs by counting the number of microspheres in selected regions.

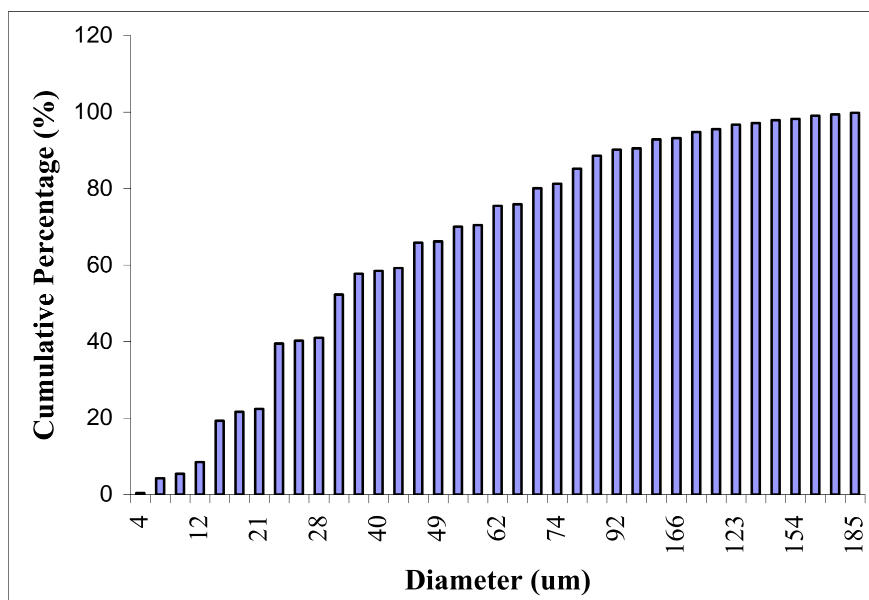

Figure 3.11 Size distribution of methotrexate loaded microspheres (ranged between 15-185 µm) obtained from SEM micrographs by counting the number of microspheres in selected regions.

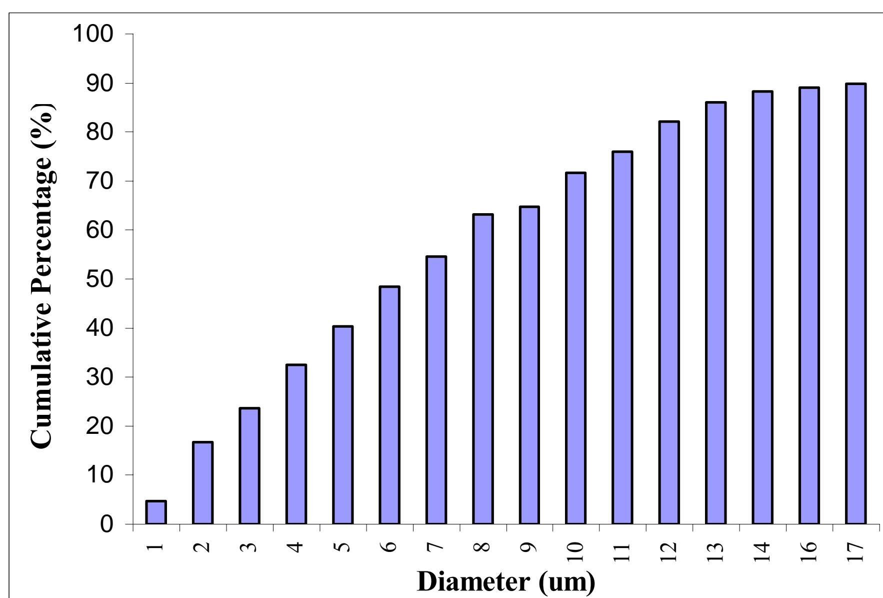

Figure 3.12 Size distribution of tamoxifen loaded microspheres (ranged between 1-17 µm) obtained from SEM micrographs by counting the number of microspheres in selected regions.

When the size of microspheres were compared it was seen that 5-FU and methotrexate loaded microspheres were generally larger in size than tamoxifen loaded microspheres. This difference could be originated due to their preparation method (solvent evaporation technique either with double emulsion or single emulsion). Coalescence of aqueous microdroplets into one final microdroplet in the solvent evaporation with double emulsion procedure (Rosca *et al*, 2004) could cause the formation of larger microspheres. Since coalescence does not occur in single emulsion procedure, smaller microspheres could be generated. However, in addition to this, other experimental factors affected the size of microspheres such as, type and rate of mixing, properties and initial concentration of drug and polymer, volumes of organic and aqueous phases, type and amount of emulsifier. In the literature size of drug loaded particles ranged from nanospheres to microspheres according to changed experimental parameters.

Microparticles of size up to 250µm and ideally less than 125µm are suitable for injectable biodegradable polymer microparticles to use them as controlled release dosage forms (Tice *et al*., 1991). In this study; when the cumulative size distribution graphs were examined, 60 % of the 5-FU loaded PLGA microspheres, 95 % of the methotrexate loaded PLGA microspheres and all of the tamoxifen loaded PLGA microspheres were smaller than 100µm.

3.2 Determination of Drug Content of Microspheres

Encapsulation efficiency is the ratio of the amount of encapsulated drug to that of the drug used for microsphere preparation. Encapsulation efficiencies were calculated according to the formula that was described in Section 2.2.2.2. Encapsulation efficiencies were calculated as 13% for 5-FU, 11% for methotrexate and 9.7% for tamoxifen.

In the study of Gupte *et al*. (2004), 5-FU and paclitaxel loaded PLGA microspheres and their separete formulations were prepared. They have reported the encapsulation efficiency for paclitaxel as 90%, which was attributed to hydrophobic nature of drug and polymer. Also they have reported the encapsulation efficiency of 5-FU alone as 19%. On the other hand encapsulation efficiency of 5-FU increased to 30% when it was combined with paclitaxel.

In this study, encapsulation efficiency that was calculated for 5-FU (13%) is close to the value in the literature. Low encapsulation efficiency for 5-FU could be attributed to hydrophilic nature of this drug. Since 5-FU had high water solubility, some of the drug may escape into the external aqueous phase during the preparation. Also there may be some drug lost in the washing solution,which reduced the encapsulation efficiency. Also, study of Liggin and coworkers (Liggin *et al*., 2000) attributed the high encapsulation efficiency of hydrophobic drug paclitaxel to its retention in the organic phase.

In the study of Muvaffak (2003), the calculated encapsulation efficiency for 5-FU loaded gelatin microspheres was between 55.85-68.21% and the calculated encapsulation efficiency for methotrexate loaded gelatin microspheres was between 60.28-77.01%. These observed higher encapsulation efficiencies might be due to differences in the preparation of drug loaded microspheres. In the study of Muvaffak (2003), drug loaded microspheres were prepared by performing water in oil emulsion (single emulsion). Drug was put into aqueous phase together with the gelatin, which could be the cause of higher encapsulation efficiency. Also the type of the polymer and some other parameters such as mixing, centrifugation etc. could be the cause of different encapsulation efficiencies.

On the other hand, in the study of Cascone *et al.* (2003), encapsulation efficiency for methotrexate loaded gelatin nanospheres was between 5.6% and 15.6% according to changed initial drug content. Although methotrexate loaded gelatin nanospheres were prepared by water in oil emulsion (single emulsion) formulation as in the study of Muvaffak (2003), quite different encapsulation efficiencies were found by these two groups. This difference may originate from preparation of nanospheres rather than micospheres and differences in the experimental parameters and conditions.

In the study of Brigger *et al*. (2001) encapsulation efficiency was calculated approximately as 80% (+/-10) for tamoxifen loaded poly (PEGCA-co-HDCA) nanoparticles. In this study, encapsulation efficiency was calculated as 9.7% for tamoxifen. In this case, although a hydrophobic drug was added to organic phase, a low encapsulation efficiency was found compared to other studies. Use of different type of polymer and experimental parameters and conditions in other studies could cause the appearance of different results.

3.3 Drug Release Studies

Release of 5-FU from PLGA microspheres were determined by incubation in PBS buffer (0.01M, pH 7.4) at 37°C as described in Section 2.2.2.3. Released drug amount was determined by spectrophotometer at 266 nm wavelength and cumulative percent average drug release versus time graph was obtained.

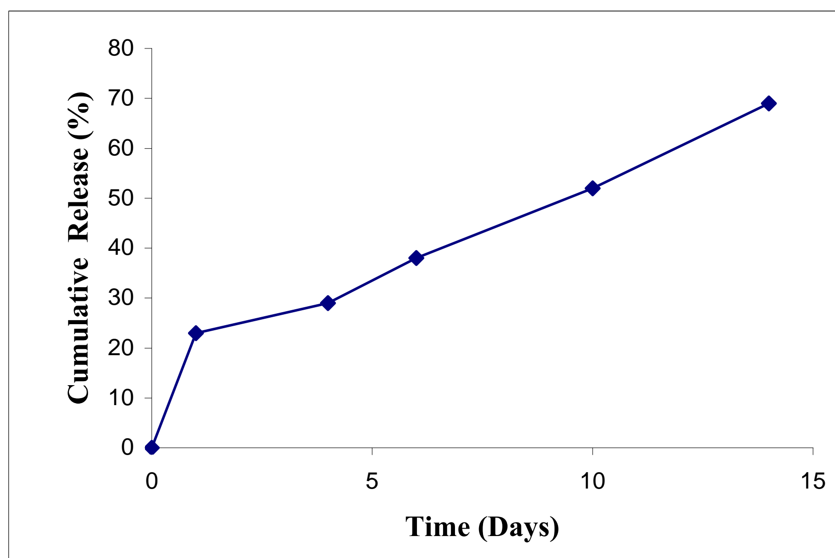

Figure 3.13 Release profile for 5-Fu from PLGA microspheres

In the release profile of 5-FU, early rapid release phase was followed by a more controlled slower release phase as shown in Figure 3.13. There was an initial release of approximately 23% for 5-FU by the end of the first day. This initial rapid release of the drug could be explained by surface associated drug, release of some poorly entrapped drug near to the surface of the microspheres and/or higher water solubility of this drug. The slower more controlled release after the initial fast release could be caused by diffusion of the drug through the polymeric pores of the microspheres and/or polymer degradation.

Release of methotrexate from PLGA microspheres were determined by incubation in PBS buffer (0.01M, pH 7.4) at 37°C. Released drug amount was determined by spectrophotometer at 258 nm wavelength and release profile was obtained as described in section 2.2.2.3.

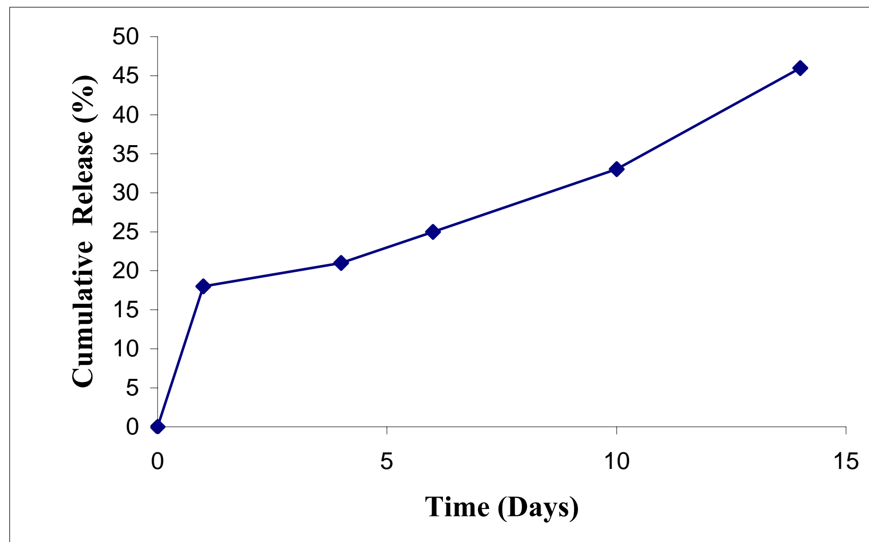

Figure 3.14 Release profile for methotrexate from PLGA microspheres

In the release profile of methotrexate (Figure 3.14), there was an initial rapid release of 18% by the end of the first day. Following the initial fast release, more controlled slower release was observed as in the case of 5-FU as explained above. Approximately 50% of the drug was released in 15 days.

In the case of tamoxifen the released drug amount was determined by spectrophotometrical measurement at 274 nm and release profile is seen in Figure 3.15.

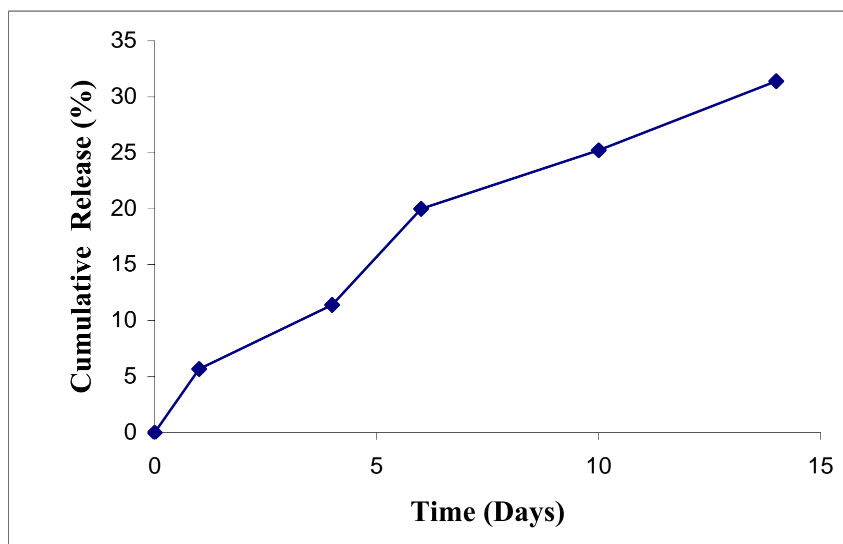

Figure 3.15 Release profile for tamoxifen from PLGA microspheres.

In the release profile of tamoxifen, there was controlled slower release as shown in Figure 3.15. On the other hand, in the study of Brigger *et al.* (2001) very high initial burst release (62%) of tamoxifen within a few minutes into the culture medium was observed. In their study, tamoxifen was loaded to poly(ethylene glycol) modified cyanoacrylate nanoparticles. In addition to this, initial burst release in the first hours into the release medium was observed in the study of Chawla and Amji *et al.*(2002) in which tamoxifen entrapped poly(epsilon caprolactone) nanospheres were prepared.

Although higher encapsulation efficiencies were observed in these studies, tamoxifen release was too fast and most of the entrapped drug were released as an initial burst release. On the contrary, in this study although encapsulation effiiciency was found lower, a prolonged slower release of tamoxifen was achieved with a low initial release.

5-FU and methotrexate release from the microspheres to the release medium followed the biphasic kinetics. Early rapid release phase was followed by a more controlled slower release phase. This early rapid release can be defined as “burst release” (Huang and Brazel, 2001) due to surface associated drug on the microspheres and poorly encapsulated drug near to the surface of the microspheres. Release of drug during the slower release phase may result from diffusion of the drug from polymer microspheres to the release medium due to concentration difference and/or from polymer degradation (Makino *et al*., 2000).

However, there were differences in the release profiles of 5-FU, methotrexate and tamoxifen. There were higher percentage of initial burst release in the release profiles of 5-FU and methotrexate compared to tamoxifen. Higher percentage of initial burst release could be attributed to the preparation of drug loaded microspheres by double emulsion formulation (Rosca *et al.,* 2004).

In addition to this, there were differences in the release rate of 5-FU and methotrexate from PLGA microspheres. The release rate of 5-FU from PLGA microspheres was faster than release rate of methotrexate from PLGA microspheres. Because 5-FU has the highest water solubility and has more tendency to release into aqueous medium from hydrophobic PLGA microspheres. Also in the study of Muvaffak (2003), release rate of 5-FU from gelatin microspheres was faster than release rate of methotrexate from gelatin microspheres. Smaller molecular structure of 5-FU than methotrexate molecular structure which were shown in Section 1.3.1 and Section 1.3.2 may also contribute to the faster release of 5-FU than methotrexate. On the other hand, there was more slower release of tamoxifen with 5.7 % initial release by the end of the first day from tamoxifen loaded PLGA microspheres which are prepared by single emulsion formulation (o/w). Tamoxifen was highly hydrophobic and also polymer PLGA was hydrophobic, which could be the cause of slower release rate of tamoxifen

In the study of Gupte (2004), while approximately 45% of the paclitaxel that was highly hydrophobic drug was releasing from PLGA microspheres in 21 days, 64% of the 5-FU was released from 5-FU loaded PLGA microspheres in the same time period. Initial fast release of 5-FU was higher than the initial fast release of paclitaxel.

Also similar release profile was reported in the study of Hussain *et al*. (2002). They reported the approximately 50% release of 5-FU from PLGA microspheres in 15 days.

In addition, while 50% of methotrexate was released approximately in 15 days in this study, 97% of methotrexate was released approximately in 10 days in the study of Cascone *et al.* (2002). This difference may be resulted from differences in the properties of polymers and releasing of methotrexate from gelatin nanospheres instead of microspheres.

CHAPTER IV

CONCLUSION

Biodegradable poly(D,L-lactide-co-glycolide) copolymers have been increasingly utilized for controlled release of anticancer drugs due to their excellent biocompatibility and controllable biodegradability. In addition, since they are biodegradable there is no need for the surgical removal of the drug carrier after the drug release is completed.

Controlled release of anticancer drugs from polymeric carriers has advantages such as reduced systemic toxicity, reduced side effects, decrease or prevention of drug resistance, increase in the efficiency of treatment, delivery at the required rate and in a required time.

In this study, poly(D,L-lactide-co-glycolide) microspheres were prepared and characterized for controlled release of three anticancer drugs, namely 5-fluorouracil, methotrexate and tamoxifen which are commonly used in the treatment of breast cancer.

Analysis of samples by inverted light microscopy confirmed the formation of microspheres for both empty and drug loaded samples. Scanning electron microscopy revealed the morphological surface properties and size of drug loaded poly(D,L-lactide-co-glycolide) microspheres. It was observed that use of either single emulsion formulation or double emulsion formulation caused significant difference in morphology, surface properties and size of microspheres.

5-FU and methotrexate loaded PLGA microparticles were mostly spherical in shape although there were some rare nonspherical ones. Some microspheres had smooth surfaces and some had rough surface. Also there were porous ones. Tamoxifen loaded microspheres were spherical in shape and they had smooth surfaces.

In this study, 60 % of the 5-FU loaded PLGA microspheres, 95 % of the methotrexate loaded PLGA microspheres and all of the tamoxifen loaded PLGA microspheres were smaller than 100µm. Size of 5-FU loaded microspheres were in a range of 14-314µm and methotrexate loaded PLGA microspheres were in a range of 15-185µm. On the other hand, tamoxifen loaded PLGA microspheres were smaller compared to 5-FU and methotrexate loaded PLGA microspheres. They ranged between 1-17µm.

Drug content of microspheres were changed according to the use of either single emulsion formulation or double emulsion formulation in the preparation of microspheres. Encapsulation efficiencies were calculated as 13% for 5-Fu, 11% for methotrexate and 9.7% for tamoxifen.

For the drugs that followed biphasic kinetics, initial fast release was followed by a more controlled slower release phase. It was concluded that initial fast release of 23% for 5-FU and 18% for methotrexate by the end of the first day was caused by surface associated drug and poorly entrapped drug. Following slower more controlled release was due to diffusion of the drug from PLGA microspheres and/or degradation of the PLGA copolymer and releasing of the drug. On the other hand there is more slower rate of release with 5.7% initial release for tamoxifen from tamoxifen loaded PLGA microspheres which were prepared by single emulsion formulation.

It could be concluded that use of either single emulsion or double emulsion formulation significantly affected the properties of the generated microparticles. In double emulsion formulations (w/o/w), polymer microcapsules in which drug is encapsulated by a polymer coat are obtained. These microcapsules can have spherical or nonspherical shape with drug containing aqueous interior part. However, in single emulsion formulations (o/w), microspheres in which drug is entrapped within polymer network are obtained Therefore microspheres that are prepared by different emulsion formulation (single emulsion or double emulsion) exhibit different properties in terms of morphology, drug content and release rate

Also formation of microspheres and so their properties are influenced by number of other factors such as properties and concentration of drug being encapsulated and polymer, volume of phases, type and concentration of emulsifier and experimental conditions.

Controlled release of anticancer drugs from polymeric carriers is a promising strategy to eliminate the problems of chemotherapy. It is expected that in further studies of cell line experiments of MCF7 breast cancer cells with optimized polymeric controlled drug delivery system with poly(D,L-lactide-co-glycolide) may decrease or prevent the development of multiple drug resistance by cancer cells. Development of drug resistance will be tested in breast cancer cell line MCF7 to compare direct drug application and drug release from poly(D,L-lactide-co-glycolide) carriers. Cell line experiments are important because they provide preliminary information about the in vivo performance of the release system. Optimization of the poly(D,L-lactide-co-glycolide) controlled release system according to the required dose for human breast cancer treatment will be promising strategy in the future.

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