DEVELOPMENT AND CHARACTERIZATION OF CORTISONE DERIVATIVE DRUG CARRYING POLYMERIC MICROSPHERES

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DEVELOPMENT AND CHARACTERIZATION OF CORTISONE DERIVATIVE DRUG CARRYING POLYMERIC MICROSPHERES

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ABSTRACT

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In this study, it is aimed to develop an injectable controlled release system of PCL and P(L,DL)LA microspheres loaded with TA and/or Ral for local treatment of rheumatoid arthritis which will avoid from systemic side effects of traditional administration and eliminate problems caused by direct local injections.

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disorder that most commonly causes inflammation and tissue damage in joints and tendon sheaths. Current strategies for the disease are mainly towards relieving symptoms and increasing mobility. The microsphere form drug delivery systems were developed to enhance the treatment success of rheumatic diseases by providing these agents alone or together for long terms without causing systemic or local site effects upon injection to the RA joints. Microspheres were prepared with s/o/w solvent evaporation technique and optimized to achieve a suitable

size for joint application, to sustain the delivery of the drug(s), to provide required amount of the agent with feasible amount of microsphere. In order to manage these, microspheres prepared with different combinations of polymers and drugs were examined for particle size analysis, surface and structural characterizations, time related drug release properties, and drug loading capacities. In vitro cytotoxicity tests using 3T3 fibroblast cells were done to evaluate the biocompatibility of drug loaded PCL microspheres. The degradation of polymers were conducted and evaluated by GPC analysis.

In PCL:TA microspheres, as polymer:drug ratio decreased (from 10:1 towards 10:4), namely as the drug partition increased, it was seen that encapsulation efficiency and loading percentages increased. Meanwhile, percent release of the drug decreased, indicating more prolonged release. Among all microspheres, PCL:TA 10:4 and PCL:Ral 10:2 were found to be the most appropriate for dual release in terms of release values (ca 21% and 0.09%, respectively), loadings (ca 27% and ca 13%, respectively) and mean particle size values (ca 100 μm and ca 95 μm, respectively). After release studies, microspheres preserved their sphericity. These selected polymer:drug groups also represented no cytotoxic effect. The microspheres for dual drug study (PCL:TA:Ral 10:4:2) released app. 55% of its TA and 0.29% of Ral at the end of 4 weeks. Drug loading capacities of these microspheres were found to be ca 14% for TA and 8% for Ral. Furthermore, with dual loading case, smallest mean particle size (68 μm) could be obtained among all studied groups.

P(L,DL)LA microspheres caused high viscosity problems during microsphere preparation steps and resulted in the slowest release, which was unfavorable for the aim of the study. To our knowledge there is no microsphere study reported with P(L,DL)LA in literature.

The TA and Ral delivery systems with PCL and P(L,DL)LA were developed and studied for the first time in literature and they were optimized for RA treatment purposes. The potential of these systems, should be further tested in experimental animal models of RA.

Keywords: Controlled Drug Release, Dual Drug Release, Polycaprolactone, Poly(L-lactide-co-D,L-lactide), Microsphere, Triamcinolone acetonide, Raloxifene

ÖZ

KORTİZON TÜREVİ İLAÇ İÇEREN POLİMERİK MİKROKÜRELERİN HAZIRLANMASI VE İNCELENMESİ

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Bu çalışmada, romatoid artritin lokal tedavilerinde kullanılmak üzere, geleneksel tedavi yöntemlerinden kaynaklanan sistemik yan etkilerden ve direkt lokal enjeksiyon uygulamalarının sebep olduğu problemlerden kaçınmak için TA ve/veya Ral yüklü PCL ve P(L,DL)LA mikroküre lerinin enjekte edilebilir kontrollü salım sisteminin geliştirilmesi amaçlanmıştır.

Romatoid artrit, genellikle eklemlerde ve tendonlarda enflamasyon ve doku hasarı meydana getiren kronik, sistemik ve otoimmün bir rahatsızlıktır. Hastalığa karşı mevcut yöntemler genelde semptomları rahatlatıcı, azaltıcı ve hareket kabiliyetini yükseltici yöndedir. Mikroküre şeklindeki ilaç salım sistemleri, sistemik ya da romatoid artritli eklemlere enjeksiyon sonucu oluşan lokal yan

etkilere neden olmaksızın bu ajanları tek başına ya da birlikte uzun dönem için sağlayarak, romatoid hastalıkların tedavilerindeki başarıyı yükseltmek amacıyla geliştirilmişlerdir. Mikroküreler s/o/w çözücü uçurma yöntemi ile hazırlanmış ve eklem uygulaması için uygun boyut, ilaç(lar)ın uzun süreli salınması ve makul miktarda mikroküre ile yeterli bioaktif ajan sağlama özellikleri yönünden optimize edilmiştir. Bu değerlendirmeler için farklı polimer ve ilaç kombinasyonları ve oranları ile hazırlanan mikroküreler, parçacık boyut analizi, yüzey ve şekil karakterizasyonları, zamana karşı ilaç salım özellikleri ve ilaç yükleme kapasiteleri açısından incelenmiştir. İlaç yüklü PCL mikrokürelerin biyouyumluluğunu değerlendirmek için 3T3 fibroblast hücreleri kullanılarak in vitro sitotoksisite testleri yapılmıştır. Polimerlerin degredasyonu GPC analizleri ile değerlendirilmiştir.

PCL:TA mikrokürelerde, polimer:ilaç oranı düştükçe (10:1'den 10:4'e) yani ilaç kısmı arttıkça, enkapsülasyon verimi ve ilaç yüklenme yüzdeleri artmıştır. Bununla birlikte, ilaç salım yüzdeleri düşmüştür. Bu da ilaç salım süreçinin uzatılmış olduğunu göstermektedir. Bütün mikroküreler arasında, PCL:TA 10:4 ve PCL:Ral 10:2 grupları, salım değerleri (yaklaşık %21 ve %0.09), ilaç yüklenmeleri (yaklaşık %27 ve %13) ve ortalama parçacık boyutu değerleri (yaklaşık 100 µm ve 95 µm) açısından ikili salım için en uygun bulunmuştur. Salım sonrasında mikroküreler küreselliklerini korumuştur. Seçilen polimer:ilaç grupları sitotoksik etki göstermemiştir. İkili ilaç salımı için olan mikroküreler, PCL:TA:Ral 10:4:2, 4 hafta sonunda TA'nın yaklaşık %55'ini ve Ral'ın yaklaşık %0.29'unu salmıştır. İlaç yükleme kapasiteleri TA ve Ral için yaklaşık %14 ve 8 olarak bulunmuştur. Ayrıca, ikili yükleme ile bütün gruplar içerisindeki en düşük boyutta mikroküreler (68 µm) elde edilmiştir.

P(L,DL)LA mikroküreleri, mikroküre hazırlama aşamasında yüksek vizkozite sorununa sebep olmuş ve bu çalışmanın amacına göre istenmeyen düzeyde, en yavaş salımla sonuçlanmıştır. Bilgilerimize göre, literatürde P(L,DL)LA ile rapor edilmiş mikroküre çalışması yoktur.

Literatürde ilk defa TA ve Ral için PCL ve P(L,DL)LA salım sistemleri hazırlanıp geliştirilmiş ve RA tedavisi için optimize edilmiştir. Bu sistemlerin potansiyeli RA'li deneysel hayvan modelleri üzerinde de incelenmelidir.

Anahtar Sözcükler: Kontrollü İlaç Salımı, İkili İlaç Salımı, Polikaprolakton, Poli(L-laktit-ko-D,L-laktit), Mikroküre, Triamsinolon asetonid, Raloksifen

To My Beloved Family

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INTRODUCTION

In all higher vertebrates, the skeleton, which is highly specialized form of connective tissue, provides internal support and movement to the organism. In this complicated function there are two main types of structures bone and cartilage. These connective tissues also serve protection to the important body organs. While both tissues present some common properties, cartilage tissue is more uniform in terms of cellular composition which covers mainly chondrocytes. Bone tissue, however, has osteoblasts, osteoclasts and bone lining osteocytes. Joints are composed of cartilage, calcified cartilage and bone tissue and can resist a great amount of repetitive physical stress (Meyer et al. (2006), Lohmander (2003)).

1.1 Joint and Cartilage Structure

The part of the body at which two or more bones contact is called joint. Joints allow movement and provide mechanical support. They can be classified according to their connection to each other such as fibrous, cartilaginous, synovial joints (Module - Introduction to Joints, anatomy.med.umich.edu, last visited on January 2011), their function such as synarthrosis, amphiarthrosis,

diarthrosis (Module - Introduction to Joints, anatomy.med.umich.edu, last visited on January 2011), or anatomically (elbow, wrist, knee, hip joints). A typical joint structure can be seen in Figure 1.1.

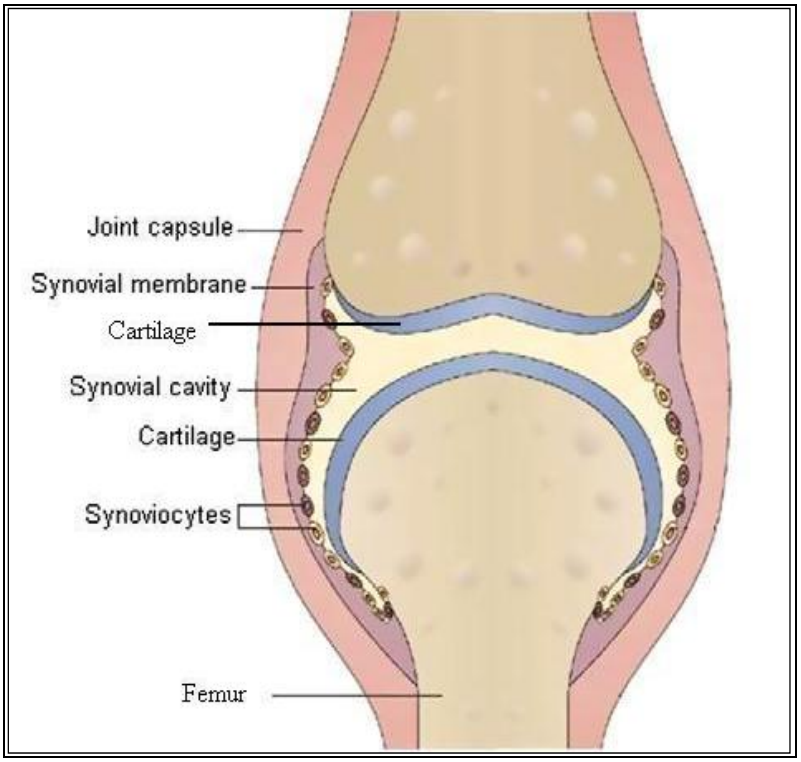

Figure 1.1. A typical joint (modified from Butoescu et al. 2009)

In adult body cartilage tissue is found on articular surface of long bones and in trachea, bronchi, nose, ear, larynx, and intervertebral discs. Cartilage is briefly composed of cells, fibers and ground substances (Meyer et al. (2006)). Providing a lubricating covering material to articular ends of bones at synovial

joints is the main purpose of adult articular cartilage (Freeman (1973)). When examined in detail, cartilage is found to be made up of a rich extracellular matrix with thinly dispersed chondrocytes. These cells constitute only 1-2% of the tissue volume in an adult. Heinegard et al. (1998) stated "The matrix, being and underhydrated gel of proteoglycans and matrix proteins reinforced by a three-dimensional network of collagen fibrils, is directly responsible for the unique functional properties of the cartilage and provides shape, resilience and resistance against compression and shear". Cartilage tissue can be classified as three types; hyaline, elastic and fibro cartilage. The most common and widely distributed cartilage type in human body is hyaline cartilage, which is a bluish, opalescent tissue. Essentially, hyaline cartilage is seen on articular surface of bones. In the junctions between large tendons and in articular cartilage of large joints fibro cartilage is seen. Elastic cartilage is present in the auricle of the external ear, the walls of the external auditory meatus, the eustachian tube, and parts of the larynx. The elastic cartilage mainly provides brightness of tubes by surrounding this structure (Meyer et al. (2006)).

Cartilage has a zonal structure. These are superficial, mid, deep, calcified cartilage, subchondral bone plate and subchondral trabecular bone zones (Meyer et al. (2006)). Zonal structure of cartilage was demonstrated in Figures 1.2 and 1.3.

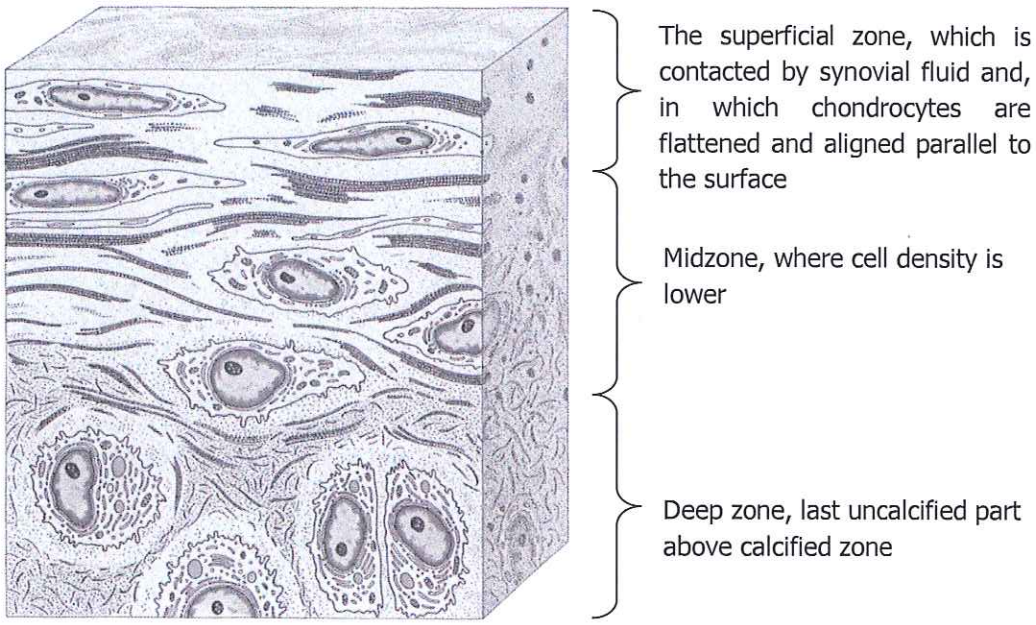

Figure 1.2. A schematic representation of zonal structure of articular cartilage cross section (modified from Meyer et al. (2006))

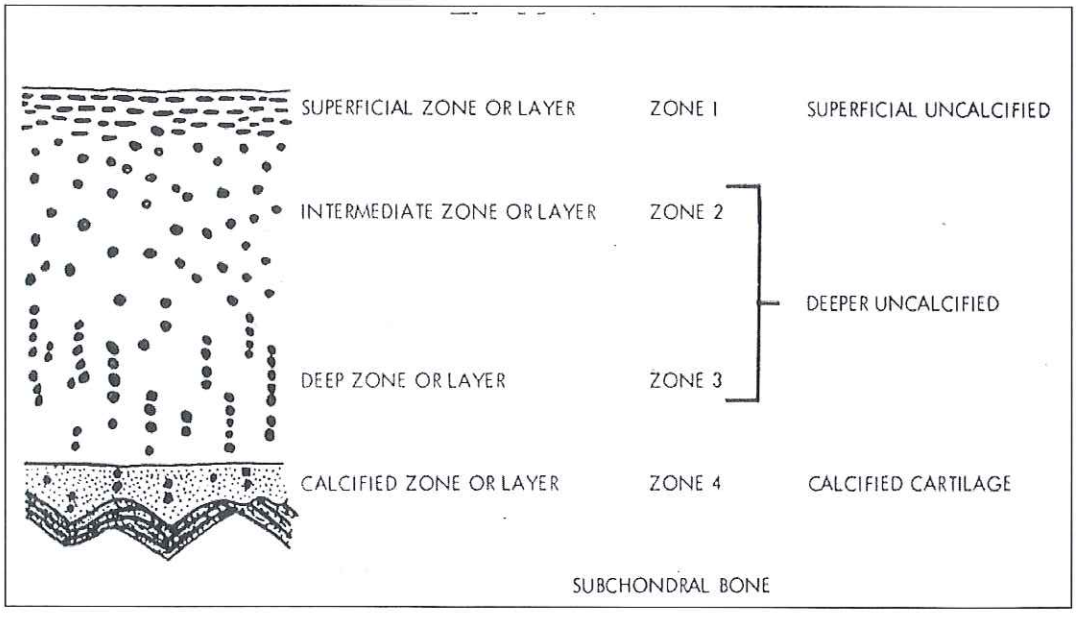

Figure 1.3. The zones of adult articular cartilage (modified from Freeman (1973))

In synovial fluid contacting superficial surface, chondrocyte cells are flattened and aligned parallel to the surface. Midzone is below the superficial zone and here cell density is lower and cells are less flattened. The deep zone is between the midzone and the layer of calcified cartilage. A separate layer of calcified cartilage is below the articular cartilage. Due to the fact that calcified cartilage is not vascular normally, remodeling process is not effective. Cartilage tissue lacks blood vessels and nerves. Therefore, no regeneration is seen in cartilage tissue, cells are nourished by diffusion from synovial fluid. In this zone, cell density is the lowest. In deep zone, aggrecan content and fibril diameter are the highest, whereas collagen content is the lowest. Subchondral bone plate is below the calcified cartilage and subchondral trabecular bone lies beneath subchondral plate (Meyer et al. (2006)).

The extracellular matrix of hyaline cartilages consists of collagen fibrils and non-collagenous proteins. Type II and type IX collagens are main fibrils (Meyer et al. (2006)).

Among non-collagenous proteins, which are ground substance of articular cartilage aggrecan is highly important due to the fact that high aggrecan content is responsible for the tissue to resist compression (Meyer et al. (2006)). Aggrecan is a long protein modified with large carbohydrates. Aggrecan consists of two large globular structural domains and these main domains are separated by another domain called glycosaminoglycan. Glycosaminoglycans (GAG) are large polysaccharides, which are very important for connective tissue. That is because water sticks to GAGs and cause the resistance to pressure.

1.2 Diseases of Joint and Cartilage

Rheumatoid arthritis, osteoarthritis, juvenile idiopathic arthritis, seronegative spondyloarthropathies, infectious arthritis, crystal induced arthritis are some of the common joint and cartilage diseases. Such diseases could be caused by ageing, trauma, autoimmunity, environmental factors and genetic inheritance (Kumar et al. (2010)).

1.2.1 Rheumatoid Arthritis and Its Treatment Strategies

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease. Synovitis (inflammation of synovial membrane) of multiple joints is its main manifestation (McPhee et al. 2010). Cartilage damage, bone erosions and subsequent changes in joint integrity are characteristics of the disease caused by the synovial inflammation (Fauci et al. (2008)). General existence of the disease is 1% of world's population. It is more common in women than in men, with women to men ratio of 3 to 1. RA may start at any age, but the highest onset for the women is from fourth to fifth decades whereas for men is at sixth to eighth decades (McPhee (2010)). With age, prevalence of the disease increases and in contrast, sex difference decreases. All races throughout the world is affected by RA (Fauci et al. (2008)). The cause of RA is still unknown but autoimmunity, genetic susceptibility and environment have very important roles in development, progression and chronicity of the disease (Kumar et al. (2010)). Untreated RA causes joint destruction which is followed by disability and shortened life expectancy. In early period of RA, aggressive treatment is applied. The joint pathologic findings include chronic synovitis with formation of

pannus, which is an inflammatory pathologic formation surrounding articular cartilage. Pannus erodes bone, cartilage, tendons and ligaments (McPhee et al. 2010). A RA developed joint is shown in Figure 1.4.

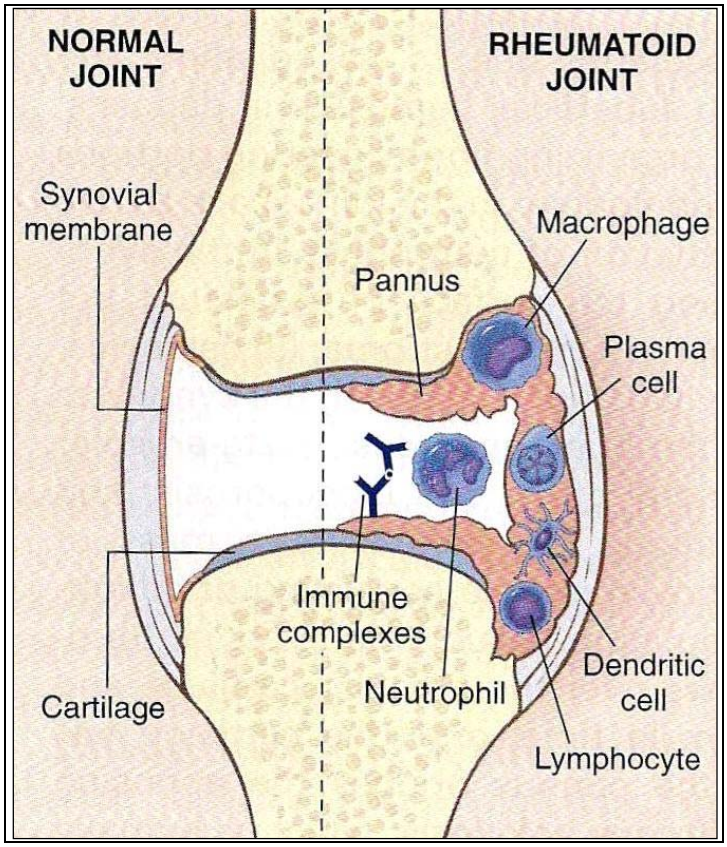

Figure 1.4. Schematic of RA lesion of an articular joint (modified from Kumar et al. (2010))

In acute phase, effusion, outflow of the fluid towards synovial cavity and other manifestations of inflammation are common. Fibrous ankylosis and loss of mobility of a joint are seen in late phase (McPhee et al. (2010)).

Figure 1.5 illustrates development of the pathogenic mechanisms and final pathologic changes played role in the stages of rheumatoid synovitis. Rheumatoid arthritis starts with nonspecific inflammation. Moreover, an amplification phase results from T-cell activation. Following these, tissue injury caused by chronic inflammation occurs. According to Harrison's Principles of Internal Medicine, 2008, "A variety of stimuli may initiate the initial phase of nonspecific inflammation, which may last for a protracted period of time with no or moderate symptoms. When activation of memory T cells in response to a variety of peptides presented by antigen-presenting cells occurs in genetically susceptible individuals, amplification of inflammation occurs with the promotion of local rheumatoid factor and other autoantibody production and enhanced capacity to mediate tissue damage."

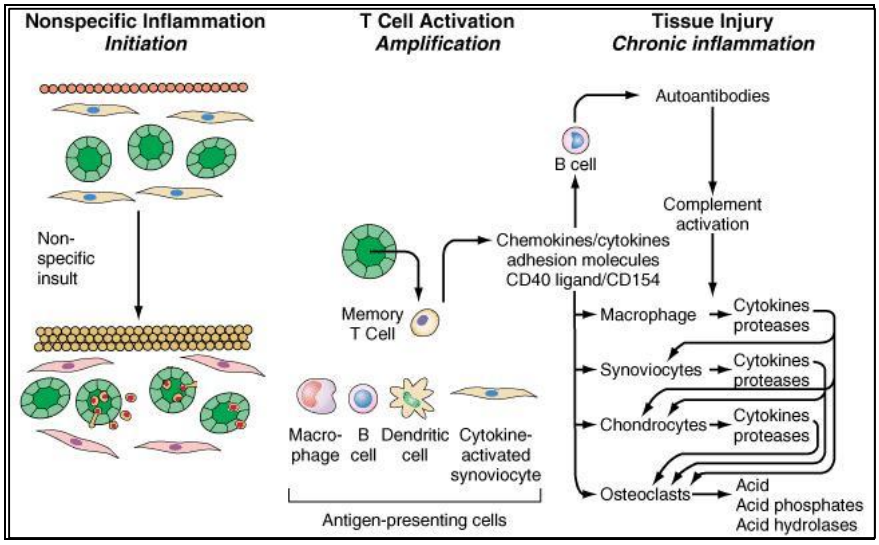

Figure 1.5. The progression of rheumatoid synovitis (modified from Fauci et al. (2008))

When clinically examined, common symptoms are pain, swelling and stiffness in joints. Symmetric swelling of multiple joints is also a typical symptom of RA. Stiffness in the morning more than one hour is another characteristic of the disease. Stiffness can be seen after day-time inactivity and can be much more severe after activity. Rupture of tendons and synovial cysts can occur. Subcutaneous rheumatoid nodules may be formed in RA joints. In advanced disease, dryness of the eyes, mouth and other mucous membranes are seen as the other signs (McPhee et al. (2010)).

For diagnosing RA no test is specified. However, at the two-thirds of adults who have RA, autoantibodies are found which are reactive with the Fc portion of IgG. Therefore, these rheumatoid factors are used to evaluate patients (Fauci et al. (2008)). In addition, radiographic images that show changes in RA joint might indicate disease state (McPhee et al. (2010)).

In treatment of RA, the therapies aim decreasing pain and inflammation, preservation of function and prevention of further deformity. Since the main cause of RA is unknown therapy remains somewhat empirical. Therapeutic applications are not curative; they are palliative, namely focused on relieving the symptoms of the disease (Fauci et al. (2008)).

Nonsteroidal anti-inflammatory drugs (NSAIDs) serve to relieve symptoms in RA. However, NSAIDs do neither protect from erosions nor alter progression. In addition, using NSAIDs may cause side effects such as gastric ulceration, perforation and gastrointestinal hemorrhage. Moreover, NSAIDs may create renal toxicity. Furthermore, some NSAIDs interact with function of platelet and delay bleeding time (McPhee et al. (2010)).

Low dose corticosteroids create a rapid anti-inflammatory effect in RA (McPhee et al. (2010)). Current evidence shows that progression of bone erosions might be delayed by low dose glucocorticoid therapy. In addition, a long term protective effect against bone damage might be provided by initial

course of low dose glucocorticoids. Monthly pulses with high-dose glucocorticoids may be useful in some patients and may fasten the response when therapy with a disease modifying antirheumatoid drug (DMARD) is initiated (Fauci et al. (2008)). Until the slower acting DMARDs effect, low dose corticosteroids are often used in order to decrease disease activity or they are used as adjunctive therapy for active disease which persists despite treatment with DMARDs. Patients which use corticosteroids for long term should take measures to prevent osteoporosis, which is a degenerative bone disease. If one or two joints are the main source of difficulty, intraarticular corticosteroids may be helpful. For symptomatic relief intraarticular triamcinolone, at a dose depending on the size of the joint, can be injected, but not more than four times a year (McPhee et al. (2010)).

Triamcinolone acetonide (TA) is a corticosteroid, which is a class of steroid hormones, and it acts as anti-inflammatory and immune-suppressive agent. Due to long and branched side chains, solubility of this glucocorticoid (a subset of corticosteroids) is highly reduced and its effects are prolonged (Ostergaard et al. (1998)). TA's chemical formula is $C_{24}H_{31}FO_6$ and its molecular weight is 434.5 g/mol. Chemical structure of TA is presented in Figure 1.6 (Triamcinolone Acetonide Product Information, sigmaaldrich.com, last visited on January 2011). TA has a wide spectrum of activity including anti-inflammatory, antiallergic and antipruritic activity (Clares et al. (2009)). TA is provided for different applications commercially in intraarticular or intraleisonal injectable suspensions, topical ointments or creams, oral tablets, inhalation aerosols, nasal sprays forms under brand names; Kenacort, Kenalog, Aristocort, Tri-Nasal, Triaderm, Azmacort, Trilone, Volon A, Tristoject, Fougera, Tricortone (Triamcinolone: MedlinePlus Drug Information, nlm.nih.gov, last visited on January 2011).

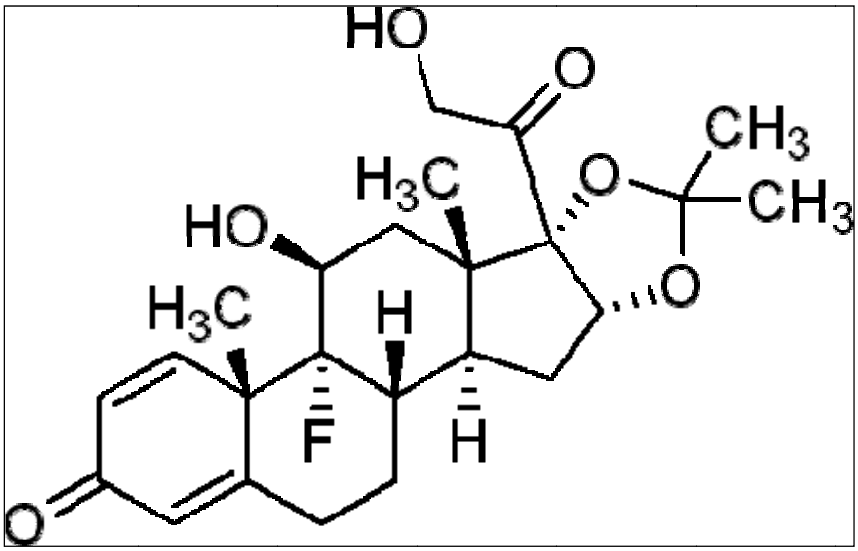

Figure 1.6. Triamcinolone acetonide (Triamcinolone Acetonide Product Information, sigmaaldrich.com, last visited on January 2011)

As it is stated in 2011 Current Diagnosis and Treatment, Ostergaard et al. (1998), Butoescu et al. (2009), Wang et al. (1998), Eberhard et al. (2004), Blyth et al. (1994), Loet et al. (2010), TA is used in treatment of rheumatic diseases like RA. However, there is no curative treatment for RA therefore, TA is used as pain reliever and anti-inflammatory agent in order to re-gain functionality and mobility of the joint and slow down the rate of bony destruction as addition to NSAIDs and DMARDs treatments mentioned above in section 1.2.1. Triamcinolone acetonide injectable suspension (both for i.a- or sub cutaneous inj.) contains 40 mg triamcinolone acetonide in its each mL of the sterile aqueous suspension with 0.99% (w/v) sodium chloride for isotonicity, benzyl alcohol as a preservative, 0.75% carboxymethylcellulose sodium, and 0.04% polysorbate 80 (surfactant). Application dose depends on the site of application, degree of disease and size of joint, if administered intra-articularly. For RA, as intra-articular administration initial dose varies from 2.5 mg to 5 mg for smaller

joints and from 5 mg to 15 mg for larger joints, depending on the specific disease entity being treated. For adults, doses up to 10 mg for smaller areas and up to 40 mg for larger areas have usually been sufficient. Single injections into several joints, up to a total of 80 mg, have been given (Kenalog-40 Prospectus).

Raloxifene hydrochloride (Ral) is a non steroid hormone based on benzothiophene (Erlandsson et al. (2000)). In addition, Ral is a selective estrogen receptor modulator (SERM), which is an agonist of estrogen on bone but an antagonist on the uterus and breast tissue (Jochems et al. (2007)). SERM is an agent that influences some of the receptors affected by estrogen and in some instances, like raloxifene, antagonizes or blocks the effects of estrogen. It behaves like estrogen in order to prevent bone loss and also has potential in breast and uterine tissues to block some effects of estrogen. Raloxifene decreases bone resorption, increasing bone mineral density and decreasing fracture incidence (Raloxifene: Drug information, uptodate.com, last visited on 2010). Its chemical formula is $C_{28}H_{27}NO_4S \bullet HCl$ and molecular weight is 510.05 g/mol. Chemical structure of Ral can be seen in Figure 1.7 (Raloxifene hydrochloride Product Information Data Sheet, sigmaaldrich.com, last visited 2010).

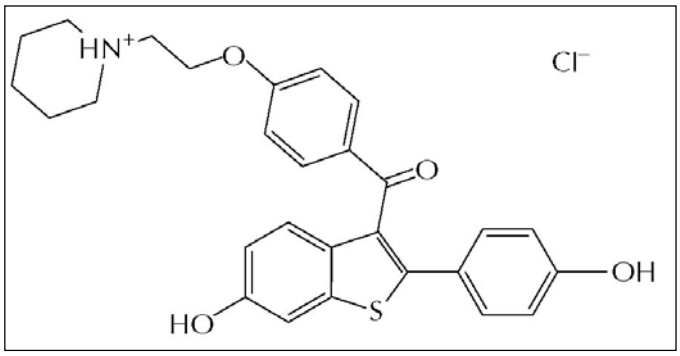

Figure 1.7. Raloxifene hydrochloride (modified from Gluck et al. 2003)

In many articles, it is stated that Ral is used in prevention and treatment of osteoporosis. Jochems et al. (2007) claimed that Ral is used in order to prevent and treat postmenopausal osteoporosis and it is safer to use Ral than estrogen containing hormone replacement therapy (HRT) due to its tissue selectivity. Moreover upon use of Ral in osteoporosis and a number of beneficial non-skeletal effects are stated by Gluck et al. 2003, Wang et al. 2008, Kovats et al. 2005, Trontelj et al. 2005, Yang et al. 2007, Bjarnason et al. 2001 and Maricic et al. 2002. Commercially available Ral is under the brand name Evista in 60 mg tablets and used as one tablet daily orally for osteoporosis treatment (for 5 years in reduction of invasive breast cancer risk) (Raloxifene: Drug information, uptodate.com, last visited on 2010). Recently, some studies claimed beneficial effects of Ral on RA. Among these, Jochems et al. (2007) stated that both estrogen scarcity and the inflammatory disease provide the development of generalized osteoporosis in postmenopausal rheumatoid arthritis. Hormone replacement therapy with estradiol preserves bone mineral density and ameliorates arthritis, but in long-term therapy significant side effects should be considered. Therefore, in order to test possible advantages of a selective estrogen receptor modulator, the raloxifene analog, LY117018, was used by Jochems et al. in the treatment of both arthritis and osteoporosis in a mouse model of RA. Their results showed that treatment with raloxifene dramatically decreased the frequency and severity of arthritis. In raloxifene treated mice effective preservation of bone and cartilage was seen, as determined by increased bone mineral density and decreased serum levels of cartilage oligomeric matrix protein compared with controls. It was stated that decreased levels of mRNA for both tumor necrosis factor α and RANKL in spleen cells from raloxifene treated arthritic mice indicated an immunosuppressive action of this SERM (Jochems et al. (2007)). In addition, Carlsten et al. (2008)

stated that raloxifene treatment was beneficial in long term treatment of established collagen-induced arthritis. It protects against the development of osteoporosis. Both anti arthritic and anti osteoporotic effects were seen in long-term treatment with raloxifene in established experimental postmenopausal polyarthritis.

DMARDs are clinically experienced to have capacity to alter the course of RA. Because they exert minimal direct nonspecific anti-inflammatory or analgesic effect, anti-inflammatory agents must be continued during administration of DMARDs (Fauci et al. 2008). Some of the DMARDs used are methotrexate, sulfasalazine, leflunomide, antimalarials and minocycline. However, DMARDs cause side effects such as gastric irritation and stomatitis. In addition, neutropenia, thrombocytopenia, diarrhea, rash, reversible alopecia, hepatoxicity and weight loss might occur due to synthetic DMARDs. Biologic DMARDs such as tumor necrosis factor inhibitors (anti-TNF), abacept, rituximab, tocilizumab are generally used with synthetic DMARDs as combinational treatment (McPhee et al. (2010)).

In severely damaged joints which do not respond to medication and physical theraphy, surgery is the last application. The most successful surgeries are done at hips, knees and shoulders. However, arthroplasties, namely fixation of a joint by surgery, which had lost its mobility, and total joint replacements could be done on a number of joints. The aim of surgeries is to decrease pain and increase mobility. However, due to the fact that RA affects multiple joints especially for the smaller joints such as fingers, surgery might not be applicable for all of them. Namely, surgery in RA is not applicable for every type of joint and not applicable for high numbers of joints, which is a characteristic of RA (Fauci et al. (2008)).

1.2.2 Other Diseases and Treatment Approaches

In addition to rheumatoid arthritis, degenerative joint disease (osteoarthritis), juvenile idiopathic arthritis, crystal induced arthritis (gouty arthritis) can also be seen in joints.

Osteoarthritis is also called as one of the 10 most disabling degenerative joint disease in developed nations. In addition to being an inflammatory disease, osteoarthritis with an origin of cartilage failure, which might be due to person's genetic susceptibility, causes biochemical and metabolic alterations and results in breakdown (Kumar et al. (2010)). The pathologic changes are considered to be related with age and occupation of the patient (Rosai et al. (2004)). Osteoarthritis, which is a multifactorial disease, is characterized by destruction of the articular cartilage, subchondral bone alterations and synovitis. Mechanism responsible for osteoarthritis is very complicated and poorly understood. The symptoms are ache, stiffness, muscle weakness, swelling, deformation in shape, reduced range of motion and loss of use of joint. Therefore, the treatment is directed to decrease symptoms, reduce pain and inflammation rather than decrease joint destruction (Alcaraz et al (2010)).

Juvenile idiopathic arthritis includes all types of arthritis before 16 years age. Most of them are different than adult onset inflammatory joints diseases. Oligoarthritis, polyarthritis with or without rheumatoid factors, and spondyloarthropathies are the nonsystemic forms and are managed quite similar to adult onset rheumatoid arthritis (Quartier (2010)).

Human lack uricase, enzyme responsible for the degradation of uric acid, and therefore develop hyperuricemia and gout. By initiation of crystallizations of urates within and about joints, gout is labeled by transient attacks of acute arthritis which results in gouty arthritis (Kumar et al. (2010)). Namely, there is accumulation of monosodium urate crystals, called tophi, in and around joint

surfaces in gout resulting in synovitis and juxtaarticular destruction of articular bone. Edema, erythema of joints, severe pain, limited articular motion and dysfunction are characteristic symptoms. Current management is with NSAIDs or with colchicine. NSAIDs are to reduce symptoms such as pain (Chu et al. (2004), Renbin et al. (2008)).

1.3 Controlled Bioactive Agent Release Systems

Traditional drug administration, which can be oral, intravenous or intramuscular, may create lower drug concentrations at the site of disease than expected. Therefore, the effect of the drug would be less pronounced at target site. In addition, traditional techniques can cause systemic side effects on different tissues and organs throughout the body. Fluctuations in the concentration of drug, which is common with traditional drug administration techniques, might create concentrations that range between toxic and insufficient levels. Compared to these approaches of medication, controlled drug release systems are more advantageous. Because controlled release systems can provide prolonged release at the site of disease (with local administration or by targeting means) they can eliminate systemic side effects. Furthermore, the concentration of the drug released might be optimized in controlled release systems by changing the parameters such as polymer type, size, shape, molecular weight, formulation method, chemical nature of the polymer matrix, etc. Matrix can be micro or nano spheres or particles and liposome (Butoescu et al. (2009)). A comparison of serum drug level following drug administration by conventional ways and after controlled release system is given in Figure 1.8 (Kydonieus (1980)).

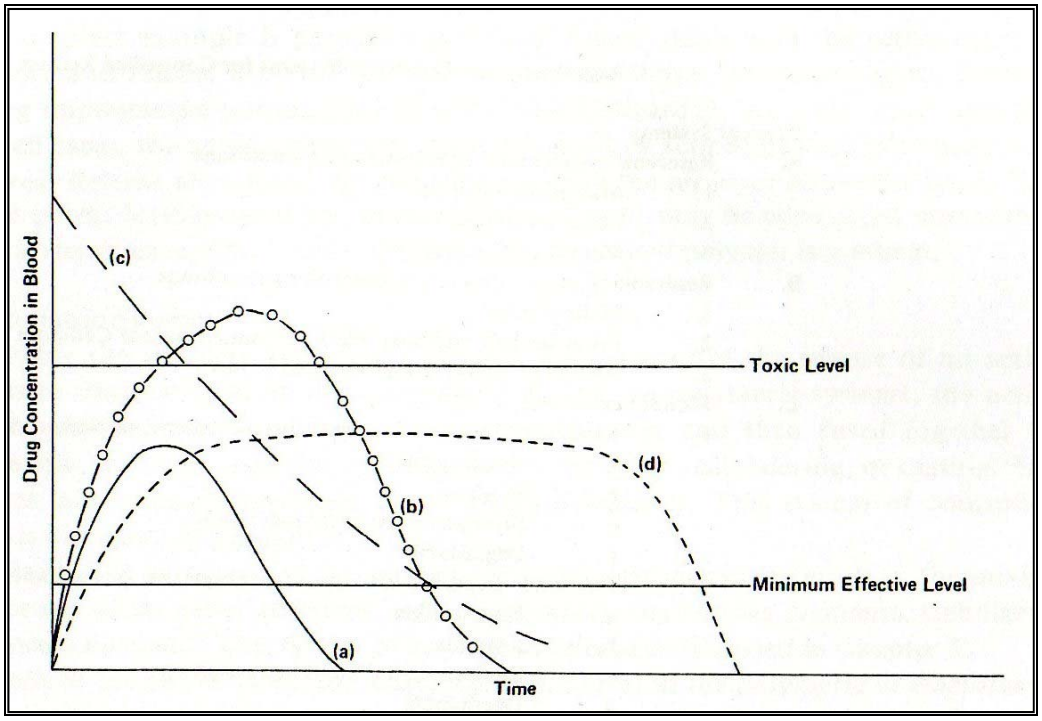

Figure 1.8. Drug level vs time. (a) Standard oral dose, (b) Oral overdose, (c) Intra venous injection, (d) Controlled-sustained release of ideal dose (Kydonieus (1980))

As stated by Vural et al. (1992) intra articular administration of a soluble bioactive agent encounters rapid clearance of the drug into circulation problem. Rapid clearance of bioactive compound may require frequent injections, which can be resulted in infection, joint disability and post injection flare (Butoescu et al. (2009)). Therefore, a controlled drug release systems of a biocompatible and biodegradable polymer or a colloidal suspension would provide more effective retention in this target site.

As it was claimed in Gluck et al. (2003) even though 60% of the drug, raloxifene, absorbed after traditional oral administration, only 2% of it was absolutely bioavailable due to absorption problems in administration route and

short half life of the drug. These kind of disadvantages might also be overcome by controlled drug release systems.

There are some disadvantages for controlled release systems especially when a non-biodegradable polymer is involved. Use of a nonbiodegradable polymer in a controlled release system as the matrix requires a second surgery in order to remove the matrix. Also in terms of cost of the medication, large scale production costs of controlled release preparation and processing might be higher than the cost of standard formulations (Kydonieus (1980)).

1.4 Polymers Used in Controlled Release

In order to benefit from advantages of controlled release systems especially biocompatible and biodegradable polymers are used. A range of suitable polymers for this purpose is documented in Table 1.1.

Table 1.1. Biodegradable polymers used in controlled release of drugs (modified from Karsa et al. (2005))

A Range of Biodegradable Polymers	
Poly(alkyl-α-cyanoacrylates)	Examples for Aliphatic Polyesters
Poly(ortho-esters)	Poly(lactic acid) or poly(lactide)
Poly(amino acids)	Poly(glycolic acid) or poly(glycolide)
Poly(dihydropyrans)	Poly(ε-caprolactone)
Poly(anhydrides)	Poly(hydroxy butyrate)

Examples of non-biodegradable polymers used in drug delivery systems involve polyvinyl alcohol (PVA), ethylene vinyl acetate (EVA), PMMA and silicon. The drug delivery systems of PVA-EVA are used as implantable drug delivery system for subconjunctival, scleral or intraocular applications (Touitou et al. (2007)).

Aliphatic polyesters are widely used in controlled drug release systems due to the fact that they degrade by bulk hydrolysis of ester bonds and nontoxic residues are removed by circulation. This property has put an enormous input into their therapeutic values (Sinha et al. (2004)). The chemical structure of some polyesters can be seen in Figure 1.9. Among these, polycaprolactone (PCL) and polylactide and their co polymers are leading.

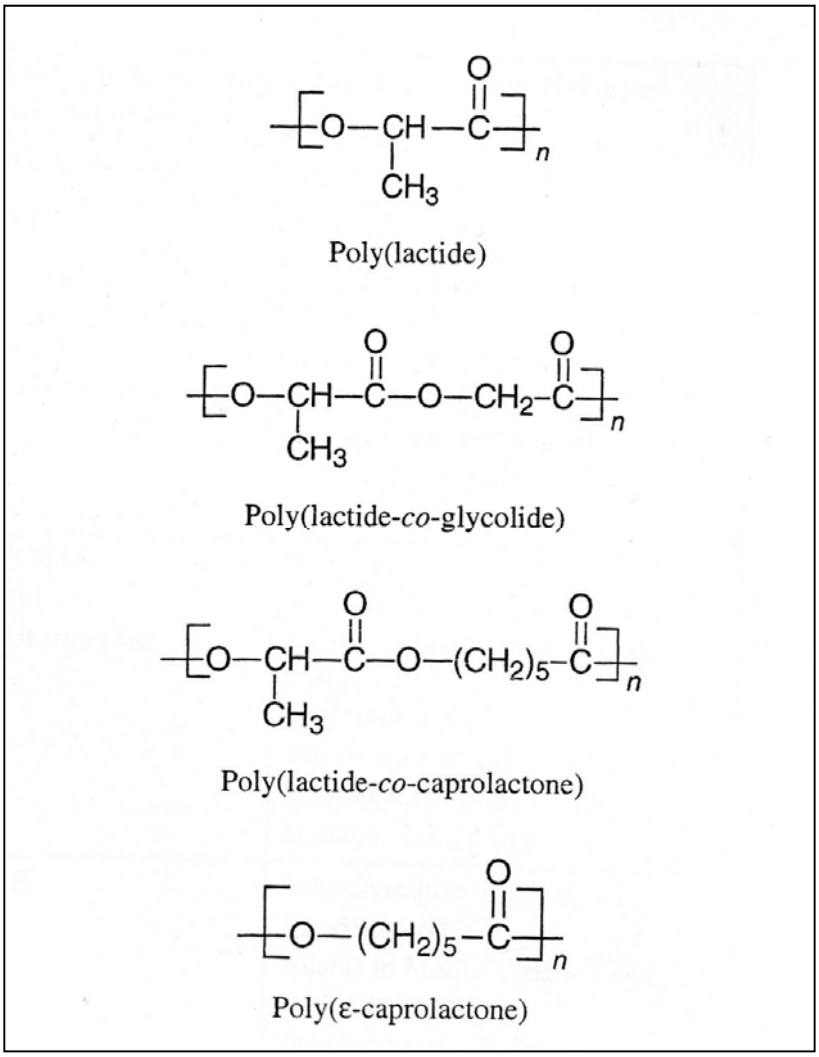

Figure 1.9. Chemical structure of some polyesters used in controlled drug release (Jones (2004))

1.4.1 Polycaprolactone (PCL)

PCL is biodegradable and biocompatible low cost polyester. It is stated as quite popular and is extensively used for implantable or injectable carriers for

the controlled release of active agents in high number of studies (Alvarez et al. (2006), Dhanaraju et al. (2006), Sinha et al. (2004), Jones (2004), Chasin et al. (1990)).

First, in 1930s ring opening polymerization of PCL was studied by Van Natta et al. However, biodegradable property of this polyester was first identified in 1973 (Sinha et al (2004)). The linear formula of PCL is $(C_6H_{10}O_2)_n$ and its chemical structure can be seen in Figure 1.10.

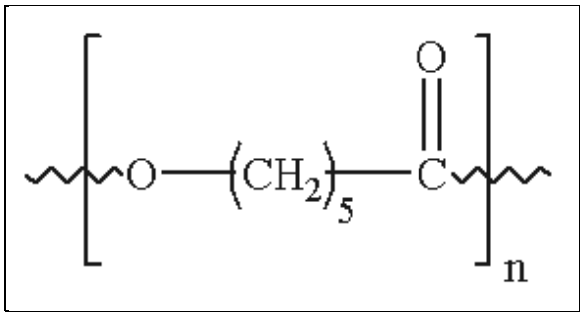

Figure 1.10. Polycaprolactone

PCL is prepared by ring opening polymerization of ε-caprolactone (Jones 2004). Polymerization of ε-caprolactone might be affected by the mechanism; anionic, cationic, coordination and radical. Different degrees of control over molecular weight and its distribution, and end group composition are provided by these mechanisms (Chasin et al. (1990)).

PCL is a semicrystalline polymer with a low glass transition temperature (Tg) of -60°C. In addition, melting temperature (Tm) ranges from 59 to 64°C according to its crystalline structure. Its solubility in chloroform,

dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature is high but it is less soluble in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile. PCL is insoluble in water, alcohol, petroleum ether and diethyl ether (Sinha et al. (2004)).

Due to slow degradation in comparison with other polymers, PCL is appropriate for long term controlled release applications. Degradation time usually extends over a year. Biodegradation of PCL can be improved by copolymerization with a fast degrading polymer (Koleske (1978)). Two-phased degradation of PCL is a bulk process. First phase is Mn loss up to 5000 Da because of chain scission. The second one is weight loss onset. Degradation is autocatalyzed (Pitt (1990)).

In pharmaceutical applications, toxicity is an important consideration that has to be taken into account. Biocompatibility of PCL has been documented in many studies. PCL has also been approved by FDA for human clinical use in vivo (Taddei et al. (2005), Pitt (1990)). For instance, Menci et al. (1994) prepared PCL microspheres by solvent evaporation technique and implanted into the brain of wistar rats in order to see tissue reaction. They concluded that the polymer resulted with no tissue necrosis. According to Nakamura et al. (1992) and Ekholm et al. (1999) implantation of PCL showed more inflammation in muscle tissue than in bone tissue. According to them, more vascularized structure of muscle tissue and more amount of implanted material might cause more inflammatory reaction.

What makes PCL more advantageous than the other polymers is its high permeability to small drug molecules and degradation without creating acidic environment. Moreover, having slower biodegradation compared to for instance poly(lactide-co-glycolide) is another advantage for long term release (Koleske, (1978)).

In terms of drug delivery, among various systems microspheres are at great importance. There are several studies reporting that various drugs have been encapsulated in PCL microspheres with effective loading and delivery (Sinha et al. (2004)).

1.4.2 Poly (L-lactide-co-D,L-lactide) (P(L,DL)LA)

Poly(lactide) or poly(lactic acid) was first reported by Kulkarni in 1966 to be appropriate for surgical implants due to its degradation product lactic acid which is a natural product of muscle metabolism. Following that, Schneider stated that poly lactide was suitable to prepare absorbable sutures. In 1971, Yolles reported first the controlled drug release application of poly lactide as erodible matrix. Since then, many patents and papers appeared including preparation and application of poly(lactide) for biomedical applications (Kydonieus (1980)). Lactic acid, which is an organic acid can be found as either L(+) or D(-) stereoisomeric forms (Jones (2004)). Racemic mixture of 1:1 D and L is called D,L-lactide (Bendix (1998)). Pure poly(lactic acid) is a semi crystalline polymer whereas polymers prepared by meso and racemic lactides are amorphous. Degree of crystallinity, solubility and melting temperature are dependent on molecular weight, optical purity, thermal history and copolymer ratio (Jones (2004)). By changing the quantity and nature of the catalyst poly lactic acid's molecular weight can be controlled (Kydonieus (1980)). Stereoisomer of high molecular weight poly lactic acid has a glass transition temperature of 60°C and melting temperature of 170°C (Dinh et al. (2003)). Lactic acid polymers can be synthesized by either ring opening polymerization or

by polycondensation (Jones (2004)). Its copolymers are synthesized by ring opening polymerization of cyclic monomers (Bendix, (1998)). Chemical structure of poly lactide monomer can be seen in Figure 1.11.

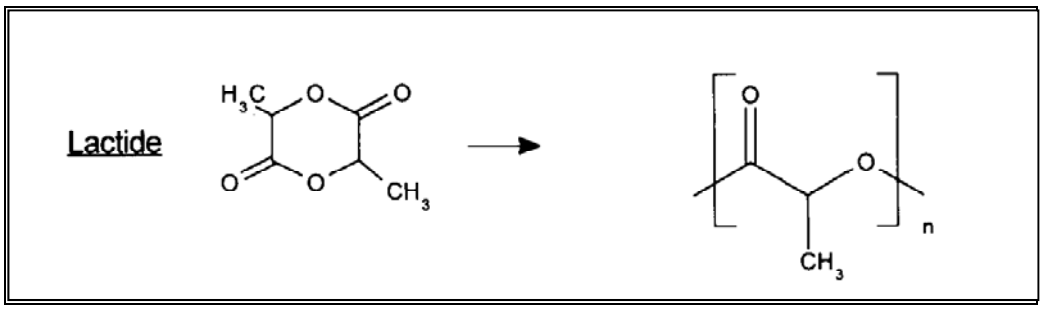

Figure 1.11. Monomer and resulting repeating unit of poly lactide (modified from Bendix (1998)).

Poly lactide and its copolymers, similar to PCL mentioned in previous section, is stated in many papers as biocompatible and biodegradable. They are being used in wound management, tissue engineering and controlled drug release applications (Vivek et al. (2007), Sinha et al. (2004), Butoescu et al. (2009), O'Donnell et al. (1997), Takei et al. (2008), Jones (2004), Kydonieus (1980), Dinh et al. (2003)).

Poly(L-lactide) (PLLA) and poly(D,L-lactide) (PDLLA) are homopolymers of poly lactide. PLLA is a semi crystalline polymer with about 70% crystallinity range which has T_m about 180°C. It has strong optical rotation and high mechanical strength properties. Therefore, PLLA is a choice for medical applications such as orthopedic devices, injection molded screw and other internal fixation devices. As a result of high crystallinity, PLLA exhibits slowest degradation rate among other resorbable poly lactides (Bendix (1998)).

PDLLA exhibits no optical activity due to being racemic polymer. Because of random distribution of L- and D- units, polymer is amorphous, glassy. Therefore, mechanical properties are lower than crystalline PLLA. On the other hand, degradation is faster in PDLLA since water can penetrate into amorphous bulk polymer and starts degradation. In case of medical applications, PDLLA is used as screws as well as membranes in dental field for guided tissue regenaration (Bendix (1998)).

In this thesis, co polymer of L- and D,L-lactide, poly(L-lactide-co-D,L-lactide) P(L,DL)LA is used. By addition of D-stereoisomer to L-chain crystallinity is decreased compared to PLLA, which improves the biocompatibility of the polymer. P(L,DL)LA is used in orthopedic applications such as pins (Bendix (1998)), intervertebral implants for spine surgery (Lazennec et al. (2006)), plates and screw (Moser et al. (2003). Besides these it is used in preparation of films for food packaging (Martino et al. (2006)), and in preparation of surgical sutures. P(L,DL)LA is also used as substrate for tissue regeneration and as drug and gene carriers due to its good biodegradability and biocompatibility (Jeun et al. (2007)).

Commercially available P(L,DL)LA (Boehringer Ingelheim Pharma GmbH & Co. Germany, RESOMER LR 704 S) has polymer composition of 67:33 to 73:27 molar ratio (L-lactide : D,L-lactide) with inherent viscosity of 2.0-2.8 dl/g. Thermal properties of this specific co polymer is T_g: 56 - 62 °C and T_m: amorphous (Boehringer Ingelheim Pharma GmbH & Co Product Information Sheets). The chemical formula of P(L,DL)LA is $(C_6H_8O_4)_n$ and chemical structure can be seen in Figure 1.12.

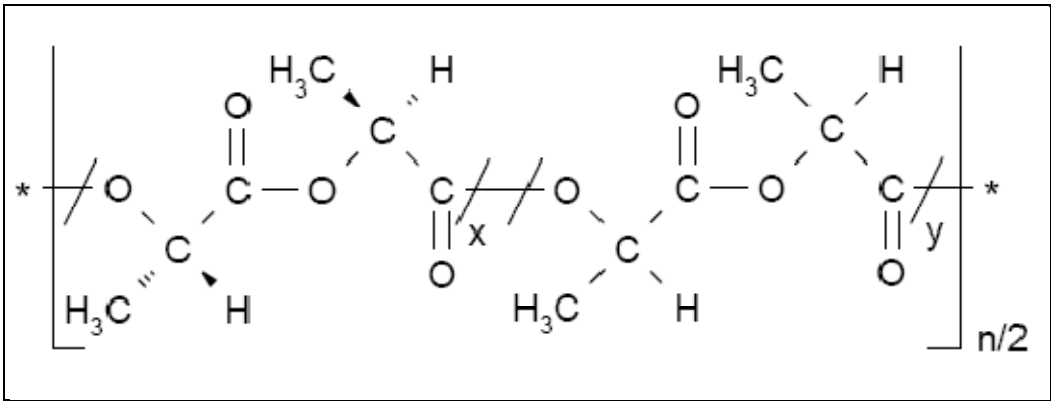

Figure 1.12. Poly(L-lactide-co-D,L-lactide) (Boehringer Ingelheim Pharma GmbH & Co Product Information Sheets)

According to a report by Wolfgang Joerg (2002) (Department of Fine Chemicals, Boehringer Ingelheim Pharma KG), after being used in living tissues or treated with water, polymer backbone of poly(L-lactide-co-D,L-lactide) 70:30 degrades by hydrolytic reaction. At the initial stage of degradation, reduction of molecular weight and changes of mechanical properties are observed. During the following stage a weight loss can be seen. The degradation products are absorbed or used by the body, as soon as sufficiently low molecular weight species (lactic acid or oligomers) have been generated.

1.5 Aim of the Study

Rheumatoid arthritis is a systemic disease that mainly attacks on and degenerates the joints. As a consequence, RA disrupts the movement and thereby the life quality of patients. Traditional treatment of the disease requires

long-term systemic drug administration which mostly results in side effects at the other tissues and organs. One other drawback of systemic treatment is lower allocation of the drugs into the synovial fluid (joint space) than the serum concentration. The new approach of treatment, thus, involves injection of drugs intraarticularly. Nevertheless, local injection requires high single-dose administration to provide the agent for long periods. This causes other problems like flare after injection, steroid arthropaty, cutaneous atrophy. In addition, intraarticular injection itself brings difficulties in terms of lack of space for needle placement. Moreover, efflux of the drug from joint cavity after instillation is another disadvantage. This route of administration requires patient rest, time and cost. At this point, in order to sustain and prolong drug retention and release at the target site, drug delivery systems such as microspheres might be a solution.

In this study, it is aimed to develop an injectable controlled release system of PCL and P(L,DL)LA microspheres loaded with a commonly used RA treatment agent Triamcinalone (TA) and/or a selective estrogen receptor modulator raloxifene (Ral) for local treatment of rheumatoid arthritis in order to avoid from systemic side effects of traditional administration and problems caused by direct local injections. It is hypothesized that this microsphere form drug delivery system when administered intraarticularly will enhance the treatment success of rheumatic diseases by providing these agents alone or together for long terms without causing systemic or local site effects upon injection to the RA joints. Microspheres were prepared with s/o/w solvent evaporation technique and optimized to achieve a suitable size for joint application, to sustain the delivery of the drug(s), to provide required amount of the agent with feasible amount of microsphere. In order to manage these, microspheres prepared with different combinations of polymers and drugs were examined for particle size analysis, surface and structural characterizations, time

related drug release properties, and for drug loading capacities. In order to evaluate biocompatibility of drug loaded PCL and P(L,DL)LA microspheres, they were analyzed with in vitro cytotoxicity tests using 3T3 fibroblast cells. Biodegradation studies of polymers were done by using GPC. The TA and Ral delivery systems were developed and studied for the first time in literature and they are optimized for RA treatment purposes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Polyvinyl alcohol (PVA), gelatin (from porcine skin, type A) and Polycaprolactone (PCL) with molecular weight 14,000 were purchased from Sigma-Aldrich Chem. Co. (USA).

Poly-(L-lactide-co-D,L-lactide) (PLLA-PDLLA, co-polymer of L-lactide: D,L-lactide in the range of 67:33 to 73:27 molar ratio) was obtained from Boehringer Ingelheim.

Triamcinolone acetonide (TA) and Raloxifene hydrochloride (Ral) were the products of Sigma-Aldrich Chem. Co.

HPLC grade methanol and chloroform were purchased from Sigma-Aldrich Chem. Co.

Dimethyl sulfoxide (DMSO) was molecular biology grade and was purchased from Applichem GmbH.

Dulbecco's modified Eagle's medium (DMEM) low glucose (1 g/l) with L-glutamine, DMEM high glucose (4.5 g/l) with L-glutamine and fetal bovine serum (FBS) were from the products of Biochrom AG.

Trypsin EDTA and penicillin/streptomycin were purchased from PAA Laboratories.

Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chem. Co. (USA).

2.2 Methods

2.2.1 Preparation of PCL Microspheres

PCL microspheres were prepared by using modified solvent evaporation technique with single emulsion; solid-in-oil-in-water (s/o/w) (O'Donnell et al. (1997)). An illustration of the preparation steps is given in Figure 2.1.

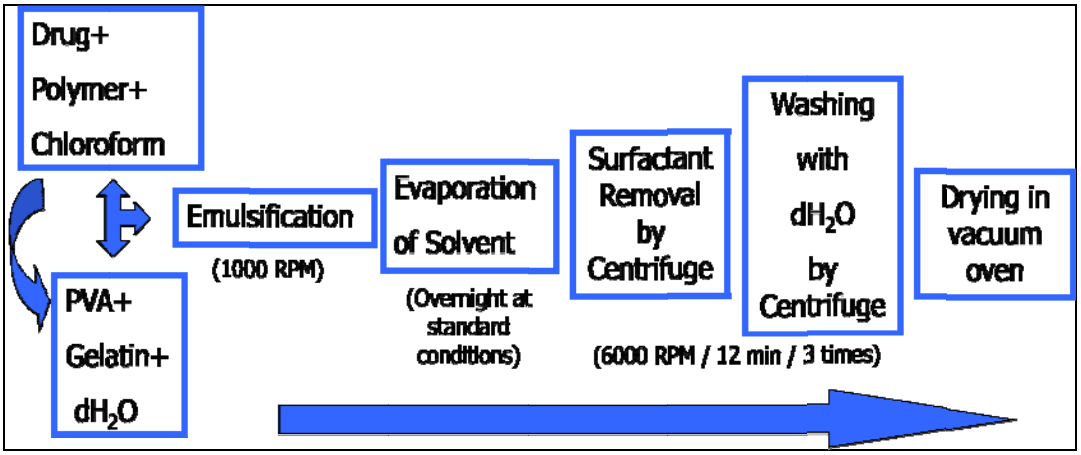

Figure 2.1. Steps of preparation of microspheres

The aqueous phase (w) was prepared using a surfactant, PVA and stabilizer agent, gelatin. First, PVA (2% w/v) was completely dissolved in water by heating at 80°C and then gelatin (1%, w/v) was added into this solution and dissolved at 70°C by stirring continuously.

For the oil phase (o), first polymer (PCL) solution in chloroform (40 mg/ml) was prepared in capped glass vials to minimize the evaporative loss of solvent during the process. Then, drug(s) (TA, Ral or both) was added into this solution, as the solid phase (s) and mixed thoroughly to disperse the solid phase homogenously. Different polymer to drug ratios were used for the drugs as explained in Section 2.2.1.1 - 2.2.1.3.

In order to form the single emulsion with these solutions, oil phase consisting of the polymer and the drug(s) were added drop-wise to the aqueous phase while stirring continuously at 1000 rpm. For the complete evaporation of organic solvent (chloroform), stirring was continued overnight and drug loaded polymeric microspheres were obtained. Formed microspheres were precipitated with centrifugation at 6000 rpm for 12 minutes. They were then washed with distilled water by adding distilled water to the microspheres and centrifuging again at the same settings to eliminate PVA, gelatin and unencapsulated drug molecules from their surfaces. For complete removal, washing process was repeated three times.

Microspheres were then transferred to a petri-plate where they were dried under low atmospheric pressure-vacuum at room temperature. They were stored in a desiccator at 4°C until use.

2.2.1.1 Triamcinolone Acetonide (TA) Loaded PCL Microspheres

In order to determine optimum polymer to drug ratio that will provide sustained drug release as well as enough local drug concentrations for the treatment period of RA, microspheres at different ratios were prepared and studied.

PCL microspheres with (10:1), (10:2) and (10:4), polymer to drug ratios were prepared using the method explained in the previous section (2.2.1).

These microspheres were labeled as #1, #2 and #3, respectively. The labeling of all microspheres is summarized in Table 2.1.

Table 2.1. Labels and compositions of the microspheres

Microspheres No	Composition of the groups	Initial Ratio of the Components
#1	PCL:TA	10:1
#2	PCL:TA	10:2
#3	PCL:TA	10:4
#4	P(L,DL)LA:TA	10:1
#5	P(L,DL)LA:TA	10:2
#6	P(L,DL)LA:TA	10:4
#7	P(L,DL)LA:Ral	10:1
#8	PCL:Ral	10:1
#9	PCL:Ral	10:2
#10	PCL:TA:Ral	10:4:2

2.2.1.2 Raloxifene Hydrochloride (Ral) Loaded PCL Microspheres

In preparation of Ral loaded PCL microspheres, two different polymer to drug ratios could be applied; 10:1 and 10:2. This was because of high viscosity of the oil phase in 10:4 ratio group (highest raloxifene composition) that

disabled the microsphere preparation process. Microspheres with lower ratios were prepared using the method described in Section 2.2.1.

2.2.1.3 Dual Drug Loaded (TA and Ral) PCL Microspheres

Dual drug loaded microspheres were prepared considering the best loading results for both drugs in single drug loading studies. Hence, the ratio for dual loading study was chosen as 10:4:2, where the polymer and the drugs used were PCL:TA:Ral, respectively. Microspheres with this initial ratio of the components were prepared using the method given in Section 2.2.1.

2.2.2 Preparation of P(L,DL)LA Microspheres

P(L,DL)LA microspheres were prepared by the same method used in the preparation of PCL microspheres with some small modifications (Section 2.2.1). Here, owing to higher viscosity of 40 mg/ml P(L,DL)LA solution compared to that of PCL solution, polymer concentration was changed to 20 mg/ml in the oil phase used in preparation of P(L,DL)LA microspheres. All other conditions were kept the same with PCL microspheres case. This change was essential to be able to add the oil phase to the aqueous phase and to get microspheres in the desired form and sizes. To be consistent with polymer to drug ratios used in PCL microspheres, drug amounts were also modified according to polymer amounts used.

2.2.2.1 Triamcinolone Acetonide Loaded P(L,DL)LA Microspheres

In the preparation of TA loaded P(L,DL)LA microspheres three different polymer to drug ratios (P(L,DL)LA:TA) were applied as given in Table 2.1 (10:1, 10:2 and 10:4).

2.2.2.2 Raloxifene Hydrochloride Loaded P(L,DL)LA Microspheres

In the case of Ral loaded P(L,DL)LA microspheres only one polymer to drug ratio could be applied (10:1) due to very high viscosity of the oil phase in other polymer:drug ratio groups despite using the polymer at lower concentration.

2.2.3 Determination of Drug Encapsulation Efficiency and Loading

Encapsulation efficiencies and loading values of drugs were calculated from the results obtained from extraction of drug(s) from microspheres.

In order to determine the encapsulated drug amounts, microspheres were either dissolved in an organic solvent that dissolves both the polymer and the drug or only the polymer component. In some cases, another solvent was added to dissolve and extract the drug for quantification.

Encapsulation efficiency (EE) was calculated as percentage of the ratio of drug amount in microspheres to the input drug used (Eqn. 1).

$$\text{EE}\ (\%) = \frac{\text{Encapsulated Drug in the Batch}}{\text{Input of Drug During Preparation}} * 100 \qquad [\text{Eqn. 1}]$$

Loading was calculated as percentage of the ratio of drug amount of microspheres to the amount of microspheres used in detection (Eqn. 2).

$$\text{Loading}\ (\%) = \frac{\text{Drug Amount in Microspheres}}{\text{Amount of Microspheres}} * 100 \qquad [\text{Eqn. 2}]$$

TA was observed to have common solvents with the polymers used in microsphere preparations. Therefore, to quantify TA present in the microspheres, first UV spectrophotometric (Hitachi U-2800A) scans of polymer and drug solutions in chloroform were done and λ_{max} value for the drug at which polymers (PCL and P(L,DL)LA) have negligible absorbances were found. Then, 5 mg of microsphere was dissolved in 10 ml of chloroform and optical density was measured at 255 nm. In order to convert this value to the drug amount, a calibration curve of TA with known concentrations of the drug in the same solvent were prepared and their absorbance values were obtained by UV spectrophotometer at the same wavelength. As a result, concentration vs. absorbance (Optical Density) calibration curve was prepared and encapsulation efficiency and loading were calculated according to equations 1 and 2, respectively.

Unlike TA, Ral had no dissolution in the same solvents with polymers. Hence, a second solvent that can solubilize this drug was used to extract drug molecules from polymer phase. At first, 5 mg microsphere was dissolved in 10

ml chloroform in order to disrupt the polymer core. After that dimethyl sulfoxide, (DMSO, 10 ml) was added to dissolve Ral suspended in the first solution. After mixing completely, this solution was used in drug quantifications. UV spectrophotometric scans were done to find λ_{max} for Ral with negligible absorbance values for polymers. In order to quantify amount of drug, known concentrations of Ral were dissolved in the same solvent mixture and absorbance values were obtained from UV spectrophotometer at λ_{max}, 300 nm. As a result, concentration vs. absorbance calibration curve was prepared and encapsulation efficiency - loading were calculated according to equations 1 and 2, respectively.

In the dual drug loaded microspheres because of differences in solubilities of the drugs (TA and Ral) drug amount detections were done in two stages. In the first stage, TA measurements were done as defined in Section 2.2.3.1 just with small difference in the λ_{max} used. Briefly, microspheres were dissolved in chloroform and O.D. values were obtained for this solution at 243 nm. In the second stage, chloroform and DMSO were used in order to dissolve the polymer of the microsphere (PCL) and the second drug, Ral. From this solution, Ral amounts were quantified following measurements of optical densities at λ_{max}, 300 nm. Encapsulation efficiency and loading values were calculated according to equations 1 and 2, respectively.

2.2.4 Determination of Drug Release Profiles of PCL and P(L,DL)LA Microspheres

2.2.4.1 Preparation of Drug Release Setup

Drug loaded PCL and P(L,DL)LA microspheres were placed into conic bottom PP centrifuge tubes (5 mg microsphere/tube) containing PBS (10 mM, pH 7.4, 5 ml). Tubes were placed in shaking water bath which was set to 37°C and 50 rpm shaking rate.

For the first 4^{th} hour of the releases, the tubes were centrifuged at 6000 rpm for 12 minutes in order to precipitate the microspheres and 1 ml samples from release media were taken. Fresh PBS (1ml) was added to release media and tubes were placed into water bath again. In the following release periods, samples were taken from release media daily and total media were refreshed with 5 ml PBS. Release studies were carried out in quadruplets. Release medium samples were refrigerated at 4°C for one week and their drug quantification was done by using high performance liquid chromatography (HPLC) system (UV/VIS Detector SPD 20-A, Degasser DGU-$20A_3$, Liquid chromatograph LC-20AT, Auto-injector SIL-10AD VP, Column oven CTO-10AS VP, all the parts are Shimadzu)

2.2.4.2 Determination of the Release Profiles of Triamcinolone Acetonide from Microspheres

The mobile phase used in HPLC analysis was MeOH:dH_2O (85:15, v/v) and the flow rate was 0.8 ml/min. The injection volume was 20 µl. The HPLC

column used had the specifications; 5 µm particle size, C18, 100Å pore size, 250x4.60 mm (Luna, Phenomenex, USA) together with Phenomenex HPLC guard cartridge system. The analyses were done at room temperature. Release media (1 ml) were mixed with 1 ml of HPLC grade methanol before injections. For the detection of TA UV/VIS detector was used and measurements were done at 240 nm at which TA had been shown to have λ_{max} by the calibration studies. Calibration curve was obtained by known concentrations of TA in PBS:MeOH (1:1) in order to quantify the release amounts of the drug.

2.2.4.3 Determination of the Release Profiles of Raloxifene Hydrochloride from Microspheres

The same HPLC column and guard cartridge system as well as other settings used in TA quantification were applied for Ral measurements in the release media. Collected release media were again mixed with equal volumes of methanol. The spectrophotometric detection of raloxifene hydrochloride, however, was done at 300 nm at which the drug has highest optical density. Calibration curve was obtained by known concentrations of Ral in PBS:MeOH (1:1) in order to quantify released amounts of the drug.

2.2.4.4 Determination of Both TA and Ral Co-Release from Microspheres

Using the same HPLC system and experimental setup with the single drug detection methods both drug amounts were measured in the same release

media. Following addition of methanol to the release samples, using dual channel of UV/VIS detector of HPLC detection of the drugs was done. Namely, at the same time, release samples were analyzed at both 240 nm for TA and 300 nm for Ral quantification.

2.2.5 Particle Size Analysis of Microspheres

Particle size analyses were done by adding distilled water to microspheres. After obtaining a suitable obscuration with the sample population, particle sizes were measured by particle size analyzer (Mastersizer 2000 Ver 5.1, Malvern Instruments Ltd., UK) and a the particle size distribution for each type of microsphere was obtained from the computer program of the device.

2.2.6 Structural Analysis of Microspheres by Scanning Electron Microscopy (SEM)

TA and Ral loaded PCL microspheres were coated with gold under vacuum and their scanning electron micrographs were obtained using JSM-6400 Electron Microscope (JEOL), equipped with NORAN System 6 X-ray Microanalysis System & Semaphore Digitizer both before and after release studies.

TA and Ral loaded P(L,DL)LA microspheres before and after release studies were coated with gold under vacuum and their scanning electron micrographs were obtained using QUANTA 400F Field Emission Microscope.

2.2.7 In Vitro Cytotoxicity Studies of Microspheres

In vitro cytotoxicity of a drug delivery systems can be evaluated considering the cell proliferation. Considering the in vivo usability/bioefficiency of the various drug delivery sytems prepared in the study, best 3 formulations based on drug release profiles and drug loading values were decided to be tested for in vitro biocompatibility,. For extraction, first 5 mg of microspheres were UV sterilized and then 5 ml of DMEM were added. Microspheres were incubated at 37°C for 24 hours.

3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with fetal bovine serum (FBS, 10%) and penicillin-streptomycin (0.3 %). Cells were seeded onto 24-well plates. The initial cell seeding density was 10^5 cells/well. Cells were incubated at 37°C under 5% CO_2 in humidified environment (Shel Lab 5215, CO_2 Incubator) for 4 hours. Then, medium was removed and samples from drug release media were added to the wells. After 1 day incubation period, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay was studied on these selected groups; PCL:TA 10:4; PCL:Ral 10:2; PCL:TA:Ral 10:4:2. Shortly, the media were removed and 500 µl MTT solutions (5 mg/ml in DMEM low glucose) was added to each well and incubated for 3 hours at 37°C in a dark environment. Following incubations, MTT solutions were removed and wells were washed with sterile PBS. Then, DMSO (500 µl) were added to solubilize the formazan crystals formed inside the cells. Cells were shaken orbitally at 200 rpm for 10 minutes. The absorbance was measured at 550 nm wavelength using µQuant™ Microplate Spectrophotometer (Biotek Instruments Inc., USA). Quadruplets were used for each group in MTT studies.

2.2.8 Polymer Degradation Studies

Polymers were placed in phosphate buffer solution (PBS, 10 mM, pH 7.4) and incubated at 37°C. Molecular weight loss was monitored 45 days later. Gel permeation chromatography (GPC) was performed to determine the molecular weight distributions by using Polymer Laboratories PL-GPC 220 at Central Laboratory, METU. Columns were calibrated with polystyrene narrow standards. Tetrahydrofuran (THF) was used as the eluent. Column temperatures were maintained at 30°C.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results of Release Studies

3.1.1 Release of TA from Microspheres

3.1.1.1 TA Release from PCL Microspheres

PCL microspheres loaded with TA at ratios, 10:1, 10:2 and 10:4 (labeled #1, #2 and #3, respectively, in Table 3.1) were studied for their TA release profiles. A calibration curve was obtained from HPLC chromatograms of known concentrations of TA and used in determination of the released amounts of the drug (Figure 3.1).

In HPLC analysis, first, pure drug was applied to the system and chromatograms were obtained for various wavelengths in order to find λ_{max} for TA. In accordance with literature (Matysová et al. (2003), Nemutlu et al. (2005), Yeh et al. (1991)) a sharp single drug peak was obtained at 240 nm at 5^{th} minute (Figure 3.2). Similar chromatograms were obtained from release studies of TA as exemplified in Figure 3.3.

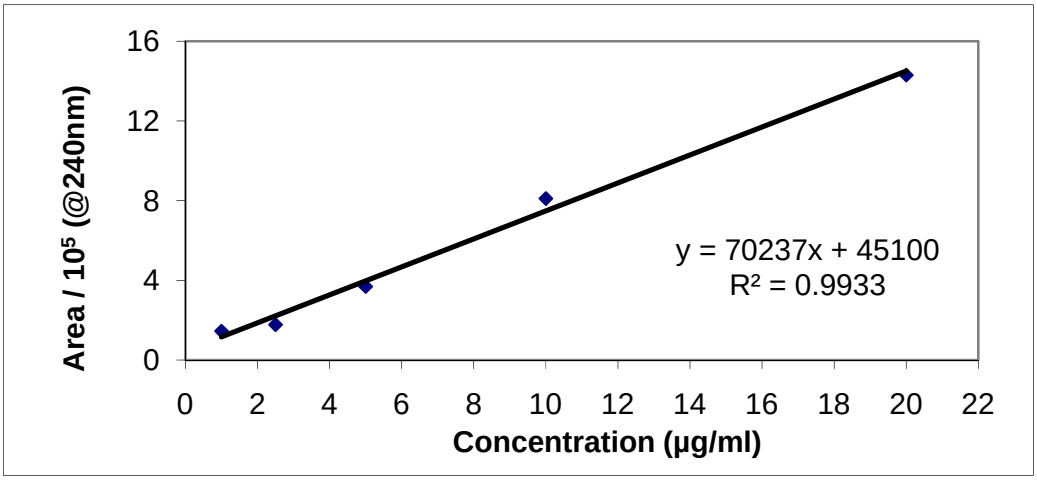

Figure 3.1. Calibration curve used for quantification of TA released from PCL microspheres

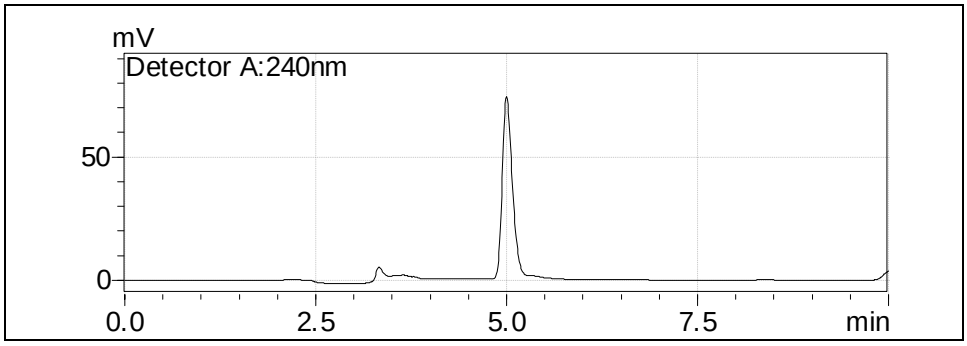

Figure 3.2. HPLC chromatogram of TA solution(10 µg/ml)

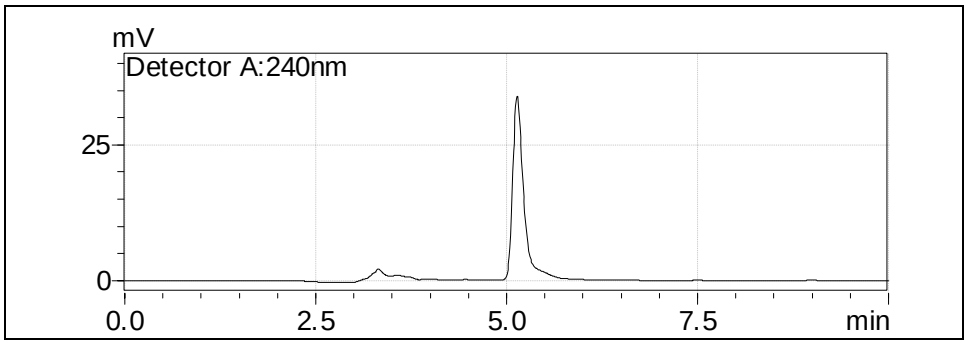

Figure 3.3. A representative HPLC chromatogram from release media of PCL:TA (10:1) microspheres

Release studies were carried out for 4 weeks. At the end of this period PCL:TA (10:1) microspheres showed complete release (100% release) of TA which corresponds to mean cumulative amount of 154.96 μg TA for these microspheres (Figures 3.4 and 3.5). The optimization studies conducted aimed to prolong the drug release period to at least 3 months for an efficient RA treatment system. Therefore, polymer:drug ratio initially used in preparation of microspheres was modified by doubling the drug content. This second set of microspheres with 10:2 ratio of PCL:TA showed a slower release profile in terms of percentage of total drug released (34.55% in 4 weeks) despite its higher corresponding mean cumulative amount, 310.03 μg TA (Figures 3.5 and 3.6). For further sustaining the TA release, polymer:drug ratio was decreased to 10:4. These microspheres had 447.10 μg TA cumulative release which was 20.87% of the loaded TA (Figures 3.7 and 3.5, respectively). Namely, as drug to polymer ratio increased (or polymer:drug ratio decreased from 10:1 to 10:4) amount of TA released increased. Similarly, Beeley et al. (2005) reported that increasing drug to polymer ratio resulted in an increase in the amount of TA released from the PCL subretinal implants of rod shaped PCL filaments with varying polymer/drug ratios (70/30 to 50/50). The group conducted their release studies both in vitro (in balanced salt solution/bovine serum albumin (1%) solution) and in vivo (rabbit eye) up to 4 weeks.

Besides this direct effect of increasing input amount of TA on release results, increased drug proportion also had significant changes on other microsphere properties which might have caused further modifications on release.

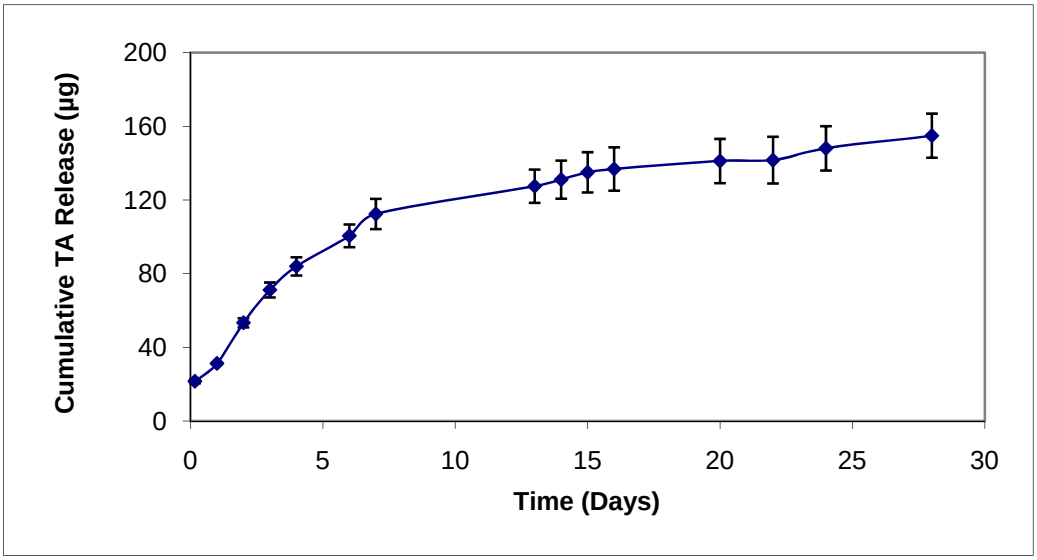

Figure 3.4. Cumulative release profile of TA from PCL:TA (10:1) microspheres (n=4)

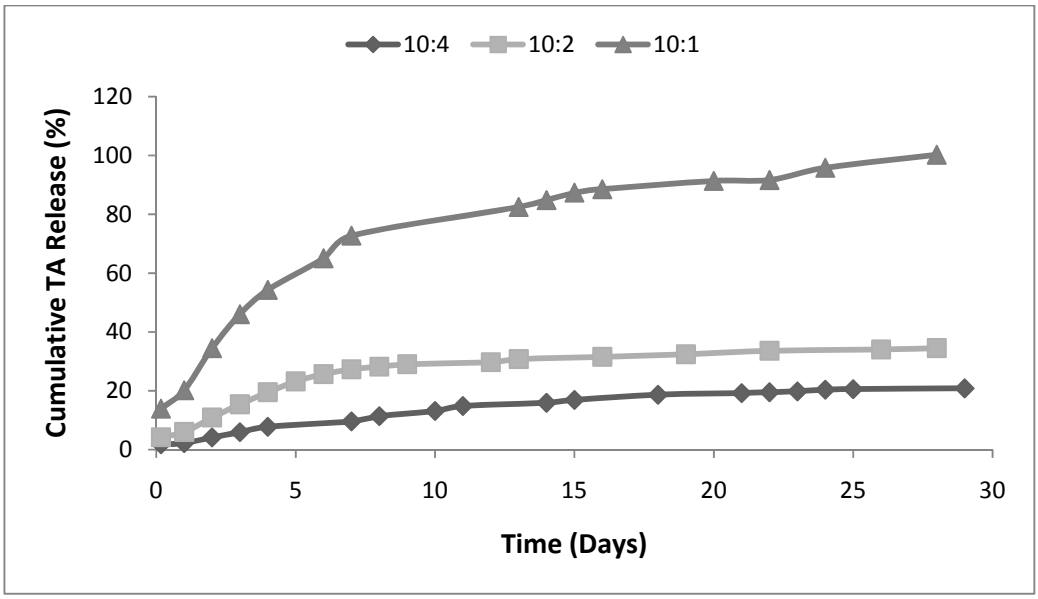

Figure 3.5. Comparison of cumulative percent release of TA from PCL:TA microspheres (n=4)

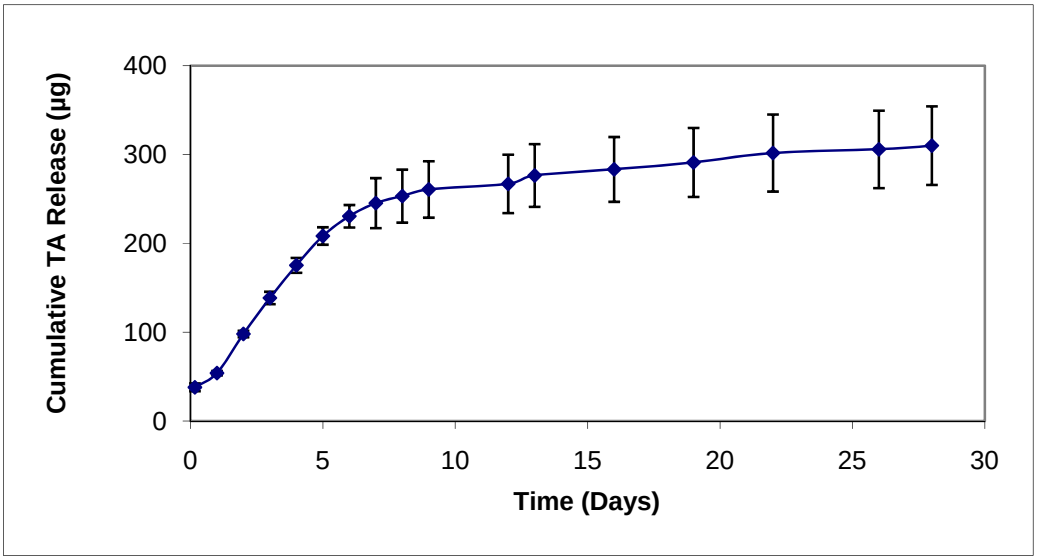

Figure 3.6. Cumulative release profile of TA from PCL:TA (10:2) microspheres (n=4)

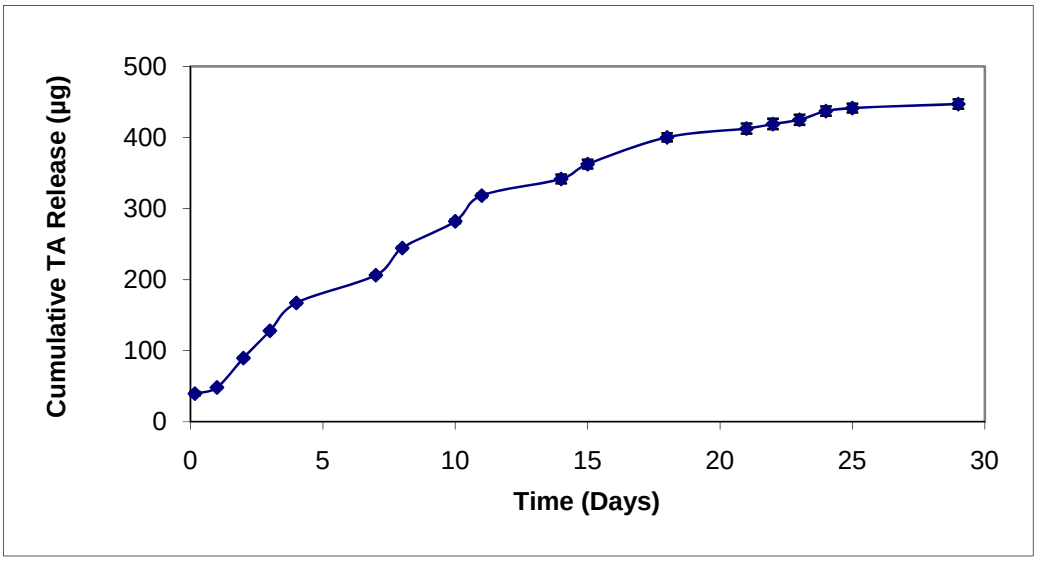

Figure 3.7. Cumulative release profile of TA from PCL:TA (10:4) microspheres (n=4)

When the results of three ratios were compared it was seen that as polymer:drug ratio decreased, the cumulative amount of the drug released increased (Table 3.1). This could be explained by the increased amount of drug within polymer carrier. When the initial drug amount was doubled the cumulative amount of drug released also doubled but further increasing the drug had the same effect with fewer trends. This might be explained by the encapsulation efficiency and loading values of microspheres. As polymer:drug ratio decreased from 10:1 to 10:2 encapsulation efficiency approximately doubled (from 23.34% to 51.33%) and loading increased approximately 6 times (from 3.09% to 17.95%). However, as polymer:drug ratio decreased from 10:2 to 10:4 encapsulation efficiency increased at smaller degree and loading had 1.5 fold increase (from 51.33% to 70.56% and from 17.95% to 27.17%, respectively).

Considering the initial aim of the release system, with the result of 20.87% release from PCL:TA (10:4) microspheres (Figure 3.5) it can be predicted that TA release could be sustained for about 4-5 months. In addition, from the biphasic release trend of TA, it can be predicted that there will be faster release and higher initial drug supply during the early phase of the treatment and a slower but continuous release with less drug concentration at later stages.

If time related release profiles were divided into two parts as initial fast release, which had larger slope, and as slower, more linear phase; first two types of microspheres (#1 and #2) completed the fast releasing initial region within approximately 7 days. After 7 days, they exhibited slower and more linear release profile for TA. On the other hand, the third set of microspheres (#3) represented same release trend with smaller change in slopes of fast and slow release phases. Also for these microspheres slow release had started after 10th day. The initial rapid release region of the release curves can be accounted for

the absorption of TA crystals on or close to the surface of the delivery system. Thus, they tend to be delivered well before and at a faster rate than the drug coming from the polymer core by diffusion. Wang et al. (2008) studied the effect of formation mechanism of PCL microspheres loaded with disodium norcantharidate (anticancer drug) on release profiles and reported the similar biphasic release profile with initial rapid release region and slower linear later region within 12 hours of total release experiments. The group conducted their release studies in different amounts of dextrose containing aqueous in vitro release media to investigate contribution of osmotic effect. Dhanaraju et al. (2006), reported a similar trend of fast initial (the first week) and thereafter more sustained in vitro release up to 20 weeks with 5:1 and 10:1 PCL microspheres loaded with water insoluble contraceptive steroids (levonorgestrel and ethinyl estradiol).

3.1.1.2 TA Release from P(L,DL)LA Microspheres

P(L,DL)LA microspheres loaded with 10:1, 10:2 and 10:4, ratios of TA, (labeled as #4, #5 and #6, respectively), were studied in the release experiments. For determination of the release profiles of TA from microspheres the linear regression equation of the curve represented in Figure 3.8 was used. A different release curve and equation than PCL microspheres were used for release studies of P(L,DL)LA microspheres. This was needed because of slower release of TA from this polymer which required detection of lower concentrations by the calibration curve.

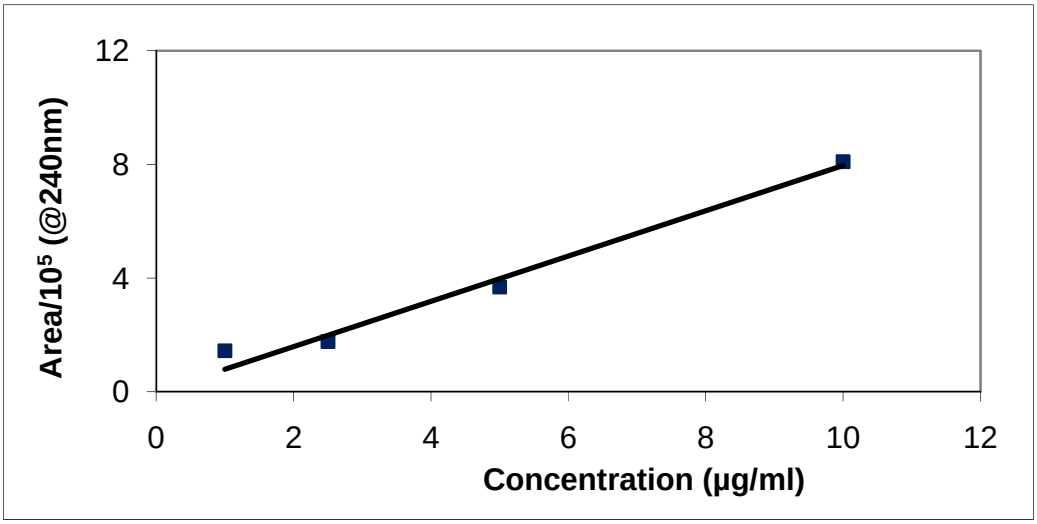

Figure 3.8. Calibration curve used for quantification of TA release from P(L,DL)LA microspheres

In Figure 3.9, HPLC chromatogram for TA release from P(L,DL)LA microspheres (10:2) is exemplified. This polymer released TA at slower rates than PCL as observed from small peak at 5 minutes and increased background peaks from PBS.

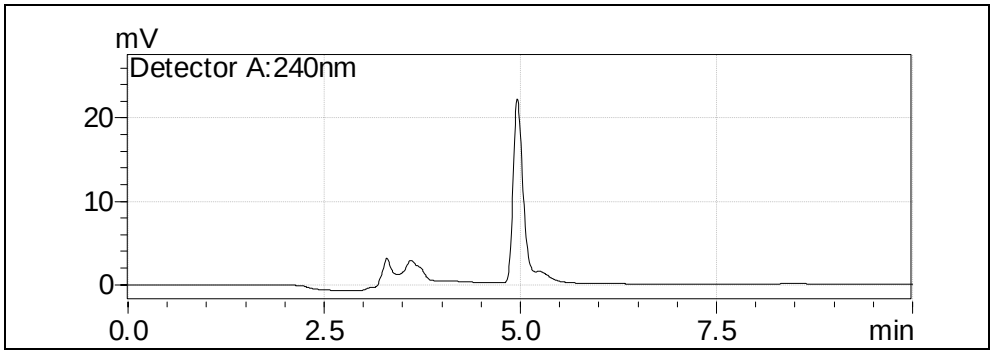

Figure 3.9. A representative HPLC chromatogram from release media of P(L,DL)LA:TA (10:2) microspheres

It was observed that P(L,DL)LA:TA (10:1) microspheres had mean cumulative amount of 30.54 µg TA released (Figure 3.10) which makes the 71.44% (Figure 3.11) of all the loaded drug. But, P(L,DL)LA:TA (10:2) microspheres, at the end of 4 weeks, released 20.5% (Figure 3.11) of all their drug content (104.5 µg, Figure 3.12). For P(L,DL)LA:TA (10:4) microspheres at the end of 4 weeks a similar percentage of drug release was observed (23.88%, Figure 3.11). However, this result corresponded to a considerably higher cumulative amount of 175.95 µg (Figure 3.13). At the end of 4 weeks, #5 microspheres released more than 3 times as much drug as that being released from #4 microspheres (Figures 3.10 and 3.12). So, the same effect of increasing initial amount of drug in PCL microspheres on release duration and amount was observed for (P(L,DL)LA:TA) microspheres. Released amount from microspheres nearly doubled with increasing the drug partition from 1 to 2 in P(L,DL)LA microspheres. The percentage of drugs being released, however, dropped from ~70% to ~20% in accordance with expectations. The change in cumulative percent releases (20.5% vs 23.88%) for the 10:2 and 10:4 groups were not in parallel with the above comparison (10:1 and 10:2 groups) and with previous release results of PCL microspheres. This was thought to be related with similarity in both encapsulation efficiency and loading values (Section 3.3) as well as particle size analysis results. When particle size results were examined (Section 3.2.1) mean diameter of the P(L,DL)LA:TA 10:2 microspheres was obtained significantly larger (489.93 µm) than both P(L,DL)LA:TA 10:1 (215.83 µm) and 10:4 (153.66 µm) microspheres. This non linear change in contrast with PCL:TA case might have contributed to the release results. Since release starts from the surface, with drug crystals at or close to surface, especially for P(L,DL)LA microspheres (seen in SEM images, Section 3.2.2), with larger particle size, which means larger surface area, might caused faster initial release than expected. In addition, when encapsulation

efficiencies of these two types of microspheres were examined, P(L,DL)LA:TA 10:2 microspheres encapsulated 49.98% of the drug input whereas P(L,DL)LA:TA 10:4 microspheres encapsulated 41.40% of the drug input. This approximately 10% difference of drug encapsulation might also be responsible for small difference in percent drug release (20.5% vs 23.88%) whereas cumulative amount release values were 104.5 µg and 175.95 µg, respectively which show the importance of loading values beyond encapsulation (higher in 10:4 group).

When the amount of drug (TA) released was compared for the two polymer types, P(L,DL)LA was found to provide less quantities of drug than PCL. The significantly lower cumulative amount and percentage release values in P(L,DL)LA ratio groups were thought to be related with the reduced polymer amounts during preparation of microspheres. This might also be explained by the drug distribution and morphological properties of the microspheres. When the SEM images of #4, #5 and #6 microspheres were examined (Section 3.2.2) it can be seen that P(L,DL)LA microspheres had more smooth surface with less pores and channels compared to PCL microspheres. Considering drug diffusion is contributed by these pores and channels, less TA release from P(L,DL)LA rather than PCL microspheres seems more reasonable. The release profiles of the microspheres prepared by P(L,DL)LA was also different than the classical biphasic release profile observed by most polymeric delivery systems and by those of PCL:TA groups in this study. Both amount and percentage TA release profiles of P(L,DL)LA microspheres did not show a distinct change in curve or significant decrease in slope after some period of release. However, there was small but recognizable turn point for slowing down the release pattern at around 10 days similar to other PCL results.

Vivek et al. (2007) compared some biodegradable polymer microspheres, PCL and poly-lactide-co-glycolide (PLGA), loaded with etoposide (poorly soluble

in pH 7.4 PBS) for their in vitro release profiles in PBS (pH 7.4 and 0.1% Tween 80 containing release medium). All microspheres in that study showed similar initial burst release followed by slower drug release pattern. It was reported that after initial rapid release the drug release was due to the passive diffusion of the drug through the pores in the polymer matrix and depends on the porosity of microspheres formed. Besides, it was shown that among all microspheres PCL showed the highest entrapment efficiency of the drug, as it is in our study when compared with TA release from P(L,DL)LA. In addition to that they also found out PCL microspheres had the least burst release and the slowest in vitro release compared to PLGA.

Table 3.1. Cumulative TA release amounts from microspheres at the end of 4 weeks (n=4)

	Cumulative TA Release (μg)	Cumulative TA Release (%)
PCL:TA (10:1) (#1)	154.96±11.97	100±7.74
PCL:TA (10:2) (#2)	310.03±44.21	34.55±4.92
PCL:TA (10:4) (#3)	447.10±6.55	20.87±0.30
P(L,DL)LA:TA (10:1) (#4)	30.54±2.63	71.44±6.17
P(L,DL)LA:TA (10:2) (#5)	104.50±3.71	20.50±0.72
P(L,DL)LA:TA (10:4) (#6)	175.95±10.00	23.88±1.35

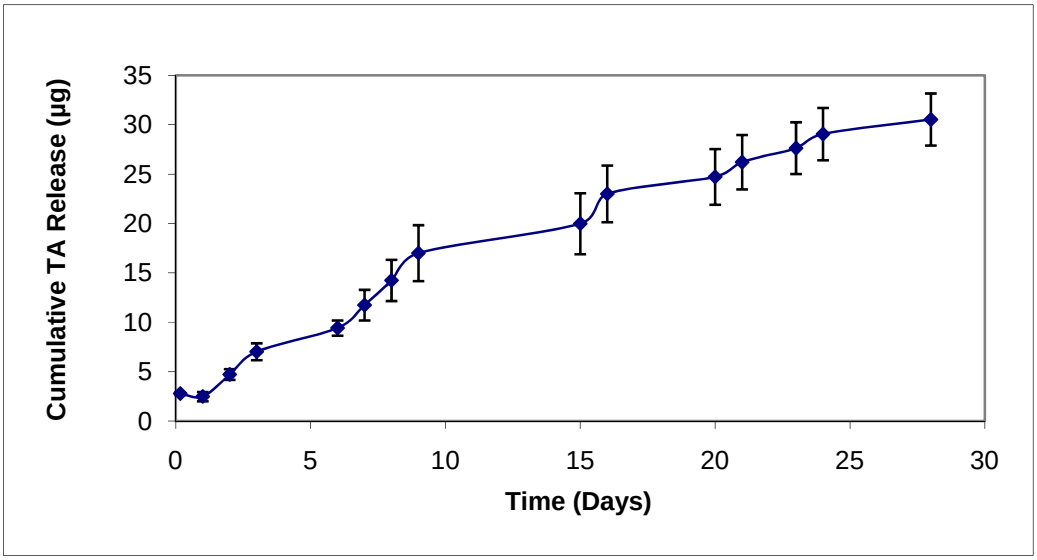

Figure 3.10. Cumulative release profile of TA from P(L,DL)LA:TA (10:1) microspheres (n=4)

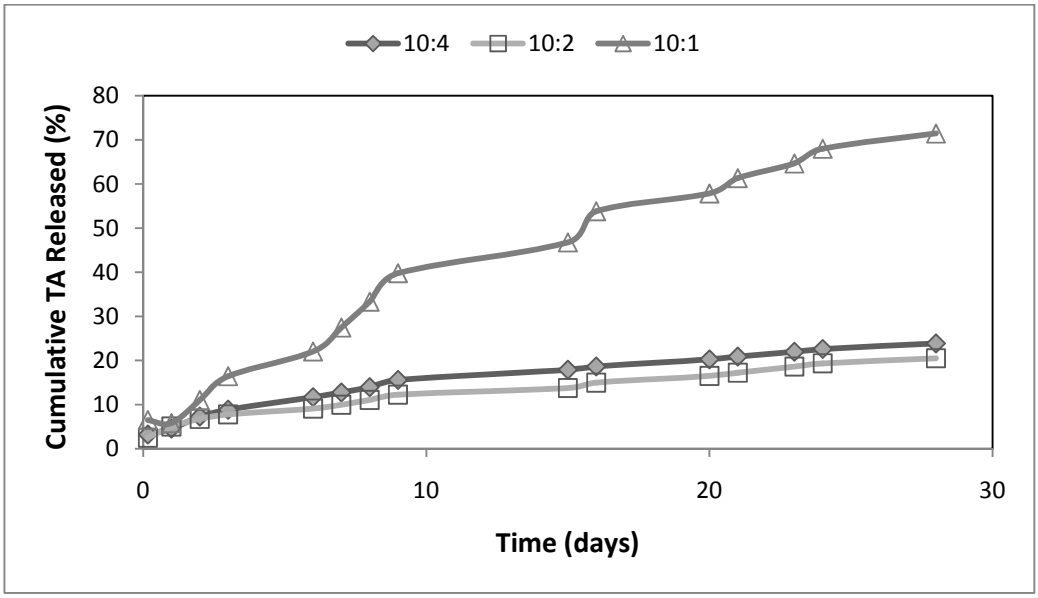

Figure 3.11. Comparison of cumulative percent release of TA from P(L,DL)LA:TA microspheres

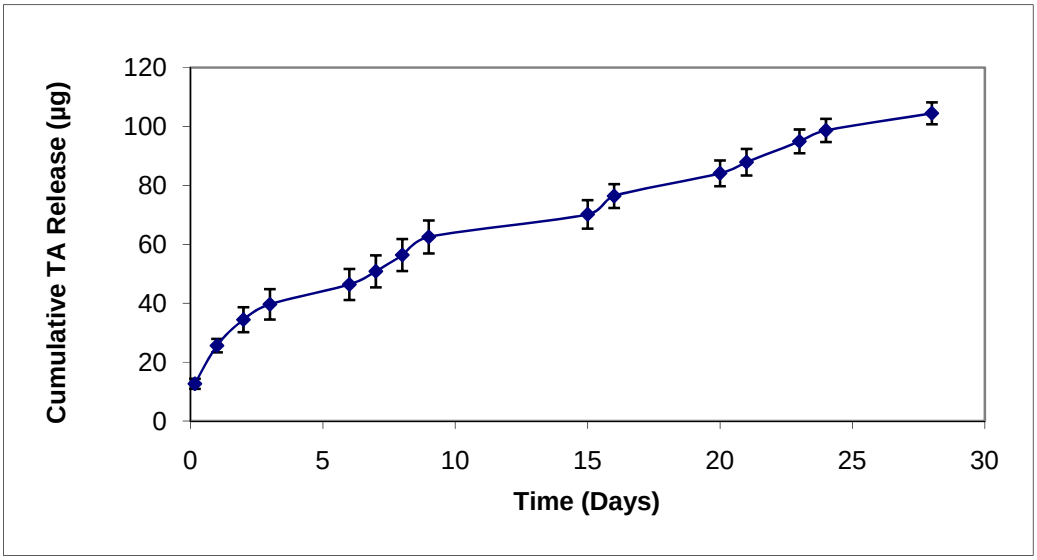

Figure 3.12. Cumulative release profile of TA from P(L,DL)LA:TA (10:2) microspheres (n=4)

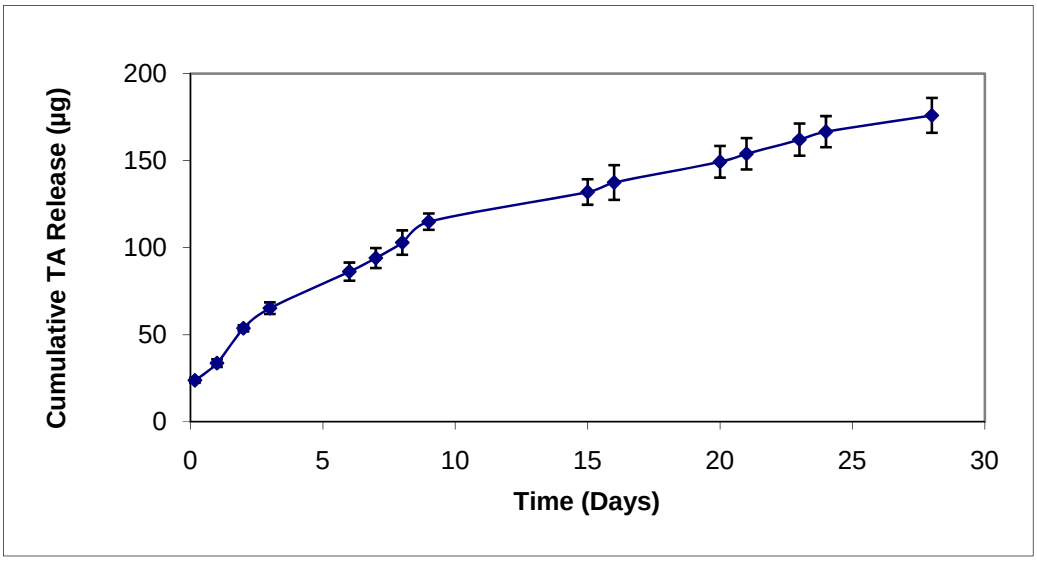

Figure 3.13. Cumulative Release Profile of TA from P(L,DL)LA:TA (10:4) Microspheres (n=4)

To our knowledge, no P(L,DL)LA microsphere release system has been reported in literature. Therefore, its homopolymer PLA could be compared in discussing the results of this system with other studies. Takei et al. (2008) investigated the effect of polymer used in preparation of microspheres on release behavior. PLA and PLA/PCL blend were used as polymers and acetamiprid, which is a pesticide, was the released agent. Release studies were done in nonionic surfactant containing water. Weight ratio of PLA to acetamiprid in microspheres varied from 40:1 to 5:1. It was reported that as polymer:released agent ratio decreased, similar to our study, cumulative percent release values increased. In addition, when the PCL content in PCL/PLA blend was changed from 50% to 80%, cumulative release amounts also increased up to ~80% cumulative acetamiprid release within 160 hours. In PCL/PLA blend cases, polymer:released agent ratio was reported to be kept at 20:1. Takei et al. (2008) claimed that increase in amount of drug release was due to the enhanced penetration of surrounding water into the microspheres compared to only PLA microspheres which had tight structure and high hydrophobic microclimate. Thus, blending with PCL, which has lower molecular weight than PLA, resulted in looser structure and less hydrophobic matrix. Also in our study, molecular weight of PCL (19239 Da, Section 3.4) was lower than P(L,DL)LA (173381 Da) and therefore higher release of TA from PCL microspheres might be reasonable compared to P(L,DL)LA.

3.1.2 Release of Ral from Microspheres

3.1.2.1 Ral Release from PCL Microspheres

PCL microspheres loaded with (10:1) and (10:2) Ral, (labeled as #8 and #9 respectively), were studied for determination of the release profiles of Ral from microspheres. After working 10:1 and 10:2 ratios, release values resulted in required molarities (0.11 μM and 0.05 μM, respectively) due to the fact that low molarity of Ral was effective in terms of cartilage regeneration (Kavas (2007)). The calibration curve used in determination of Ral concentration in these studies was presented in Figure 3.14.

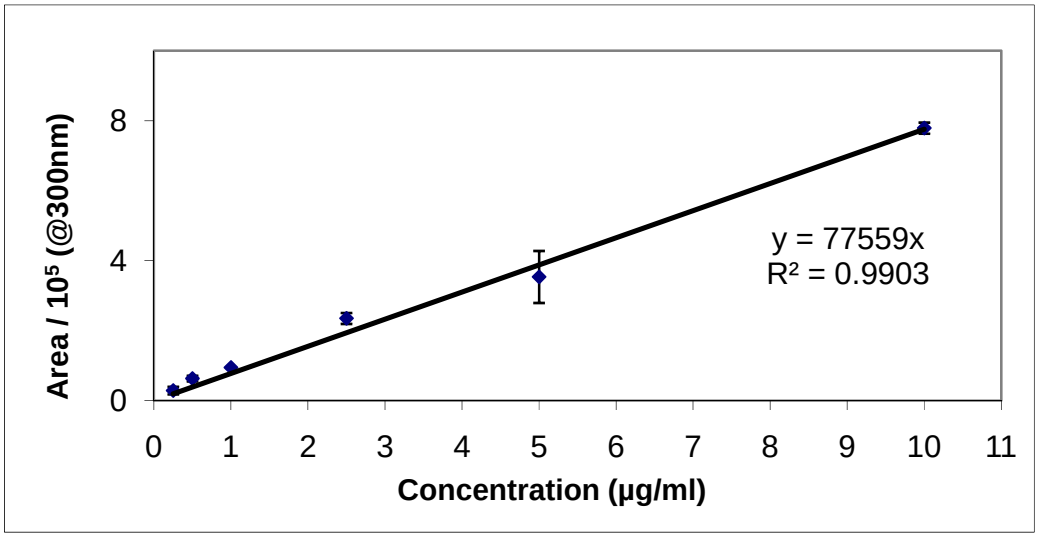

Figure 3.14. Calibration curve used for determination of Ral release profile of PCL and P(L,DL)LA microspheres

Upon preliminary HPLC experiments with pure drug solution, a sharp peak for Ral was found at about 7.5 minute at 300 nm (Figure 3.15a). Even though the pure drug peaks were sharp and narrow, those for Ral released from microspheres had wider curves. Due to slow release of Ral the size/area of released drugs' peak was considerably smaller than the free drug sample peak. Therefore, the peak that arise from PBS became more in the release samples' HPLC chromatograms. This PBS peak was seen in all microsphere types just after 2.5th minute with same characteristics, independent of drug type due to not being in mobile phase but being very dilute in injected phase.

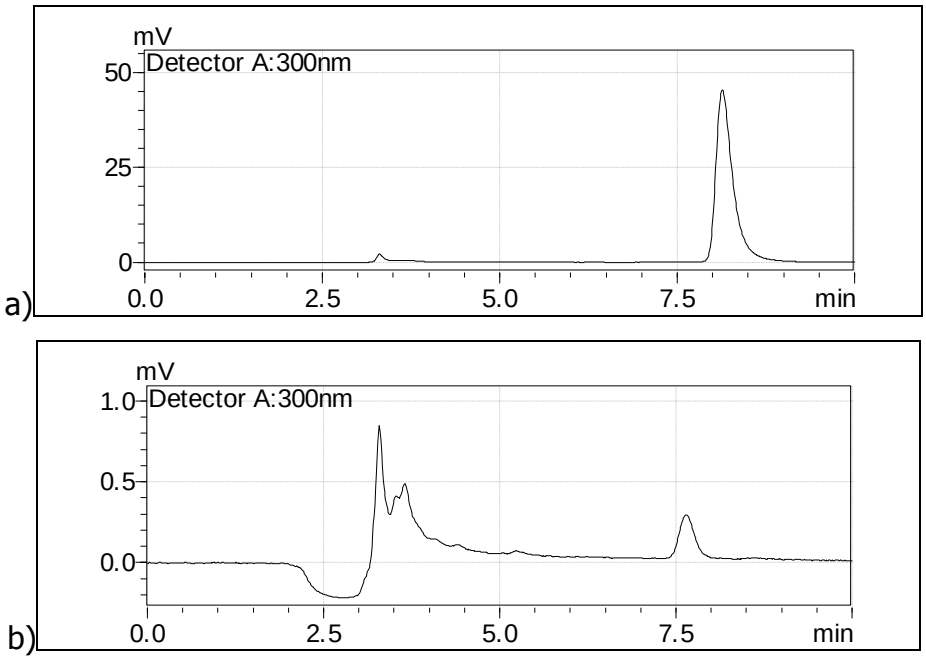

Figure 3.15. Representative HPLC chromatogram of Ral a) for 10 µg/ml Ral in MeOH:PBS 1:1. b) for the 4-Hour release samples of PCL:Ral (10:1) microspheres. Right arrows indicate the peak for Ral, left arrows indicate PBS peak

It was observed that PCL:Ral (10:1) microspheres released only 0.67 µg cumulative amount of raloxifene at the end of 4 weeks which is 0.16% of all the loaded drug (Figures 3.16 and 3.17). Similarly, PCL:Ral (10:2) microspheres had a mean cumulative release amount of 0.61 µg at the end of 4 weeks (Figure 3.18). However, owing to difference in loading, this corresponds to 0.09% (Figure 3.17).

When the initial polymer to drug ratio is considered, no significant change in the cumulative amounts of released Ral was seen between 10:1 and 10:2 ratio groups, which is another reason for not studying 10:4 ratio. However, in the case of percentage release, it was seen that the value decreased nearly to half when the drug to polymer ratio doubled. This is due to higher loading in the second group (higher number in the fraction when calculating % release) which was explained in Section 3.3.

In Table 3.2 cumulative Ral release and cumulative TA Release results were compared. It is seen that there was a drastic decrease in both amount and percentage release of the released drugs when the polymer:drug ratio were kept the same. This might be due to the size of the drug crystals and interaction of them with the polymer during preparation of microspheres. Higher molecular weight of Ral (510.05 g/mol) than molecular weight of TA (434.5) might have some contributed to this result. In addition, it is known that although solubility of both TA and Ral are poor Ral has less solubility (13 mg/L) than TA (17500 mg/L) which might be another reason of this result.

Crystallinity of Ral can be another point for slow release. Drug's crystallinity has been previously shown to slow down the release. Bikiaris et al. (2009) reported the similar slow release behavior of Ral from different polyesters (poly(ethylene succinate) and poly(propylene adipate)) and explained with drug loading in crystalline form, which was unable to leave the inner parts of the microspheres through small microchannels.

Table 3.2. Comparison of cumulative release amounts for #1, #2, #8, and #9 microspheres at the end of 4 weeks

	Cumulative Release (µg)	Cumulative Release (%)
PCL:TA (10:1) (#1)	154.96±11.97	100±7.74
PCL:TA (10:2) (#2)	310.03±44.21	34.55±4.92
PCL:Ral (10:1) (#8)	0.67±0.05	0.16±0.01
PCL:Ral (10:2) (#9)	0.61±0.11	0.09±0.01

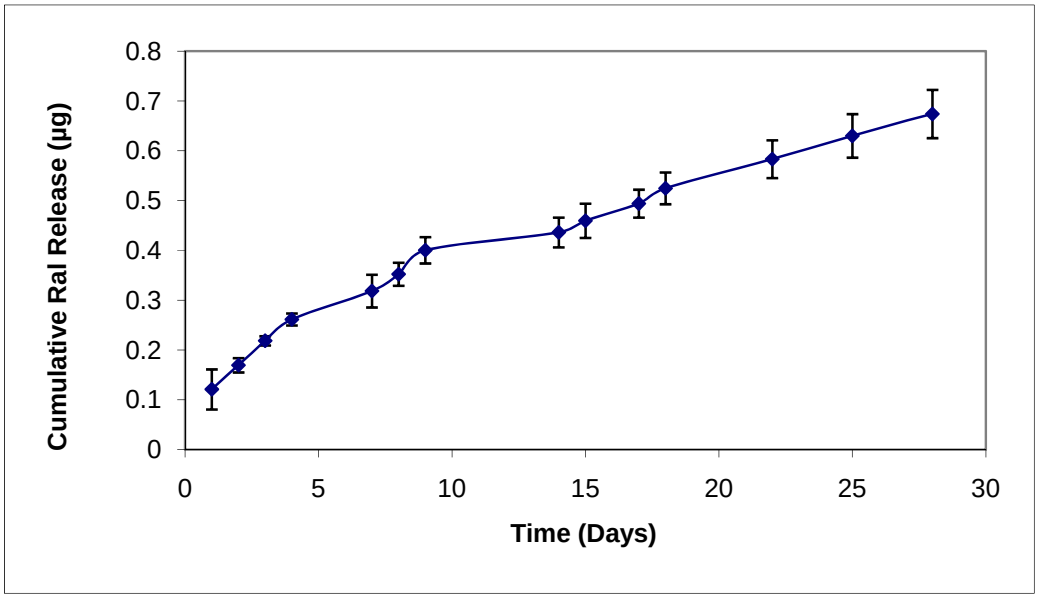

Figure 3.16. Cumulative release profile of Ral from PCL:Ral (10:1) microspheres (n=4)

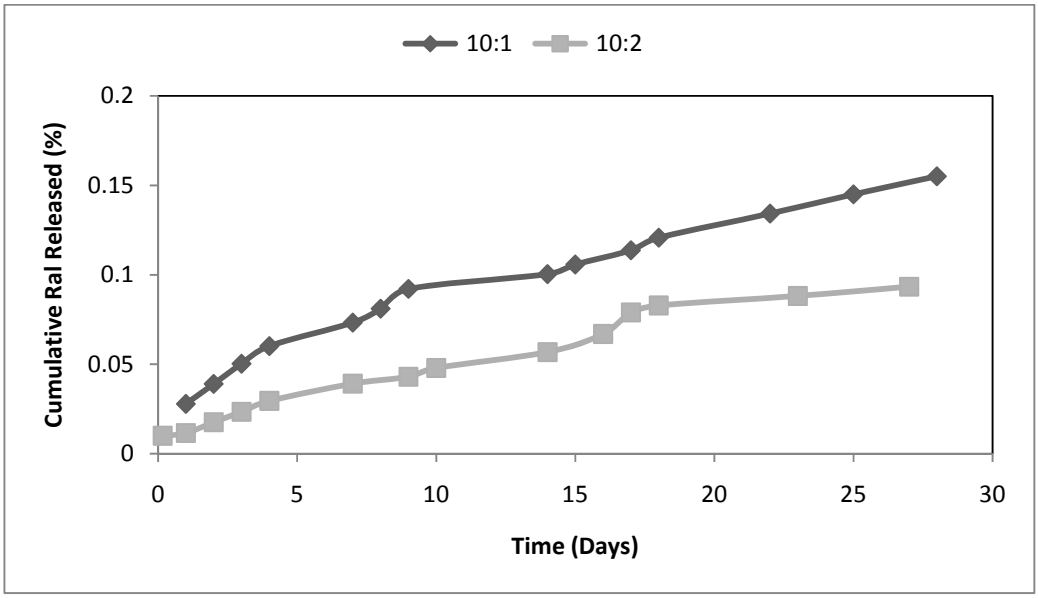

Figure 3.17. Comparison of cumulative percent release of Ral from PCL:Ral microspheres (n=4)

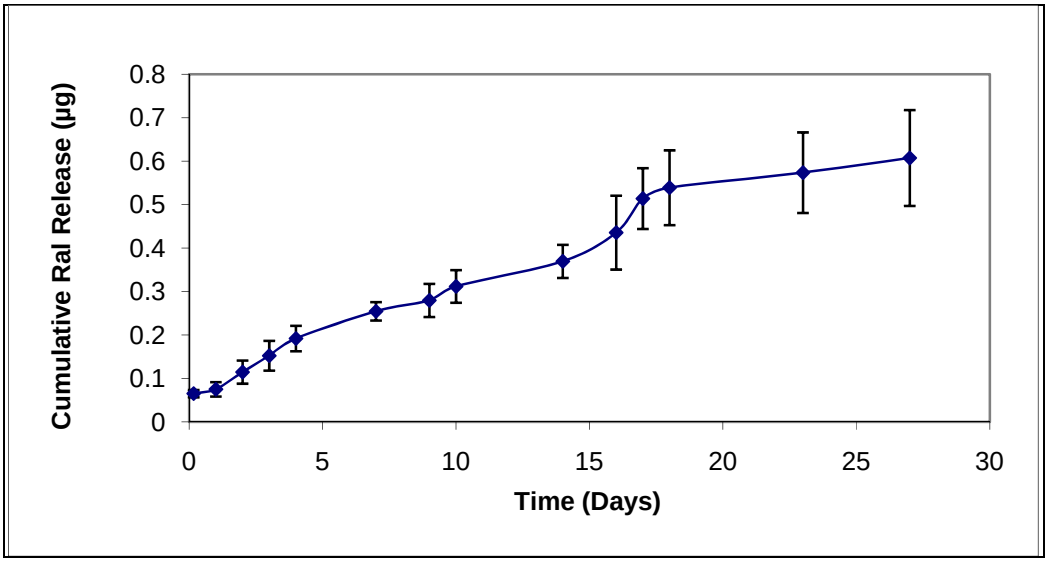

Figure 3.18. Cumulative release profile of Ral from PCL:Ral (10:2) microspheres (n=4)

3.1.2.2 Ral Release from P(L,DL)LA Microspheres

Ral release from P(L,DL)LA microspheres (labeled as #7) could be studied with 10:1 ratio group only. This was due to infeasibility of P(L,DL)LA and Ral combination for preparing microspheres at the other ratio groups. In these groups, the polymer-drug phase was highly viscous and resulted in problems in preparation of microspheres. Therefore, only 10:1 polymer to drug ratio group could be studied. Ral peak from release media of P(L,DL)LA microspheres can be seen in HPLC chromatogram (Figure 3.19).

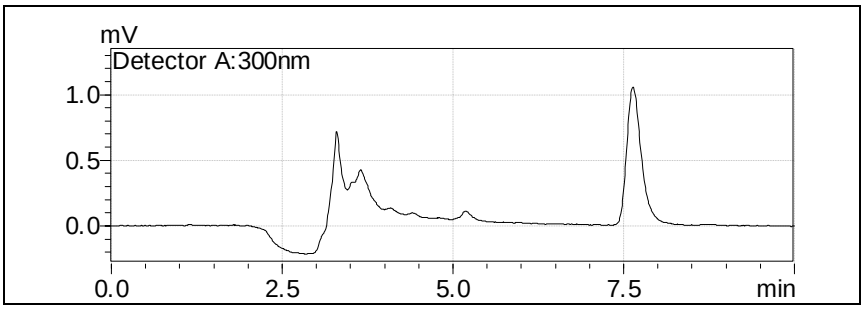

Figure 3.19. Representative HPLC chromatogram of Ral for the 4-Hour release samples of P(L,DL)LA:Ral (10:1) microspheres. Arrow indicates the peak for Ral

Release studies showed that for P(L,DL)LA:Ral (10:1) microspheres a cumulative amount of 6.93 µg which was equal to a mean value of 3.35% (Figures 3.20 and 3.21) was delivered during a 4-week period.

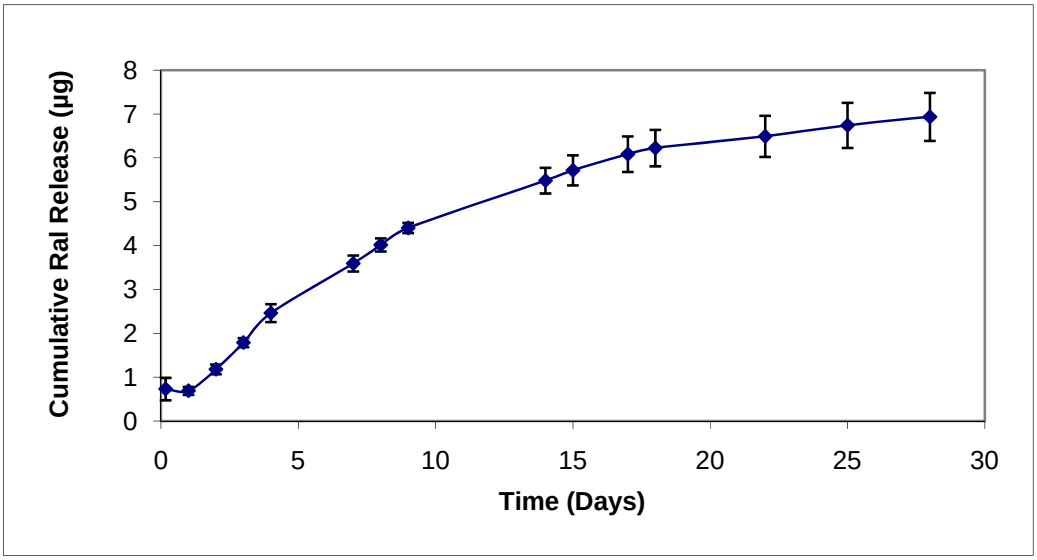

Figure 3.20. Cumulative release profile of Ral from P(L,DL)LA:Ral (10:1) microspheres (n=4)

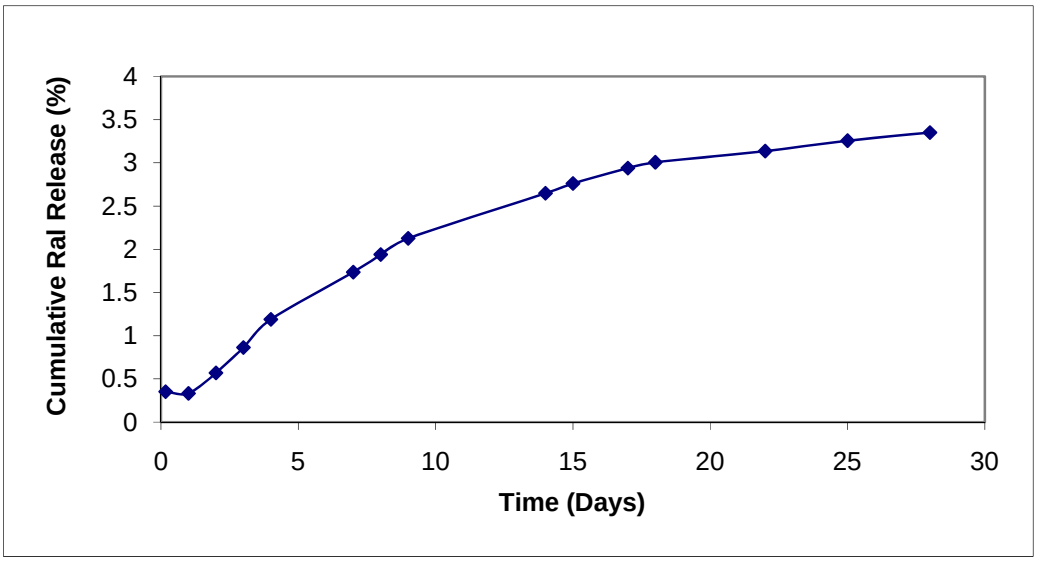

Figure 3.21. Cumulative percent release of Ral from P(L,DL)LA:Ral (10:1) microspheres (n=4)

When the effect of polymer type on release from microspheres was investigated, it was recognized that PCL released less Ral than P(L,DL)LA. This was a favorable property for the aim of this study in terms of prolonging the release of Ral. It is known that Ral is effective on cartilage tissue at low concentrations (1-5 μM) (Kavas (2007)). The difference in release amounts in relation to polymer type might have arisen from the polymer structure.

Besides, it can be said that P(L,DL)LA microspheres released less drug when loaded with Ral rather than TA. This trend was similar and even more enhanced in the release of both drugs from the other polymer (PCL) (Table 3.3). Thus, irrespective of polymer type Ral release was slower than TA owing to a combination of prospective reasons explained above.

Table 3.3. Release values for #7, #8, #4 and #1 microspheres at the end of 4 weeks (n=4)

	Cumulative Release (μg)	Cumulative Release (%)
P(L,DL)LA:Ral(10:1) (#7)	6.93±0.54 (2.95 μM)	3.35±0.26
PCL:Ral (10:1) (#8)	0.67±0.05 (0.34 μM)	0.16±0.01
P(L,DL)LA:TA (10:1) (#4)	30.54±2.63	71.44±6.17
PCL:TA (10:1) (#1)	154.96±11.97	100±7.74

3.1.3 Release of Both TA and Ral from PCL

In order to obtain the optimum release duration and drug delivery amounts among all polymer drug combinations, PCL:TA 10:4 and PCL:Ral 10:2

were chosen as suitable for considering a combinatorial treatment. Thus, they were combined in single group with 10:4:2 ratios of PCL:TA:Ral. The release profile of both drugs from these #10 labeled microspheres were studied in the same release media. For determination of the release profiles of TA and Ral from microspheres calibration curves in Figure 3.1 and Figure 3.14 were prepared as reported in Sections 2.2.4.2 and 2.2.4.3, respectively. For HPLC detection, no interference was seen from the use of two drugs (Figure 3.22). The HPLC peaks of both TA and Ral were sharp and narrow and appeared at their specific times 5th and 7.5th minutes, respectively. However, due to the fact that released Ral was much lower than released TA from #10 labeled microspheres, it was hard to quantify both of them on the same HPLC chromatogram. Hence, dual detection of release amounts could be established by using two different wavelength measurements.

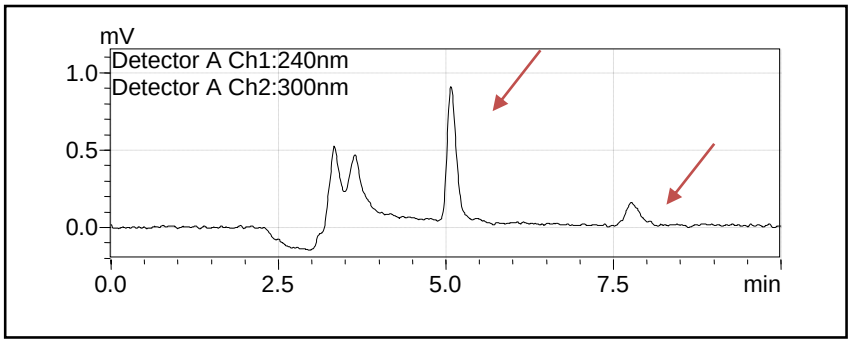

Figure 3.22. A representative HPLC chromatogram of 4 hour release of TA and Ral from PCL:TA:Ral (10:4:2) microspheres (left arrow indicates TA peak and right one indicates Ral peak)

For PCL:TA:Ral (10:4:2) microspheres it was observed that 55.82% of loaded TA was released at the end of 4 weeks, which was equal to mean cumulative amount of 396.78 µg (Figures 3.23 and 3.24). On the other hand, Ral released from PCL:TA:Ral (10:4:2) microspheres was 0.29%, which was equal to mean cumulative amount of 1.14 µg (0.08 µM) (Figures 3.23 and 3.24).

In Table 3.4 dual release values are compared with the same polymer drug combination and ratio of single drug release results.

Table 3.4. Release values for #10, #3, and #9 microspheres at the end of 4 weeks

	Cumulative TA Release (µg)	Cumulative TA Release (%)	Cumulative Ral Release (µg)	Cumulative Ral Release (%)
PCL:TA:Ral(10:4:2) (#10)	396.78±6.66	55.82±0.93	1.14±0.06 (0.50 µM)	0.29±0.01
PCL:TA (10:4) (#3)	447.10±6.55	20.87±0.30	-	-
PCL:Ral (10:2) (#9)	-	-	0.61±0.11 (0.30 µM)	0.09±0.01

According to Table 3.4, we may suggest that quantitatively close amounts of TA have been released from the dual and single drug loaded microspheres. When experimental loading ratios are compared the former (dual) would be expected to release slower amounts as observed with other loading groups (10:1 and 2). Hence, despite this fact this group had high amounts of TA and Ral release indicating the effect of corelease in terms of enhancement of release process for both drugs.

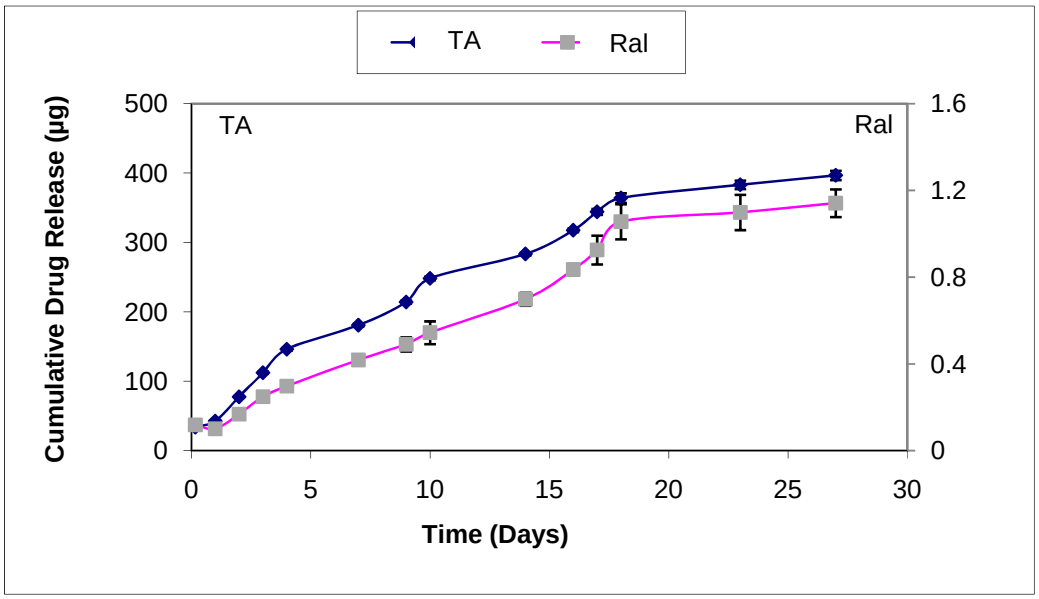

Figure 3.23. Dual release of TA and Ral from PCL:TA:Ral (10:4:2) microspheres within 4 weeks (n=4)

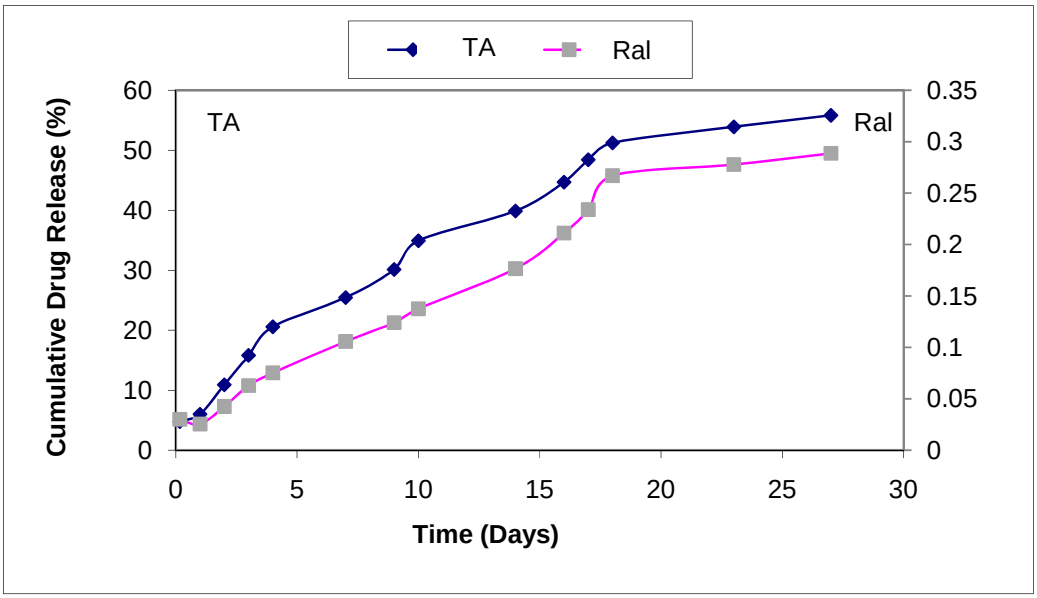

Figure 3.24. Cumulative percent TA and Ral release from PCL:TA:Ral (10:4:2) microspheres within 4 weeks (n=4)

Both TA and Ral does not show an initial rapid release phase in dual release study. Until day 18 it seems that there was a gradual increase in the release rate whereas after day 18, release became linear. This might be explained by slow surface erosion property of the polymer after the drug was released close to the surface.

3.2 Structural Characterization of Microspheres

3.2.1 Particle Size Analysis

Results of particle size analysis showed that there was no linear relationship between increasing drug to polymer ratio and mean diameters. In Table 3.5 mean, minimum and maximum diameters of each microsphere group were documented. The particle size distribution curves of related groups are also given in Figures 3.25, 3.26 and 3.27.

In the TA loaded PCL microspheres; #1, #2 and #3 (Figures 3.25, 3.26 and 3.27, respectively) as drug to polymer ratio increased an increase was observed in diameter values (mean, minimum 10% and maximum 10%). The lowest drug to polymer ratio microspheres (#1) had the smallest size among these microspheres with a mean diameter of 80.89 μm. The particle size distribution of TA loaded PCL microspheres had bell-shaped, single and narrow peaks indicating a homogeneous distribution of diameters.

Wang et al. (2009) prepared PCL microspheres loaded with another low water soluble drug p-nitroaniline with single emulsion s/o/w method. It was

reported that as the drug loading increased from 8.20% to 47.14%, the average size of microspheres also increased (from 58 to 146 μm). These results are in accordance with our loading results (Table 3.6 Section 3.3). This might be explained by the compartmentability of the drug in polymer phase. As drug is also hydrophobic, with increase in its input in the preparation step, more drug could be distributed in the hydrophobic polymer matrix increasing both the loading and size of the microspheres.

Butoescu et al. (2009) studied the influencing factors for intra articular drug delivery systems for rheumatic diseases and reported that microparticles with diameters larger than 20 μm were not internalized by macrophages. In addition, above this diameter, no important inflammatory response was seen. The mean size and minimum size values for all groups of microspheres are in accordance with these results and thus, microspheres would not be expected to be phagocytosized by macrophages.

In the TA loaded P(L;DL)LA microspheres (#4, #5 and #6, Figures 3.28, 3.29 and 3.30, respectively) as drug to polymer ratio increased an increase was observed at first in the diameter values (mean, minimum 10% and maximum 10%). However, with further increasing the drug content a decrease was observed in this parameter of microspheres. This outcome was opposite with the PCL microspheres of the same drug. The highest drug to polymer ratio microspheres (#6) had the smallest size among this group with a mean diameter of 153.66 μm. When compared with the first group, all TA loaded P(L,DL)LA microspheres were larger than all PCL microspheres irrespective of the loading ratio. Besides that, particle size distribution curves represented wider and shouldered peaks which showed nonhomogeneous distribution of the sizes. This might be due to high molecular weight distribution of the original copolymer used in the study that caused such deviations in the microsphere sizes. Another possible explanation might be clumping of the microspheres

during preparation due to insufficiency of the surfactants used or after preparation process due to inability of complete removal of the surfactants from the surface of microspheres.

Vivek et al. (2007) studied biodegradable polymer microspheres and reported that when preparation conditions such as concentration of surfactant or stirring speed were increased PCL always resulted in smaller particle sized microspheres than those of another co-polymer of PLA, poly(lactide-co-glycolide). Mean particle diameter of PCL microspheres varied between 10 and 60 μm according to preparation properties. These results are in accordance with our results which showed larger particle diameters for the co-polymer of PLA (P(L,DL)LA) compared to PCL.

When P(L,DL)LA was used as the carrier of the second drug (Ral), the largest mean diameter (825.54 μm) among all the microsphere groups was reached (#7 microspheres). Particle size distribution curve (Figure 3.31) represented a sharp peak similar to those of TA-PCL microspheres with just differentiating by a small shoulder which was seen in all P(L,DL)LA microspheres distributions. This result might be explained by the complications in microsphere preparation process or might be related to high viscosity of the P(L,DL)LA Ral solution (as mentioned before) which might have increased the stability of polymer phase against stirring forces, thus preventing their further reduction in size.

In the Ral loaded PCL microspheres (#8 and #9-Figures 3.32 and 3.33, respectively) as drug to polymer ratio increased, again an increase was seen in diameter-sizes. Considering that both drugs are hydrophobic and smaller mean diameters were obtained with PCL rather than P(L,DL)LA within the same polymer drug ratios in groups #2, #5 and #9 it might be said that this outcome mainly depended on polymer properties rather than drug. Yet, a small amount of variation (ca 10 % increase) in size was achieved by using Ral instead of TA

in PCL microspheres with the same polymer to drug ratios. This might be explained by the higher molecular weight of Ral (510.05 g/mol) than TA (434.5 g/mol). Furthermore, particle size distribution curves represented sharp homogeneous peaks similar to TA-PCL peaks with exception of small shoulder like extensions at the right.

From particle size analysis it was also observed that mean diameter of dual drug loaded microspheres (#10) was the smallest (68.77 μm) among all the microspheres. Namely, dual drug loaded PCL microspheres' mean size was smaller than single drug loaded PCL microspheres' at the same polymer to drug ratios. This might be partially due to the fact that both loading and encapsulation efficiency of the drugs were decreased in dual drug loading. The particle size distribution figure represents a sharp characteristics but with a small extension towards larger values (Figure 3.34).

Lamprecht et al. (2000) also loaded PCL microspheres (s/o/w prepared) with two model anti-inflammatory drugs (sulfasalazine and betamethasone) singly or together for oral treatment of inflammatory bowel disease. It was reported that the microspheres loaded with both drugs represented a diameter size range of 102 to 277 μm, which was slightly higher than sizes of dual drug loaded microspheres in the current study (15.91-151.37 μm).

Table 3.5. Particle size results of microspheres

Microspheres	Size Under Which 10% is Found (µm)	Mean Diameter (µm)	Size Under Which 90% is Found (µm)
#1 PCL:TA 10:1	29.97	80.89	164.77
#2 PCL:TA 10:2	26.49	86.72	178.34
#3 PCL:TA 10:4	32.44	100.44	198.71
#4 P(L,DL)LA:TA 10:1	49.26	215.83	645.44
#5 P(L,DL)LA:TA 10:2	94.54	489.93	1069.25
#6 P(L,DL)LA:TA 10:4	58.46	153.66	556.87
#7 P(L,DL)LA:RAL 10:1	96.77	825.54	1491.74
#8 PCL:RAL 10:1	32.51	88.17	238.96
#9 PCL:RAL 10:2	28.89	95.31	200.54
#10 PCL:TA:RAL 10:4:2	15.91	68.77	151.37

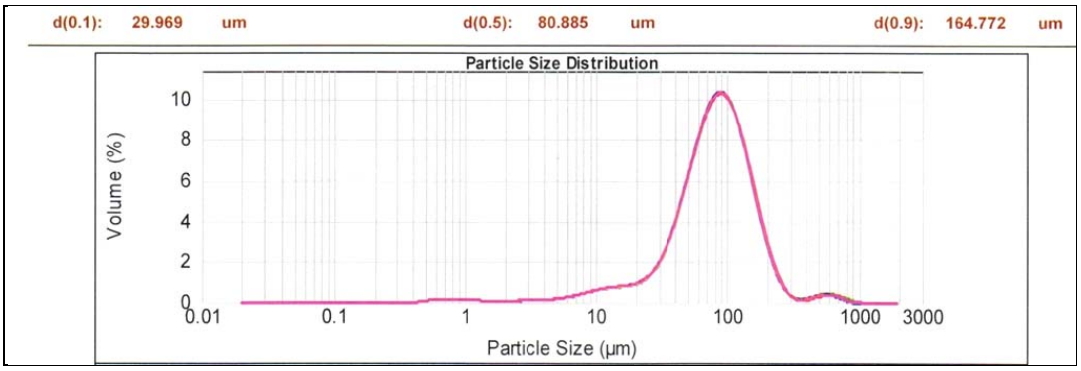

Figure 3.25. Particle size distribution of #1 microspheres

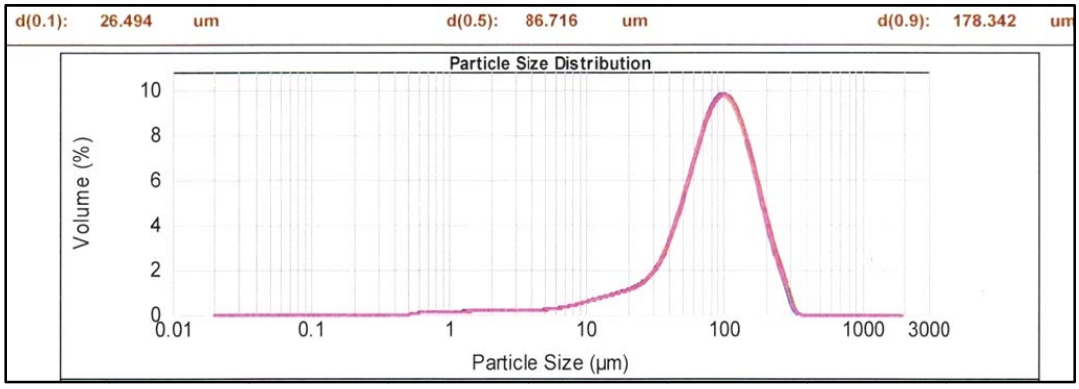

Figure 3.26. Particle size distribution of #2 microspheres

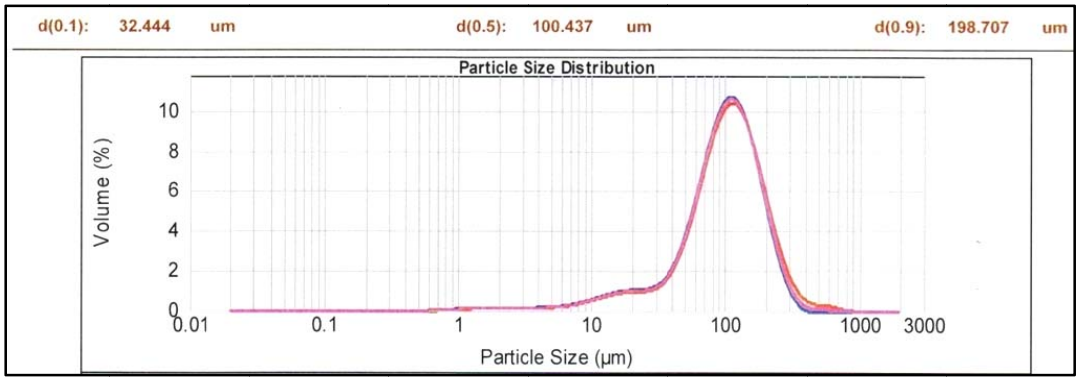

Figure 3.27. Particle size distribution of #3 microspheres

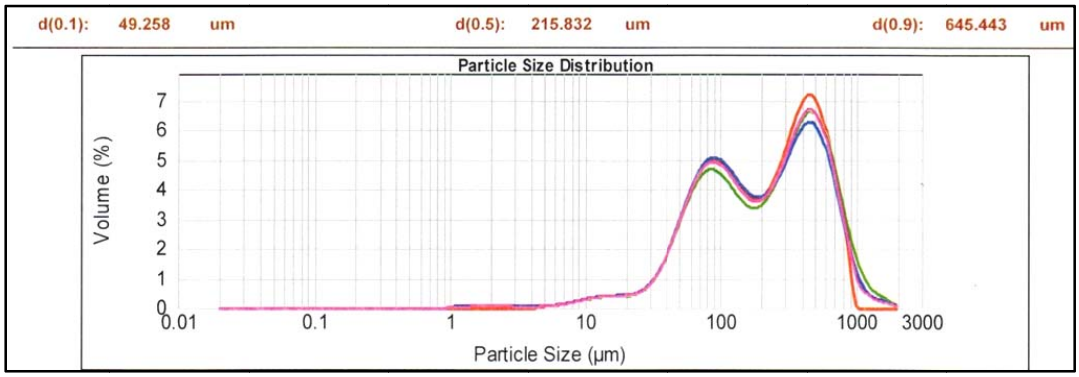

Figure 3.28. Particle size distribution of #4 microspheres

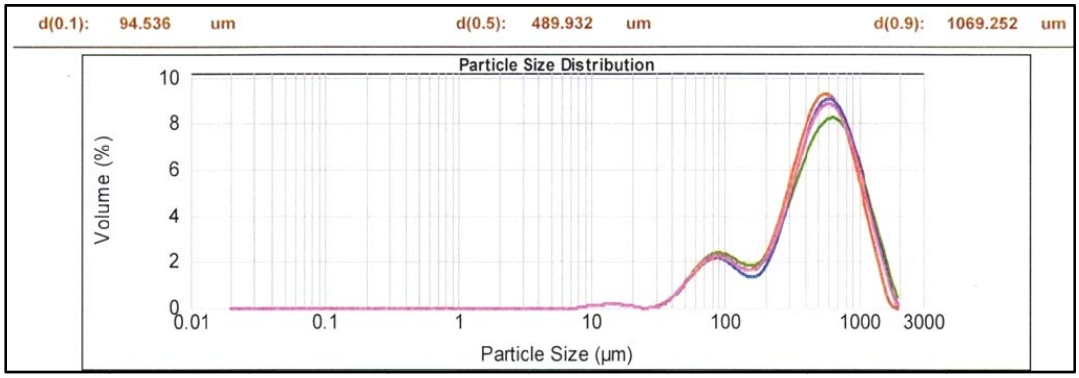

Figure 3.29. Particle size distribution of #5 microspheres

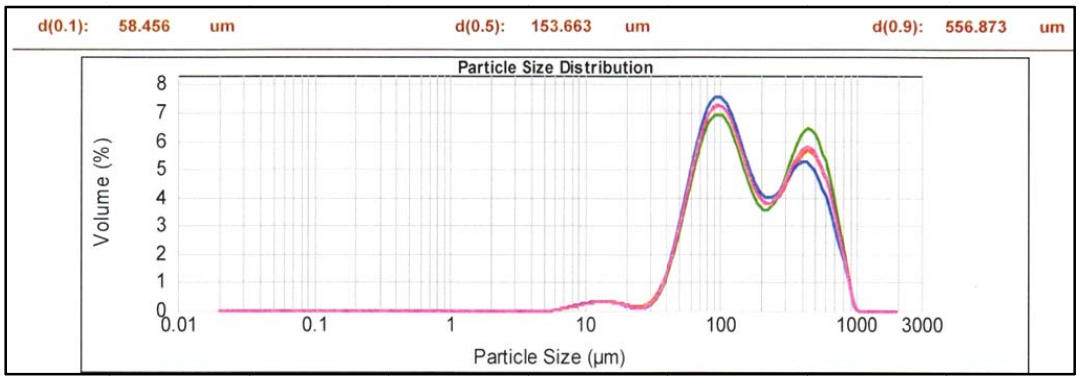

Figure 3.30. Particle size distribution of #6 microspheres

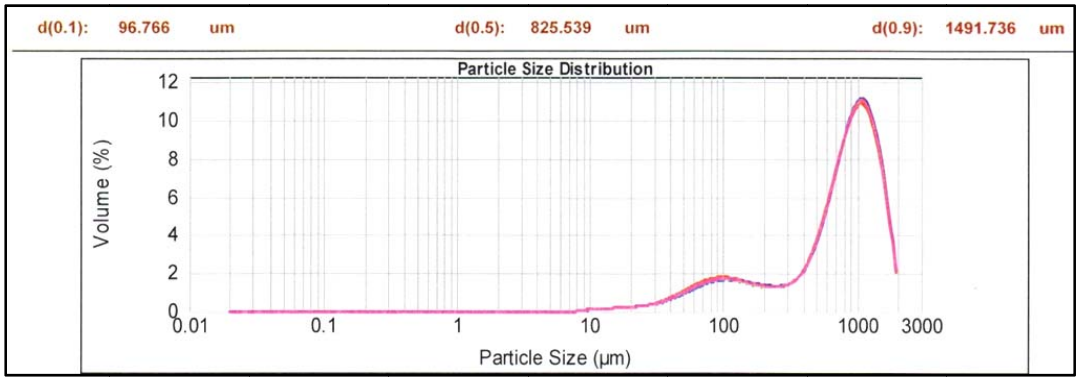

Figure 3.31. Particle size distribution of #7 microspheres

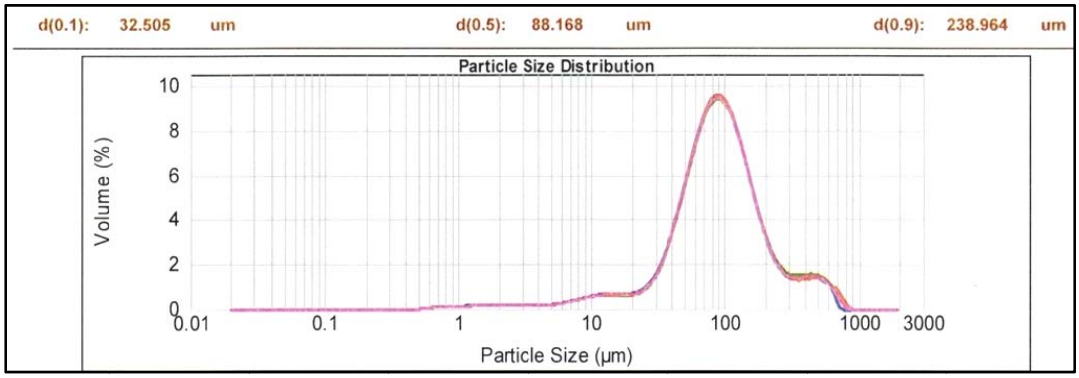

Figure 3.32. Particle size distribution of #8 microspheres

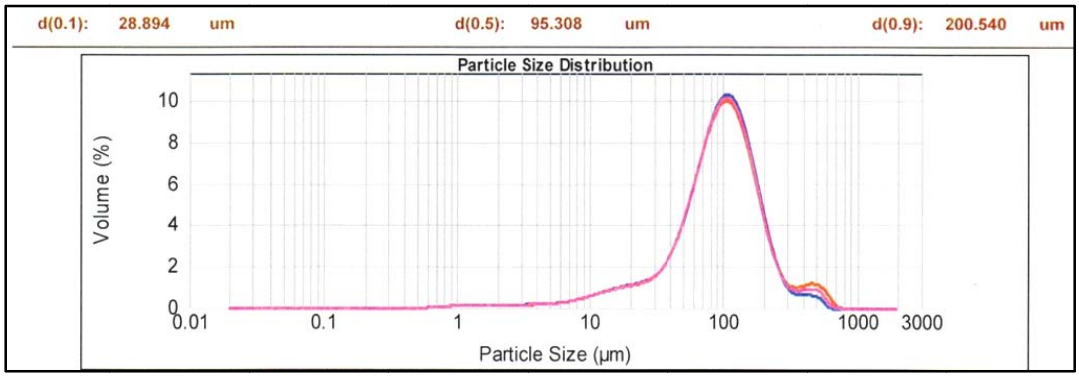

Figure 3.33. Particle size distribution of #9 microspheres

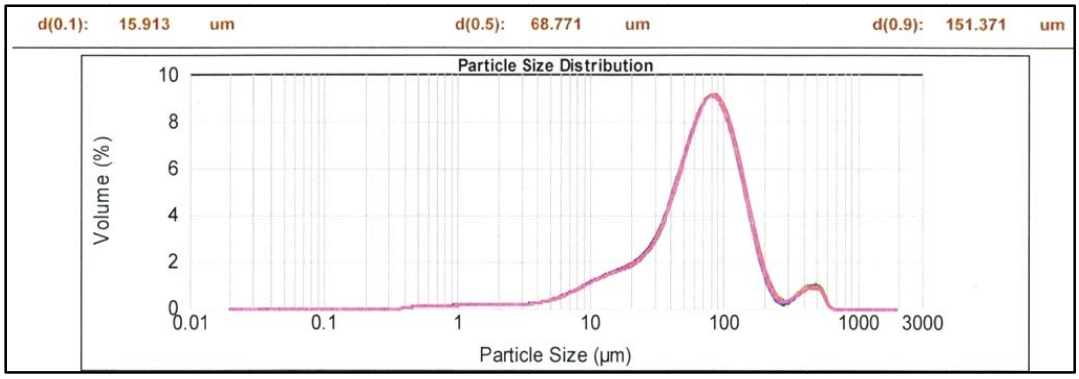

Figure 3.34. Particle size distribution of #10 microspheres

3.2.2 Scanning Electron Microscopy Analysis

Microspheres were analyzed by scanning electron microscopy before and after drug release experiments to observe both the morphology and surface topography of the microspheres. These analyses also helped for evaluating the changes in microsphere structures upon release of the drug. However, during SEM examination, all P(L,DL)LA microspheres got charged and started to melt with JSM-6400 Electron Microscope (JEOL), equipped with NORAN System 6 X-ray Microanalysis System & Semaphore Digitizer (Central Lab., METU). Therefore another device, QUANTA 400F Field Emission Microscope (UNAM, Bilkent University) was used for P(L,DL)LA microspheres whereas PCL microspheres were examined with the former one.

Surface analyses of PCL:TA 10:1, 10:2 and 10:4 microspheres (Figure 3.35 a, b, c, respectively) showed that the surfaces were smoother for lower loading ratio groups (Figure 3.35-a and b) than the highest drug loading one (Fig 3.35.c). All the microspheres had spherical shapes without much pores. Wang et al. (2009) stated that during s/o/w solvent evaporation microsphere preparation technique hydrophobic drug, such as TA in our case, dispersed within hydrophobic PCL matrix. Therefore, there was no accumulation of drug crystals on the surfaces in SEM images and also no initial sharp burst release was seen. However, as drug to polymer ratio increased (from 10:1 to 10:4) loading of the drug increased and this caused an increase in the roughness of the PCL microspheres probably owing to more drug molecules spacing between polymer chains in the microsphere coat. Similar result was seen in Wang et al.'s (2009) study in which PCL microspheres were loaded with another hydrophobic drug, p-nitroaniline. Thus, it can be concluded that excess drug crystals beyond the carrying capacity of the polymer carrier might impair the smooth surface of microspheres.

Surface morphology of same group of microspheres after release (PCL:TA 10:1, 10:2 and 10:4) presented in Figure 3.36 showed that microspheres well preserved their spherical shape but had large cavities and pores owing to loss of drug. In Figure 3.36-b the microspheres were better preserved and had smoother surfaces than those in Figure 3.36-a. This was thought to be related with the drug release rates of these microspheres. In the former group (10:1) higher percentage of drug was released than the later one (10:2). This effect of drug release was pronounced in the third ratio group (10:4), which also initially had rough surface (Figure 3.36-c). For PCL microspheres, it was shown that the release was generally dominated by drug diffusion rather than polymer degradation, therefore it is reported that this polymer is more suitable for long time release (Wang et al. (2009)). The release and SEM analysis together indicated that even if the surface morphologies have changed significantly the microspheres were effective in sustaining the drug release due to this property of PCL.

SEM analyses of P(L,DL)LA:TA 10:1, 10:2 and 10:4 microspheres before release (Figure 3.37 a, b-c-d and e-f, respectively) showed that these microspheres had good contour for the spherical structure and had highly smooth surfaces with no observable pore. Especially when compared with PCL:TA microspheres, these microspheres were more regular in their structure and surface topography. As drug ratio in initial mixture of the system increased (from 10:1 to 10:4), loading of the drug increased and the drug crystals that were observed on the surface of PCL microspheres were again seen as just below the outer surface of the microspheres. One example of this observation was pointed in Figure 3.37-f by arrows. This difference in view of drug crystals by SEM was also in accordance with release experiments where these microspheres had more sustained and less initial burst release of TA than PCL microspheres.

In Figure 3.38, the SEM micrographs of P(L,DL)LA:TA 10:1 (a), 10:2 (b, c, d) and 10:4 (e, f) microspheres after release studies are presented. In the low drug containing group (#4, Figure 3.38-a) microspheres were slightly irregular in shape (not like perfect spheres) and had no cavities, holes or fragments on their surfaces. This might be due to low loading of the drug (0.86%). On the other hand, the other microspheres (#5 and #6, Figure 3.38-b, c, d and e) while preserving their spherical shape showed large ruptures and deformations on their surface due to the loss of big drug crystals from underneath the surface layer. These two groups had similar surface outcomes after release experiments probably owing to their comparable drug loading and release percentages. To our knowledge, no microsphere drug delivery system of P(L,DL)LA was reported before. Instead, it had been used as implant material for orthopedic purposes in regard to its long resorption period and its high mechanical strength properties. The SEM analysis of P(L,DL)LA suggested that this polymer owing to its high molecular weight was successful in obtaining microspheres with perfect spherical structures and smooth non porous surfaces. However, this structure was also responsible for unpredictable loading and release behavior. It was clear that only drug molecules that were close to surface could be released as the microspheres had very small porosity for water uptake and drug diffusion. These results also suggested that the remaining drug to be released will take quite a long time for it will occur only in accordance with polymer loss. Thus, although P(L,DL)LA provided better microsphere structure they were not found preferable for in vivo use in treatment of cartilage tissue.

Surface morphology and structure of P(L,DL)LA:Ral 10:1 microspheres (#7) before and after release are shown in Figure 3.39. These microspheres were also regular in their shape and had smooth surfaces similar to TA loaded microspheres of the same polymer. However, with this drug no drug crystal was observed at or near the microsphere surfaces. This might be due to low drug

loading or due missing such samples during SEM analysis. Yet, similar ruptures (indicating loss of drug crystals) could be observed after release studies (Figure 3.39-c). Too low release results were in accordance with these SEM images for the P(L;DL)LA microspheres. Having the highest particle size (825.54 μm), and being slowest in release results (6.93 μg, 3.35%), in addition to other complications and difficulties in microsphere preparation steps prevented studying further polymer:drug ratio combinations for this polymer drug couple.

PCL:Ral 10:1 (#8) and 10:2 (#9) microspheres were found to be more in spherical contour and have smoother surfaces (Figure 3.40) than TA loading of the same polymer. They, however, had more rough structures compared to P(L,DL)LA microspheres. Between two loading groups, in 10:1 microspheres there were more cavities, pores and channels than in 10:2 microspheres. So, when Ral to PCL ratio was increased, unexpectedly, less rough and less channeled surfaces were observed in contrast to PCL:TA microspheres (#1, #2 and #3). This might be due to dissolved drug molecules between smaller polymer chains of PCL. However, at the same polymer to drug ratio of PCL:Ral, more pores were seen than PCL:TA. This might be explained by characteristics of the drug, for instance the large crystal size of the Ral. Wang et al. (2008) studied the formation mechanism during s/o/w or w/o/w with different stirring rates and release behavior of PCL microspheres loaded with different agents such as disodium norcantharidate, 5-fluorouracil, bovine serum albumin and NaCl. When investigating cause of porosity they reported that porosity was a result of characteristic of the drug salt.

The SEM figure of PCL:Ral 10:1 (#8) microspheres (Figure 3.41-a b) showed that there were deeper and enlarged channels on surface after release. This was in accordance with above suggestion on drug dissolution and more alignment within polymer molecules. In Figure 3.41-c and d (10:2 ratio group), pores rather than deep channels and big cavities were observed after release

(#9). A smoother surface of 10:2 than 10:1 ratio group was thought to be related with too slow release rate (0.09%) in the former group.

The last studied group which had combination of both drugs (PCL:TA:Ral 10:4:2, #10) in PCL carrier showed that microspheres before and after (Figure 3.42 a-b, c-e) release had spherical shape with a diameter range in accordance with particle size analysis. Very small beads of microspheres, around and on top of large ones were observed in SEM micrographs. Similar small microspheres adhered to large ones were also shown by Dhanaraju et al. (2006) with SEM images of steroid loaded PCL microspheres. When PCL:TA:Ral 10:4:2 microspheres were compared with single drug loaded microspheres, PCL:TA 10:4 (#3) and PCL:Ral 10:2 (#9), combined group was observed to have smoother surface than the single drug cases. Lamprecth et al. (2000) studied two anti-inflammatory drugs, sulfasalazine and betamethasone, loaded PCL microspheres, prepared by s/o/w solvent evaporation, for inflammatory bowel disease and found out that internal morphology of these microspheres was a matrix structure with entrapped drug crystals. A similar matrix structure inside the microspheres was thought to be formed in the present study owing to high crystallinity of both drugs and their hydrophobic carriers. After release study, microspheres retained their spherical shape. However, some porosity and a fissured structure was seen probably as the effect of release of two drugs together. More fissured structure with still retaining the spherical form was reported by Dhanaraju et al. (2006) after much longer in vitro release time (8 to 20 weeks). Wang et al. (2008) investigated the reason of porosity in PCL microspheres by loading different drugs and claimed that stirring rate or embedment of small microspheres in large one (similar image can be seen in Figure 3.42-e, inside small microsphere showed by an arrow) might had caused porosity.

Overall microspheres showed that they retained their intact structure and spherical form after release studies. The considerable changes on their surfaces were observed only for P(L;DL)LA groups in the form of large pores-cavities upon release of drug from the surface layers.

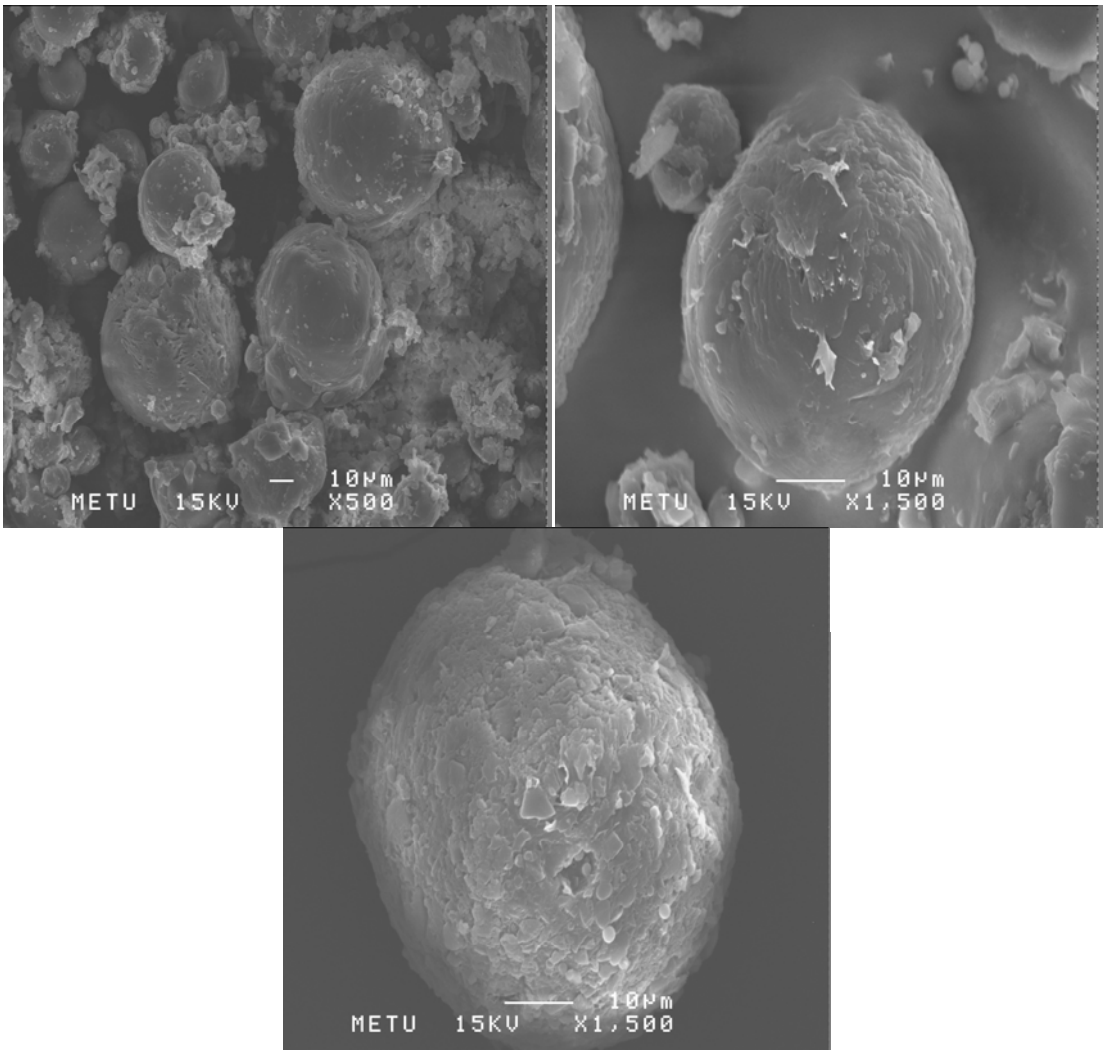

Figure 3.35. SEM images of PCL:TA 10:1 [#1] (a-500X), 10:2 [#2] (b-1500X) and 10:4 [#3] (c-1500X) microspheres before release

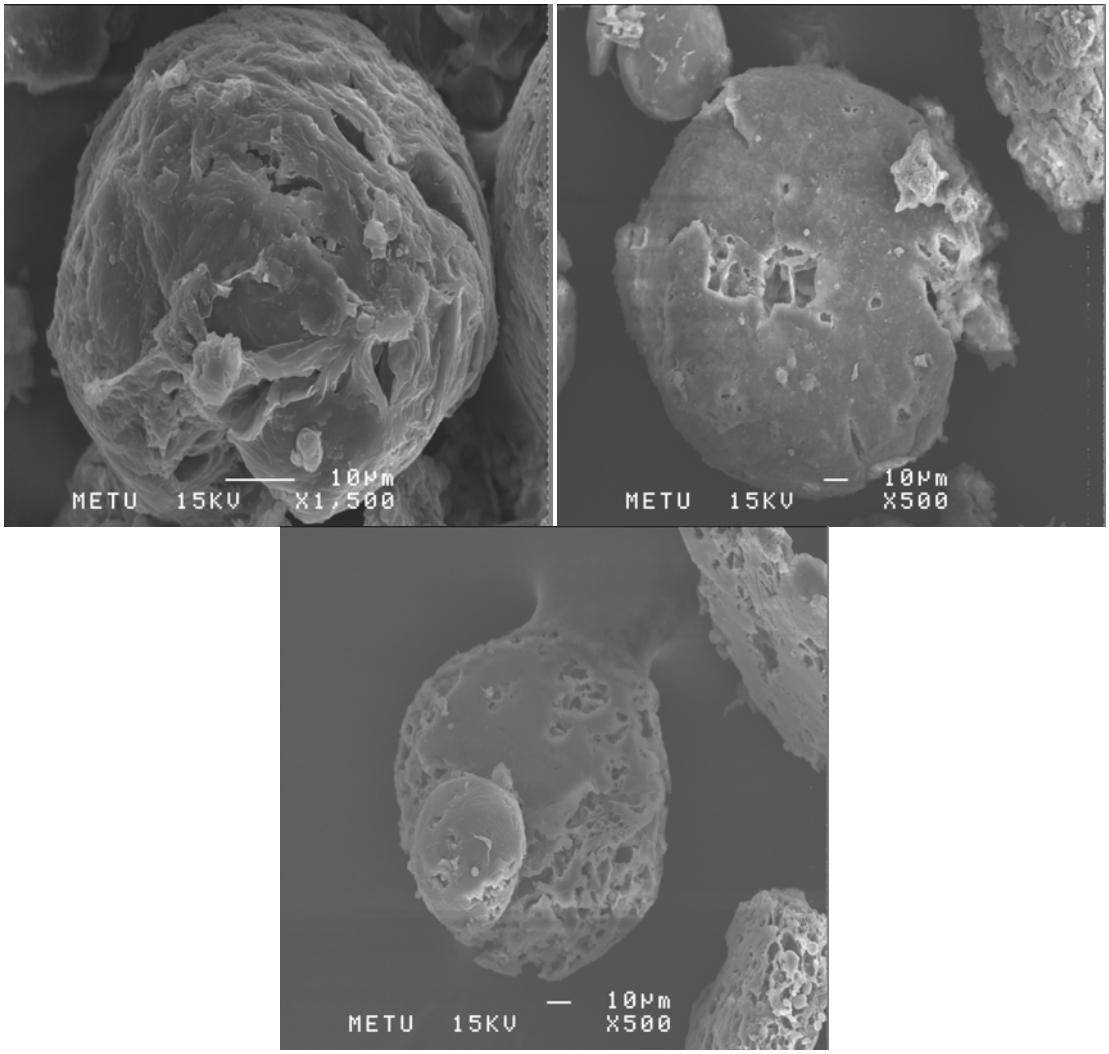

Figure 3.36. SEM images of PCL:TA 10:1 [#1] (a-1500X), 10:2 [#2] (b-500X) and 10:4 [#3] (c-500X) microspheres after release

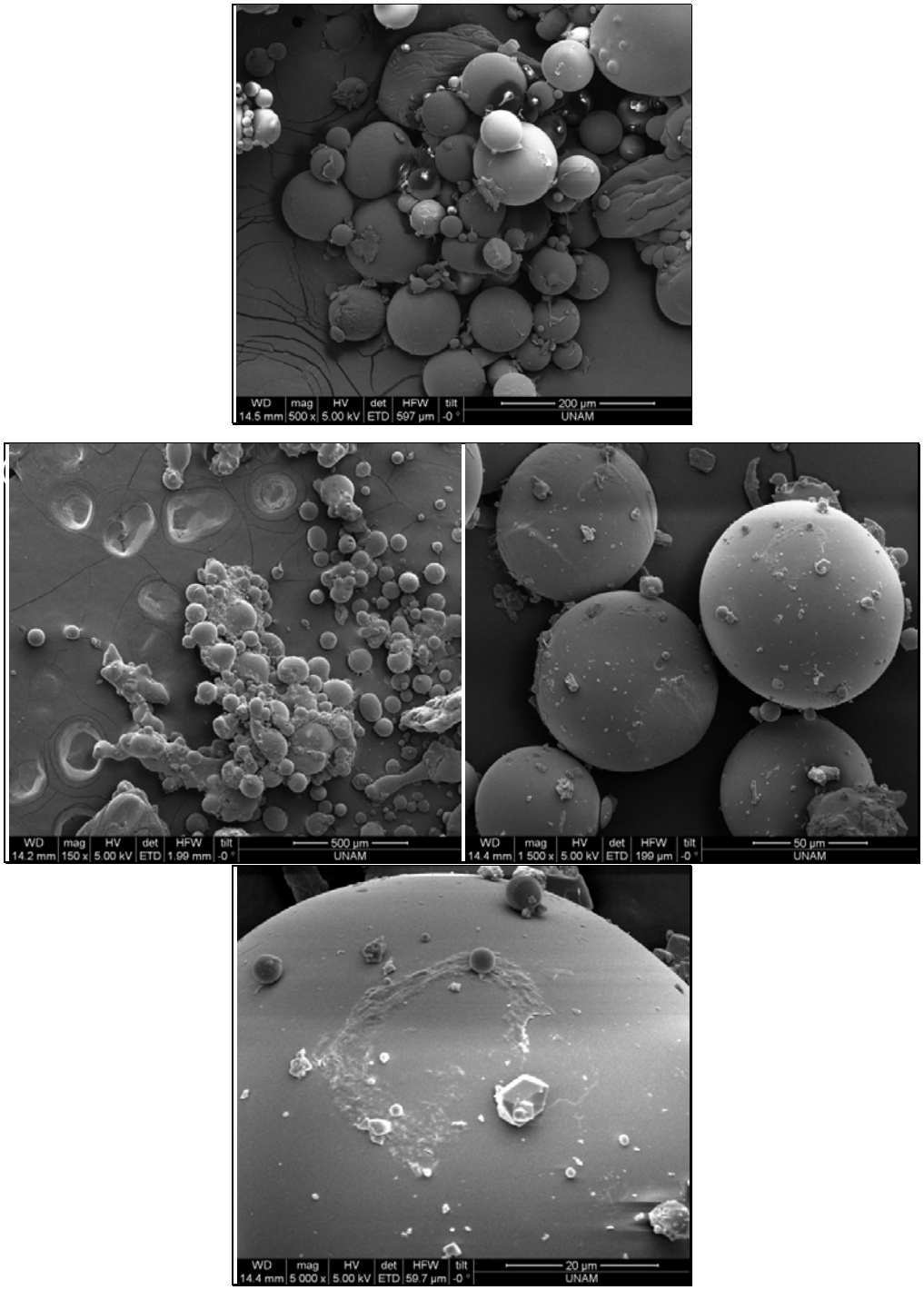

Figure 3.37. SEM images of P(L,DL)LA:TA 10:1 [#4] (a-500X), 10:2 [#5] (b-150X, c-1500X, d-5000X) and 10:4 [#6] (e-1500X, f-5000X) microspheres before release

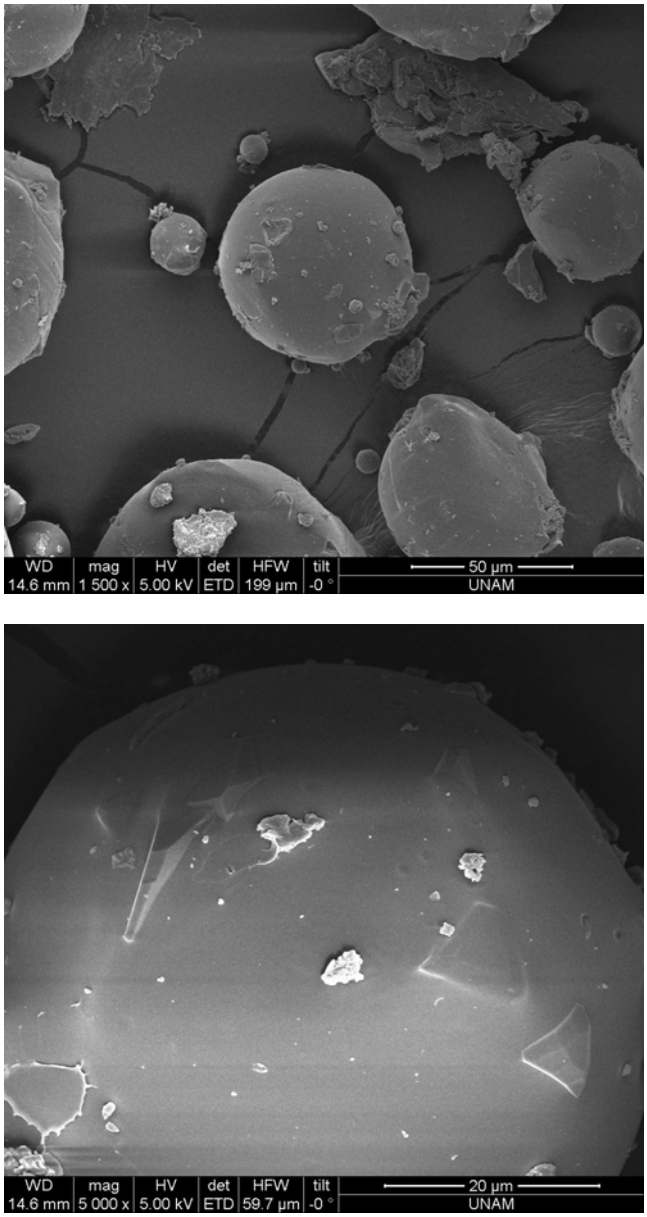

Figure 3.37. (continued) Arrows indicate drug crystals at or just below the surface

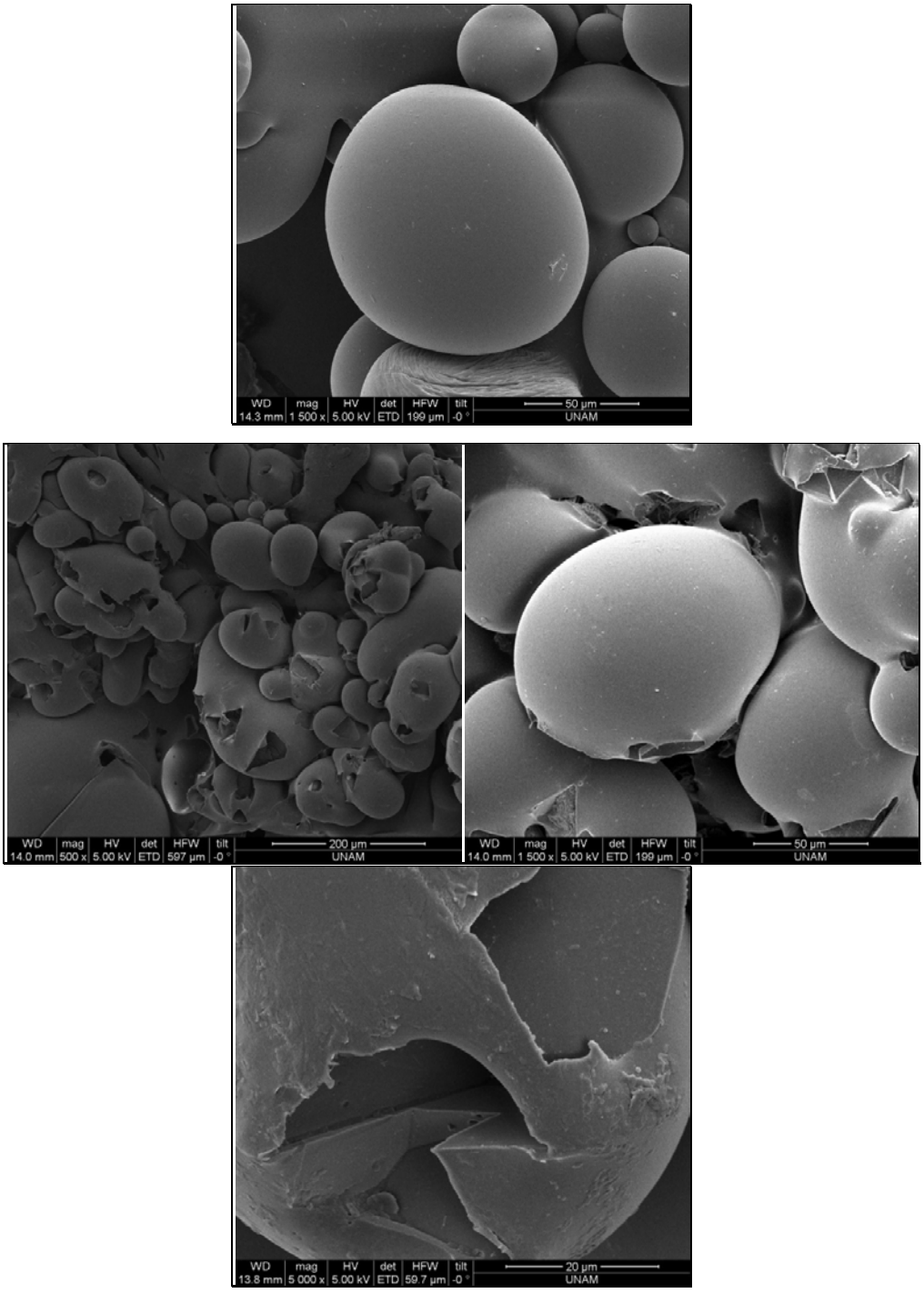

Figure 3.38. SEM images of P(L,DL)LA:TA, 10:1 [#4] (a-1500X), 10:2 [#5] (b-500X, c-1500X, d-5000X) and 10:4 [#6] (e-5000X) microspheres after release

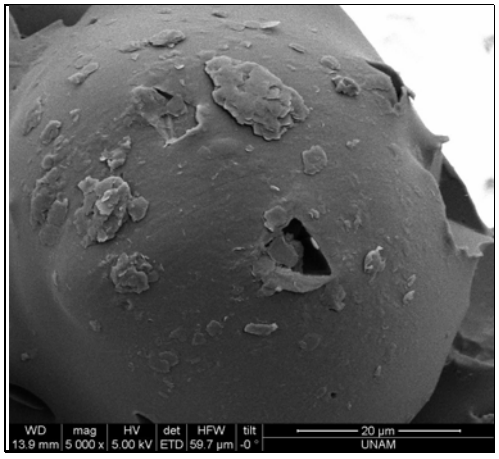

Figure 3.38. (continued)

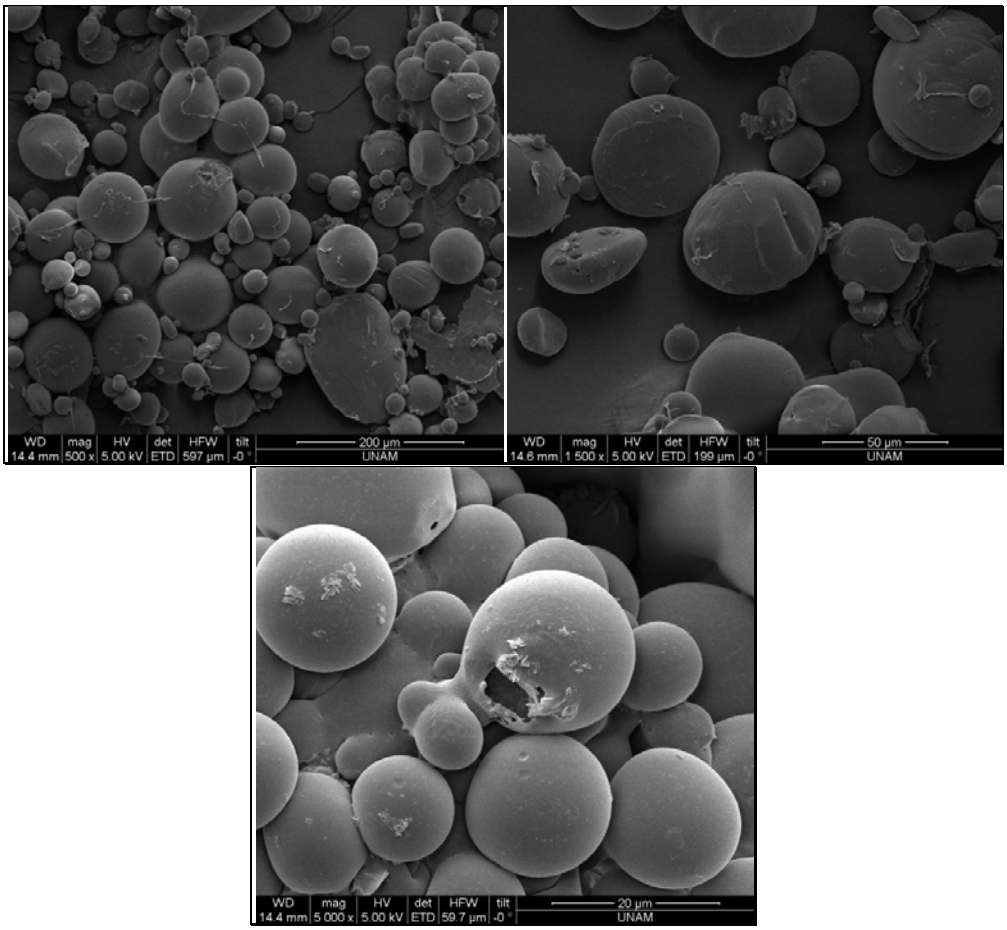

Figure 3.39. SEM images of P(L,DL)LA:Ral 10:1 [#7] (a-500X, b-1500X) microspheres before and (c-5000X) after release

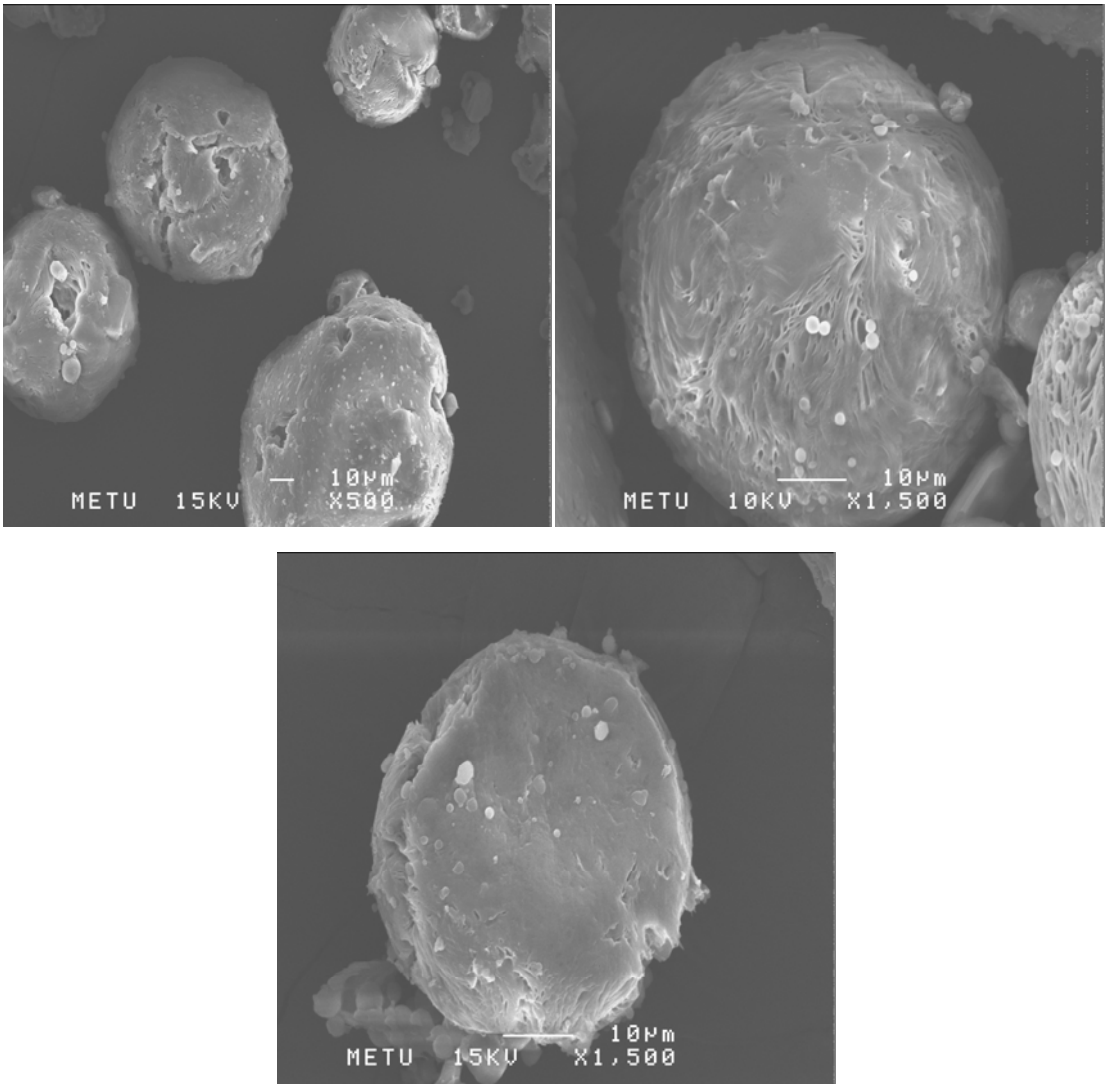

Figure 3.40. SEM images of PCL:Ral 10:1 [#8] (a-500X, b-1500X) and 10:2 [#9] (c-1500X) microspheres before release

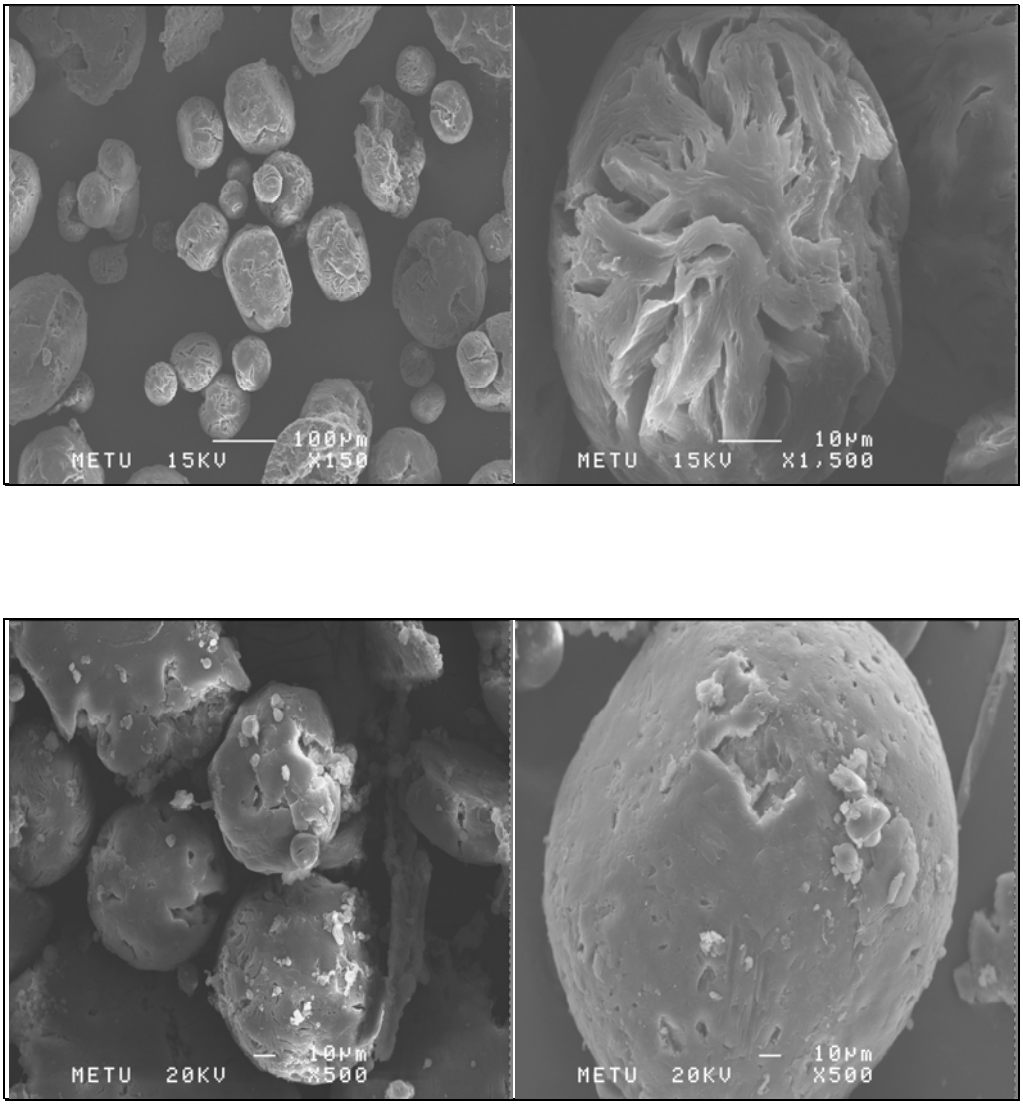

Figure 3.41. SEM images of PCL:Ral 10:1 [#8] (a-150X, b-1500X) and 10:2 [#9] (c-500X, d-500X) microspheres after release

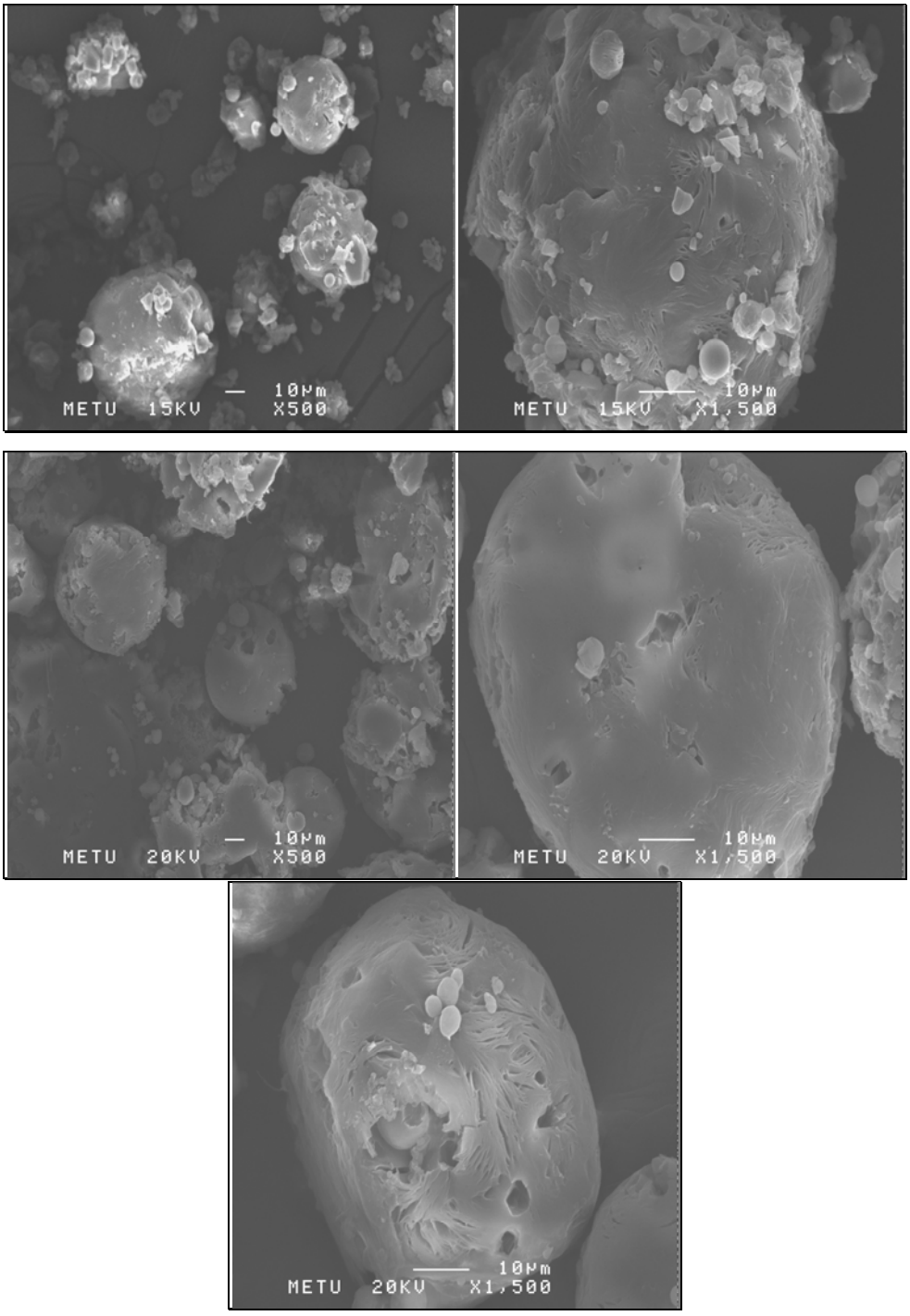

Figure 3.42. SEM images of PCL:TA:Ral 10:4:2 [#10] (a-500X, b-1500X) microspheres before and (c-500X, d-1500X, e-1500X) after release. (Arrow indicates small microsphere imbedded in larger one)

3.3 Results of Encapsulation Efficiency and Loading

Encapsulation efficiency and loading are two important parameters affecting drug release properties of delivery systems. Many factors are known to change these two parameters such as properties of polymer and drug, their initial ratio, or variables of preparation conditions (temperature, stirring rate and evaporation rates etc.). The effect of carrier and drug related factors on EE and loading have been studied as presented in Table 3.6.

In the groups #1 to #3, the changing parameter was polymer to drug ratio. It was observed that as the drug proportion increased, loading and encapsulation efficiency increased accordingly. Among these microspheres, the maximum loading and encapsulation efficiency were obtained in the group with PCL:TA, 10:4 ratio (Table 3.6). This might be explained by the fact that the drug (TA) was initially added into polymer phase and the higher the amount added the more will be kept within the oil phase during preparation of microspheres. Similar trend was observed by Blatsios et al. (2010). The group reported that increase in drug input resulted in increased drug loading and encapsulation for the same drug, TA, in PLA microspheres. These microspheres were than tabletted into sclera implant form in that study. Furthermore, Wang et al. (2009) reported the same behavior with another less water soluble drug, p-nitroaniline using PCL microspheres. PCL is a hydrophobic polymer that requires use of organic solvents. Drugs with low water solubility, thus, tend to be in relation with the dissolved polymer phase rather than aqueous phase. Hence, the more drugs are used, the more they will be entrapped in the matrix.

Beeley at el. (2005) related the loading with release rate and stated that as the drug loading gets lower, TA release rate gets lower from PCL microparticles. Similarly, the release experiments of the present study showed

decrease in cumulative amount of drug released with decrease in loading values.

In the groups, #4 to #6, again the changing parameter was polymer to drug ratio with difference in polymer used, P(L;DL)LA. From #4 to #5 an increase was observed in both encapsulation efficiency and loading in accordance with increase in drug to polymer ratio (Table 3.6). But further increase in drug to polymer ratio led to a slight decrease in EE while loading still increased. Among these microspheres, the maximum loading was obtained when the drug to polymer ratio was the highest 10:4 and the maximum encapsulation efficiency was obtained when the polymer to drug ratio was 10:2. As mentioned before, Blatsios et al. (2010) claimed that increasing TA input increased drug loading and encapsulation with PLA microspheres. One reason for this might be the change in viscosity of the drug/polymer phase beyond a ratio during preparation.

On the other hand, when #4, #5 and #6 microspheres were compared with #1, #2 and #3 microspheres, decrease in both loading and encapsulation efficiency values was observed by changing the polymer type from PCL to P(L,DL)LA. So, for higher encapsulation and loading values of TA, PCL was more efficient.

In the #7 group, only one P(L,DL)LA:Ral (10:1) polymer:drug ratio was applicable due to high viscosity of oil phase during preparation of microspheres. Interestingly, this group had better encapsulation efficiency and loading than for same ratio TA and the P(L,DL)LA polymer. These values were also slightly higher than TA loading of PCL microspheres.

In the groups #8 and #9, a decrease in encapsulation efficiency was observed in contrast to increasing drug to polymer ratio. Conversely, an increase in loading was observed in accordance with increase in drug to polymer ratio. This phenomena suggested the idea that raloxifene with its solid crystals

incompatible with polymer phase might interfere with the formation of micropsheres properly above a certain ratio and thus, decreased the total amount of encapsulated drug. Meanwhile the successfully formed microspheres might have been loaded with the highest possible amount of this drug.

When the loading and encapsulation efficiency results of PCL:Ral (10:1) (#8) were compared with P(L,DL)LA:Ral (10:1) (#7) it was observed that there was a significant decrease for P(L,DL)LA case. Therefore, PCL was thought to be a more suitable polymer instead of P(L,DL)LA in terms of encapsulation and loading of Ral.

In the dual drug loaded PCL microspheres (#10) encapsulation efficiency and loading values of both drugs were observed to be less than the corresponding values for single drug loading of PCL at the same initial ratio (#3 and #9). This could be due to less polymer per drug in #10 microspheres than in #3 and # 9 ones to encapsulate drug(s). In a study, Liu et al. (1999) loaded ionic polysaccharide microspheres with chemosensitizers and anticancer drugs. It was reported that for both a single agent and dual agents high drug loading was achieved (28%). However, the equilibrium drug loading was dependent on the ratio of the microspheres to the drug. In addition, the equilibrium drug loading was dependent on the relative affinity of the agents to the microspheres in the case of dual agents. In our case the affinity of the drugs to the polymer was good as known from initial encapsulation efficiency values of drugs alone.

Table 3.6. Encapsulation efficiency and loading values of microspheres (n≥2)

	EE (%)	Loading (%)
#1 PCL:TA (10:1)	23.34±0.01	3.09±0.01
#2 PCL:TA (10:2)	51.33±0.01	17.95±0.01
#3 PCL:TA (10:4)	70.56±0.01	27.17±0.01
#4 P(L,DL)LA:TA (10:1)	6.33±0.35	0.86±0.01
#5 P(L,DL)LA:TA (10:2)	49.98±0.01	10.20±0.01
#6 P(L,DL)LA:TA (10:4)	41.40±0.03	14.73±0.01
#7 P(L,DL)LA:Ral (10:1)	29.23±0.01	4.14±0.01
#8 PCL:Ral (10:1)	63.57±0.02	8.69±0.01
#9 PCL:Ral (10:2)	56.52±0.17	13.01±0.01
#10 PCL:TA:Ral (10:4:2)	42.04±0.07 TA 47.72±0.02 Ral	13.94±0.01 TA 7.91±0.01 Ral

3.4. Polymer Degradation Studies

In order to evaluate polymer degradation during the release period, decrease in polymer molecular weight was examined by gel permeation chromatography (GPC).

In Table 3.7, GPC results for P(L,DL)LA are compared for weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity (Mw/Mn) values before and after incubation in PBS at 37°C for 45

days. As usual for polymer molecular weights, Mw values were found to be higher than Mn values. At the end of incubations, P(L,DL)LA showed significant decrease in both Mw and Mn values. In a previous study, screws and plates have been prepared from same copolymer (weight-average molecular weight (Mw) of 165,000 g/mol). The plates showed decrease in Mw approximately 31% at 13 weeks and approximately 43% at 26 weeks. The screws demonstrated similar decreases in Mw (32% and 50%, respectively).

The decrease in molecular weight, in the current study, however, is thought to not cause any significant effect on release properties as the values of Mw were still quite high. This was also supported by the SEM analysis in which morphology of the microspheres was demonstrated to be preserved. Moreover, when the chromatograms were examined (data not shown), there was only a slight shift caused by the small decrease in molecular weight values and the shape of the polymer peaks before and after incubations were the same. There was also no shoulder or appearance of new peak in the chromatograms. Besides, polydispersity of P(L,DL)LA was reduced to 2.40 (from 2.46) after incubation in PBS. Saez et al. (2008) reported that GPC results represented high molecular weight values (19700 Da) after degradation (28 days) of poly (D,L-lactide-co-glycolide) microspheres, thereby allowing drug to be released from the pores throughout the microspheres. In the present study, even higher molecular weight for P(L,DL)LA (Table 3.7) was achieved after incubation and this was in accordance with sustained slow release outcomes for both TA and Ral. Toth et al. (2002) used the same polymer P(L,DL)LA as resorbable interbody fusion cage for in vivo experiments and reported that significant degradation was seen after 24 months. In addition, they reported that P(L,DL)LA, a racemic form of PLA, was amorphous and degradation occured from surface by dissolution. In another study in which P(L,DL)LA (L:DL – 95:5) was used as medical screw and plate it was reported that, molecular weight of

43700 Da decreased approximately 75% from its initial weight after 12 weeks in vitro aging and decreased 80% from the initial value after 24 weeks (Mainil-Varlet et al. (1997)). In addition, according to Moser et al. (2003), as the portion of DL-lactide relative to L-lactide in co-polymer increased the rate of degradation increased too. Moser et al. (2003) also used P(L,DL)LA (70:30) as implantable screw and platelet and compared their results with Mainil-Varlet et al. Mainil-Varlet et al. (1997) also investigated the pH effect of buffer solution used in degradation studies by using two groups. In the first group the PBS used for degradation study was changed when the pH drop was seen. In the second group, PBS was not refreshed. Finally, it was concluded that the pH of the buffer solution had no evident effect on the mechanical properties or the rate of degradation as estimated from the drop in molecular weight of the aged samples. These results were supportive for our study where PBS was not refreshed during 45 days of incubation and its pH dropped to 6.95 from the starting pH of 7.4. After working with P(L,DL)LA (70:30) as implantable screw and pin, Landes et al. (2006) concluded that according to extrapolation from in vitro hydrolysis results complete degradation would take 2 to 3 years.

Table 3.7. GPC results for polymers before and after incubations

	Mn (Da)	Mw (Da)	Polydispersity Index
P(L,DL)LA before incubation	70476	173381	2.46
P(L,DL)LA after incubation	17357	41718	2.40
PCL before incubation	8451	19239	2.28
PCL after incubation	7059	17044	2.41

In Table 3.7, GPC results for PCL were also presented. Before incubation result represents the original molecular weight of the polymer. After incubation result represents incubation of polymer in PBS at 37°C for 45 days. Similar to P(L,DL)LA, a decrease was observed in M_w and M_n, upon incubation. However, it was not such significant. In addition, the shapes of the microspheres were shown to be preserved by SEM analysis. Besides, polydispersity index increased to 2.41 (from 2.28) after incubation of PCL in contrast with P(L,DL)LA. As it was reported by Barbato et al. (2001), in an aqueous medium, PCL microspheres degrade at longer times due to its semi crystallinity and hydrophobicity. When a polymer is hydrophilic and has more amorphous structure, water easily penetrates within this structure and degradation by hydrolysis starts. Vivek et al. (2007) studied degradation and release of microspheres with different ratios of polylactide-co-glycolide and PCL. It was reported that the slowest drug release as well as the slowest degradation (as surface erosion) were seen after 28 days for PCL microspheres. Slow degradation by random hydrolytic chain scission of ester linkages and by hydrophobic structure of PCL was also reported by Ha et al. (1997). Besides polymer properties, shape, size, porosity, and surface to volume ratio and site of implantation might also contribute to the rate of degradation. When the two polymers are compared for degradation results, P(L,DL)LA can be suggested to have higher decrease in molecular weight thus showing more susceptibility to degradation than PCL. This might be related with P(L,DL)LA having larger chain length which when degraded would have higher impact on change in mean molecular weight than the other polymer. For instance, 178/2=69 kDa, change might be 69 kDa for P(L,DL)LA whereas, 18/2=9, change might be just 9 kDa for PCL.

3.5 Results of In Vitro Cytotoxicity Tests

In vitro cytotoxicity was conducted by incubating cells in DMEM in which selected groups of microspheres had been incubated for 24 h. The group selections for in vitro studies were based on their in situ results (i.e. loading, drug release properties etc.) with considering their suitability for future in vivo applications. The MTT results of these groups (PCL:TA (10:4) (#3), PCL:Ral (10:2) (#9) and PCL:TA:Ral (10:4:2) (#10) microspheres) and controls were presented in Figure 3.43.

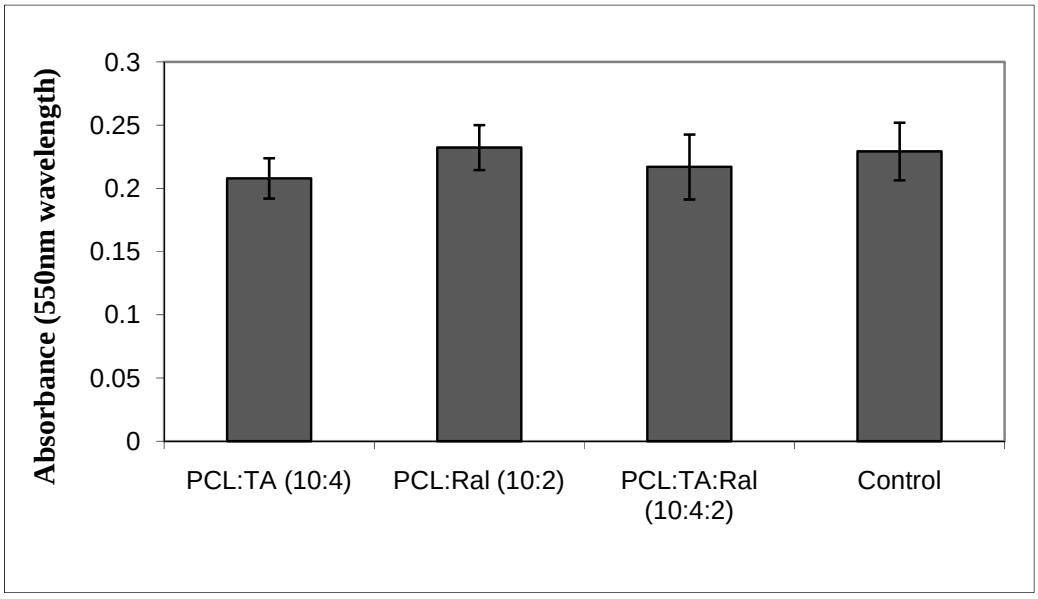

Figure 3.43. Comparison of cell viabilities upon 48 h incubation of cells in DMEM in which the selected groups of microspheres were incubated for 24 h. (control groups are cells incubated in cell culture media)

No significant difference in the cell viability of microsphere groups and control was observed (Figure 3.43). MTT assay results showed that no toxic material was released from microspheres for the 24 h release period. Although the biocompatibility of PCL is known and it had been approved by U.S Food and Drug Administration (FDA) for in vivo human clinical use (Taddei et al., 2005) the processing methods and materials could have caused harmful effects on the cells. So, the in vitro cytotoxicity tests provided information on the absence of these potential unwanted outcomes of microspheres in short term. Besides these, TA and Ral which are two commercial drugs could have had some negative effects owing to high local extracellular concentrations with their release from the microspheres in cell culture media upon 48 h incubation. However, this negative effect on the viability of the cells was also not observed.

CHAPTER 4

CONCLUSION

Rheumatoid arthritis is a systemic, chronic, inflammatory autoimmune disease which affects mainly joints as swelling, inflammation and loss of movement function. RA usually requires long term drug treatments to gain the healthy state of the affected joints. In most cases these drugs are immunosupressive agents which can cause unwanted systemic side effects. Yet, there is no local drug delivery system for this disease. The two drugs selected for development of local RA treatment system in this study involve: i) an anti-inflammatory immunosuppressive glucocorticoid, triamcinolone acetonide, which is effectively used in treatment of most rheumatoid arthritis cases and ii) a well known selective estrogen receptor modulator, raloxifene hydrochloride, which is used in osteoporosis treatment but has recently been recognized for its regenerative property in cartilage tissue. Therefore, in this study injectable controlled release system of PCL and P(L,DL)LA microspheres loaded with TA and/or Ral were investigated, in order to evaluate their usability in rheumatoid arthritis local treatment. The success of such a system has prospective advantage of avoiding from systemic side effects of traditional administration and discarding the problems caused by direct local injections of the drugs.

In PCL:TA microspheres, as polymer:drug ratio changed in the way that drug partition increased (from 10:1 towards 10:4), higher encapsulation efficiency and loading percentages were obtained. In addition to this, release of drug was prolonged to a level of therapeutic usability of the systems for long

term treatments. Biphasic release profiles seen in all microspheres were in accordance with release profiles of similar studies with polymers and indicated a suitable treatment approach as early period faster release of drugs (about 10 days) and slower at the later stages. SEM chromatograms showed sphericity of the microspheres before and after release studies. Mean particle sizes of the PCL microspheres were found to be in suitable range (20-100 μm mean particle size) for intra articular injections.

Even though P(L,DL)LA microspheres represented better spherical shape and more smooth surface than PCL microspheres in SEM micrographs, these microspheres showed complications in preparation due to high viscosity of polymer phase. Especially in the case of Ral loaded P(L,DL)LA microspheres, only one polymer to drug ratio could be applied (10:1) owing to very high viscosity of the polymer-drug (oil) phase in other ratio groups. In addition, drug loading percentages were lower in both triamcinolone acetonide and raloxifene hydrochloride loaded P(L,DL)LA microspheres compared to PCL microspheres, which also affected release results of these microspheres. Besides these, mean particle sizes of both TA and Ral loaded P(L,DL)LA microspheres were significantly larger than those of PCL microspheres indicating difficulty of their intraarticular application. The highest particle size, too low release results, complications and difficulties during microsphere preparation steps prevented studying further polymer:drug ratio combinations for this polymer drug couple. To our knowledge, no microsphere study was reported with P(L,DL)LA in literature. With the results in the current study we may suggest that upon further modifications this polymer might give better results for long term delivery of a hydrophilic drug and together with lower concentrations of the polymer. The slow drug release with P(L,DL)LA and Ral was thought to be related with combination of strong hydrophobicity of both

drug and polymer as well as high crystallinity of the drug and high molecular weight of the polymer.

When P(L,DL)LA microspheres were compared with PCL microspheres at polymer: drug ratios of 10:1, 10:2 and 10:4, lower loading and encapsulation efficiency values were observed in the former polymer. Considering all these results, PCL was found more suitable for dual drug release and for dual release PCL:TA:Ral 10:4:2 microspheres were prepared considering single drug results of this polymer. Both single drug loaded PCL:TA 10:4 and PCL:Ral 10:2 microspheres, and dual drug loaded PCL:TA:Ral 10:4:2 microspheres did not show cytotoxicity in MTT studies. In PCL:TA:Ral 10:4:2 microspheres, even though encapsulation and loading values were slightly decreased compared to PCL:TA 10:4 and PCL:Ral 10:2 microspheres, release results were still sufficiently sustained and suitable for in vivo applications. When examined separately, TA release decreased slightly while Ral release increased slightly with co-release. Besides, the lowest mean particle size was obtained with dual drug loaded PCL:TA:Ral 10:4:2 microspheres, which is advantageous for applications in intraarticular injections.

In conclusion, the TA and Ral delivery systems with PCL and P(L,DL)LA were developed and they were optimized for successive RA treatment purposes for the first time in literature. The bioeffectiveness of these systems should be further tested with in vivo experimental models of RA.

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