IN SITU, IN VITRO AND IN VIVO EVALUATION OF EFFECTIVENESS OF NEW TREATMENT APPROACHES INVOLVING CONTROLLED DRUG DELIVERY SYSTEMS IN CARTILAGE DEGENERATIONS

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ABSTRACT

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Osteoarthritis (OA) is a degenerative joint disease which has yet no complete treatment with medication. Doxycycline, a well-known antibiotic, has been shown to prevent matrixmetallopreoteinases-MMPs, indicating potency on OA treatment. However, long term systemic use can cause side effects on other tissues. This study aimed to develop controlled drug delivery systems of doxycycline/doxycycline-chondroitin sulfate (D/D-CS) in the form of PCL microspheres for providing a better and new treatment approach via local application. After optimization studies for size, loading efficiency, surface/structure and release properties, microspheres of low Mw PCL (14 kDa) was decided to be more suitable than those of high Mw (65 kDa). The release profile of former was also more compatible with diffusion model than that of latter. The bio-effectiveness of the microspheres was evaluated with three-dimensional *in vitro* model; osteoarthritic-rabbit chondrocytes embedded in agarose and subjected to interleukin-1β throughout incubations. In vitro treatments with D/D-CS microspheres showed significant reduction in MMP-13 activity compared with untreated OA controls for 15 and 24-day incubations. Although collagen and GAG analysis results showed no enhancement of synthesis with MS treatments, significant decrease in GAG and collagen release from D/D-CS MS treated groups and from D MS treated ones

respectively. Overall evaluations of the efficacy using *in vivo* rabbit OA model showed better radiographic scores and histological outcomes for D/D-CS MS groups compared to only hyaluronan injected and/or untreated controls in 8 weeks. The ex-vivo biomechanical properties of cartilages demonstrated improved hardness with values comparable to healthy group upon application of D-CS MS.

Keywords: PCL, doxycycline, chondroitin sulfate, drug delivery, interleukin 1-β, osteoarthritis

ÖZ

KONTROLLÜ İLAÇ SALIM SİSTEMLERİ İLE KIKIRDAK HASARLARININ TEDAVİSİNE YÖNELİK YENİ YAKLAŞIMLARIN ETKİNLİĞİNİN İN SİTU, İN VİTRO VE İN VİVO DEĞERLENDİRİLMESİ

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Osteoartrit, henüz ilaçlar ile tam tedavisi olmayan bir dejeneratif eklem hastalığıdır. Çok iyi bilinen bir antibiyotik olan doksisiklinin (D) matriks metalloproteinazları-MMP'leri engellediği gösterilmiş ve bu ilacın OA tedavi potansiyalinin olduğunu belirtmiştir. Ancak, doksisiklinin uzun süreli sistemik kullanımı diğer dokularda yan etkiye sebep olabilmektedir. Bu çalışmada doksisiklin/doksisiklin-kondroitin sulfat (D/D-CS) içeren kontrollü ilaç salım sistemlerinin PCL mikroküreler formunda geliştirilerek lokal uygulanmasıyla daha iyi ve yeni bir tedavi yaklaşımı sağlanması hedeflenmiştir. Boyut, yükleme etkinliği, yüzey/yapı ve salım özellikleri için yapılan optimizasyon çalışmaları sonrasında, düşük molekül ağırlıklı PCL (14 kDa) ile hazırlanan mikrokürelerin yüksek molekül ağırlıklı PCL'e (65 kDa) göre daha uygun olduğuna karar verilmiştir. Bu mikrokürelerin salım profilinin difüzyon modeli ile daha uyumlu olduğu da görülmüştür. Mikrokürelerin biyolojik etkinliği, üç boyutlu in vitro modelde değerlendirilmiştir. Bu modelde osteoartritik-tavşan kondrositleri agarose gömülmüş ve inkübasyonlar boyunca interlökin 1-β'ya maruz bırakılmıştır. 15 ve 24 günlük in vitro uygulamalar, D/D-CS mikrokürelerle tedavilerin MMP-13 aktivitesini tedavi edilmemiş kontrollere göre anlamlı derecede azalttığını göstermiştir. Collagen ve GAG analiz sonuçları MS tedavilerinin bu moleküllerin sentezlerini arttırmadığını göstermiş olmasına rağmen, D/D-CS

mikroküre lerinin ve sadece D mikroküre lerinin sırasıyla salınan GAG ve kollajen miktarlarını azaltığı gözlenmiştir. In vivo tavşan OA modeliyle yapılan, 8 haftalık araştırmaların tüm değerlendirmeleri sonucunda D/D-CS mikroküre gruplarının radyografik skorları ve histolojik bulgularının hiyaluronan enjekte edilmiş ve/veya tedavi edilmemiş kontrollere göre daha iyi olduğunu göstermiştir. Çıkarılan eklemlerdeki kıkırdağın biyomekanik özelliklerinin D-CS mikroküre lerle tedavi sonrasında sağlıklı gruplara benzer sertlik değerlerine ulaştığı belirtmiştir.

Anahtar sözcükler: PCL, doksisiklin, kondroitin sülfat, ilaç salımı, interlökin-1 β, osteoartrit

To My Parents

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3-D…….	Three-dimensional
ASTM…….	*American Society for Testing and Materials*
CS……..	Chondroitin sulfate
D……	Doxycycline
D MS……	Doxycycline loaded microsphere
D-CS MS……..	Doxycycline-chondroitin sulfate co-loaded microsphere
DMEM…….	Dulbecco's Modified Eagle's Medium
DMMB…….	1,9 dimethyl methylene blue
DMSA…….	Dimercaptosuccinic acid
DMSO……..	Dimethyl sulfoxide
DNA…….	Deoxyribonucleic acid
DSC……	Differential Scanning Calorimeter
DSNC…….	Disodium norcantharide
ECM……	Extracellular matrix
ELISA…….	Enzyme-linked immunosorbent assay
FCS……..	Foetal calf serum
FTIR……	Fourier Transform Infrared
GAG……	GlycosaminoglycN
GPC……	Gel Permeation Chromatography
HE……	hematoxylin and eosin
HA……	Hyaluronan
HYL……..	Hyaluronan (Adant)
HYP……	Hydroxyproline
IL-1β……...	Interleukin-1 beta
JIS…….	Japanase International Standards
MMP……..	Matrix metalloproteinase
MT……..	Masson's trichrome
MTT……	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mw……..	Molecular weight
MS…….	Microsphere
NSAIDs……..	No-steroidal anti-inflammatory drugs
OA……..	Osteoarthritis
OARSI……	Osteoarthritis Research Society International
PBS……..	Phosphate buffered saline
PCL……	Polycaprolactone
PLA…….	Poly(lactic acid)
PLGA…….	Poly(lactic-co-glycolic acid)
PVA……	Poly(vinyl)alcohol
RPMI-1640……	Roswell Park Memorial Institute Medium
SEM…….	Scanning electron microscopy
SO…….	Safranin O
Trypsin-EDTA……..	Trypsin-ethylenediamine tetraacetic acid

CHAPTER 1

INTRODUCTION

1.1. Controlled Drug Delivery Systems

Controlled release technology has become one of the most important topics in the pharmaceutical sciences since 1980s. Polycaprolactone (PCL), polylactide (PLA) and poly (lactide-co-glycolide) (PLGA) gained interest at the same time for development of bone implants using polymers. Nowadays, use of these polymers in investigations of new dosage forms is the new trend in this technology. Controlled drug delivery aims to supply drugs (or other bioactive agents) within biocompatible carriers to the living system either by controlling the site and/or controlling the rate of drug released. Control on the application site can be achieved by drug targeting (passive or active) to the injured region of the body. In literature, there are many researches on polymeric drug delivery systems (Slots and Rams, 1990; Sendil et al, 1999; Keskin et al., 2005; Dhanaraju, et al., 2006; Shukla et al., 2007; Hnaien et al; 2011; Jiang et al., 2011). The form of drug delivery systems can be sphere, capsule, rod, membrane, slab etc. Their size may vary from nano, micro or to milli levels depending on the aim.

Three mechanisms are involved in the release of bioactive agents from controlled release formulations: “Fickian diffusion through the polymer matrix, diffusion through pores in the matrix and drug liberation by polymer erosion.” (Jeong, et al., 2003). However, it is difficult to provide a general exact release profile/rate for these systems since it depends on several system related factors; namely polymer and drug properties and proportions, size and form. Polymer related factors are molecular weight, crystallinity, and hydrophilicty etc. Factors that depend on drug are solubility of the drug, molecular weight polymer-drug interactions, and etc. Among the system associated factors are drug loading efficiency, physical state of the drug in the matrix, particle size and distribution, porosity and internal structure of the particles (Jeong, et al., 2003).

“Controlling the release is an attainable and desirable characteristic for drug delivery systems. The factors affecting the drug release rate revolve around the structure of the matrix where the drug is contained and the chemical properties associated with both the polymer and the drug “(Freiberg and Zhu, 2004). Controlled release dosage form is the most important concept of the issue. Drug property, route of administration, delivery vehicle property, drug release mechanism, targeting ability and biocompatibility determine the drug release dosage (Figure 1.1). Apart from drugs, delivery systems can be designed for gene and protein delivery purposes. An ideal gene delivery requires more specific cellular targeting than other forms of drugs (Park and Mrsny; 2000). Because, genes should not be released till they arrive at the target cell and tissue.

In drug delivery studies, polymers, generally regarded as safe (GRAS), are used. Food and Drug Administration (FDA) approved use of a limited number of polymers in clinical studies.

Important classes of drugs have to benefit from advances in drug delivery technology to enhance or facilitate the action of their therapeutic agents. Choosing the suitable delivery systems is important to maximize the potential therapeutic impact of each drug candidate.

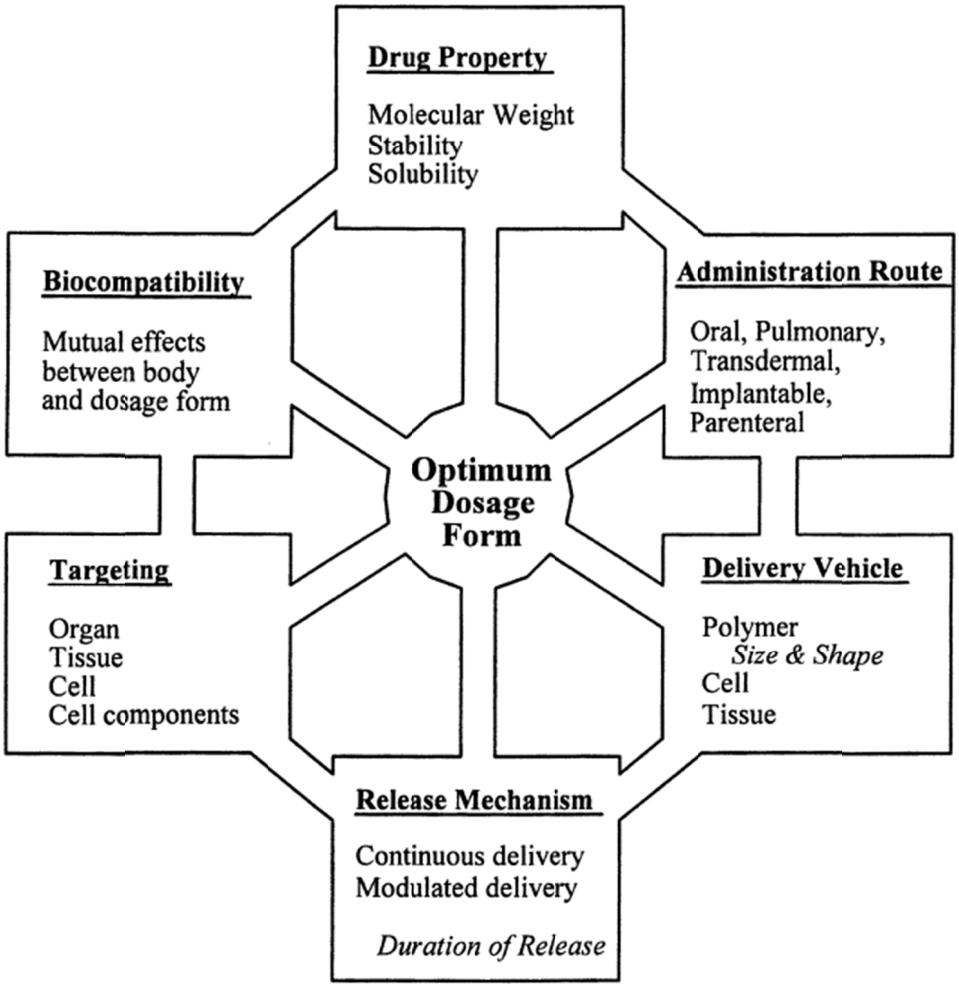


Figure 1.1. "Interdependent factors important in the design of controlled drug delivery .systems" (Park and Mrsny; 2000).

Since the initiation of pharmacological therapy, maintaining steady therapeutic drug concentration levels while minimizing side effects has been a major challenge. For the traditional drug delivery systems, typically tablets or intravenous injections, the administration of the entire dose of drug in one portion would result in high, sometimes close to toxic, plasma concentrations of the drug, leading to adverse reactions. By effective management of the dose size and dose frequency, it is possible to achieve therapeutic steady state levels of a drug. However, the drug concentration in plasma may cause toxic side effects if the drug level swings above the maximum level or a lack of efficacy if the drug level falls below the minimum level (Figure 1.2). Therefore, the idea of having a prolonged drug delivery system which can maintain a long-term appropriate therapeutic plasma concentration by releasing drugs in a controlled fashion become appealing and popular, leading to the rise of controlled delivery.

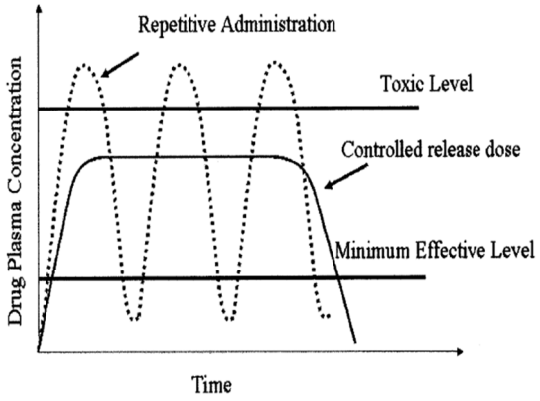


Figure 1.2. Plasma concentration of drug as a function of time after administration

As indicated in the term, controlled release is the technique to control the release of drugs so as to maintain a relatively constant, effective plasma level for an extended period of time by reproducible and predictable kinetics. A successful controlled delivery system should reduce the frequency of administrations, which would be a relief to many patients including the elderly and those chronically ill. In addition, a

steady-state level of the drug could be maintained to minimize the side effects through reducing fluctuations in drug level (Langer, 1993)

1.2. PCL

Polycaprolactone (PCL) was synthesized by the Carother's group in the early 1930s (Woodruff and Hutmacher 2010). This biodegradable polymer can be prepared from ring-opening polymerization of ε-caprolactone using either anionic, cationic and co-ordination catalysts or free radicals such as 2-methylene-1-3-dioxepane). PCL is a hydrophobic, semi-crystalline polymer whose crystallinity decreases with increasing molecular weight. Glass transition temperature (T_g) of PCL is about -60°C. Because of this, in body temperature it is expected to be at rubbery stage. Melting point (T_m) is between 59 and 64 °C. These properties of PCL enable easy shapeability at low temperatures.

During 1970s and 1980s, PCL and its copolymers were extensively used as drug delivery devices due to numerous advantages of PCL. Among these advantages tailorable degradation kinetics and mechanical properties, flexible shaping characteristics, high biocompatibility, and ease of adding functional groups for rendering the polymer more hydrophilic can be given. PCL degradation rate is slower than other frequently used polymers such as polyglycolide (PGA), poly D,L-lactide (PDLA) and also its copolymers. Its slow degradation property allows PCL to be used in long-term delivery devices, especially for applications lasting more than 1 year (Woodruff and Hutmacher, 2010). It has hydrolytically labile aliphatic ester linkages. Hydrolytic degradation makes degradation rate slow. It takes approx. 2-3 years in aqueous environment for complete degradation. Although its mechanical properties are not strong, copolymerization and blending with other polymers enhance this property of PCL (Sinha et al, 2004). During biodegradation, PCL is known not to produce an acidic environment. Thus, it is suitable to use as drug carrier in synovial area of the joint cartilage. PCL is the most widely used polymer in bone and cartilage repairs due to several advantages on uses in bulk amounts such as stability, low price and availability (Luciani, et al., 2008 and Hutmacher, 2000).

PCL also can be used in blends to improve several properties such as crack resistance, adhesion, and etc. It can be blended with cellulose propionate, cellulose acetate butyrate, polylactic acid and polylactic acid-co-glycolic acid for modifying the drug release rate of microparticles (Woodruff and Hutmacher, 2010).

PCL micro-nano/spheres can be prepared by using different types of methods such as oil in water (o/w) and water in oil (w/o) dispersions according to the type of the drug (Freiberg and Zhu, 2004). Emulsions in controlled conditions can form uniform spheres. Besides single emulsion (Perez et al, 2000) and double emulsion solvent evaporation methods (Dhanaraju et al., 2006) spray drying technique (Chu et al., 2006) can also be used for preparation of micro-nano/sphere forms (Wang, et al., 2009).

Degradation rate, high permeability of many drugs and non-toxic degradation products are also the major factors for using PCL as a long-term drug/vaccine delivery vehicle. Capronor® is a long-term contraceptive delivery device in which PCL is used as the polymer. It has been used for the long-term zero order release of levonorgestrel (Nair and Laurencin, 2007; Nair and Laurencin, 2006). In Figure 1.3, chemical structures of PCL and its monomer in cyclic form, called ε-caprolactone, are presented.

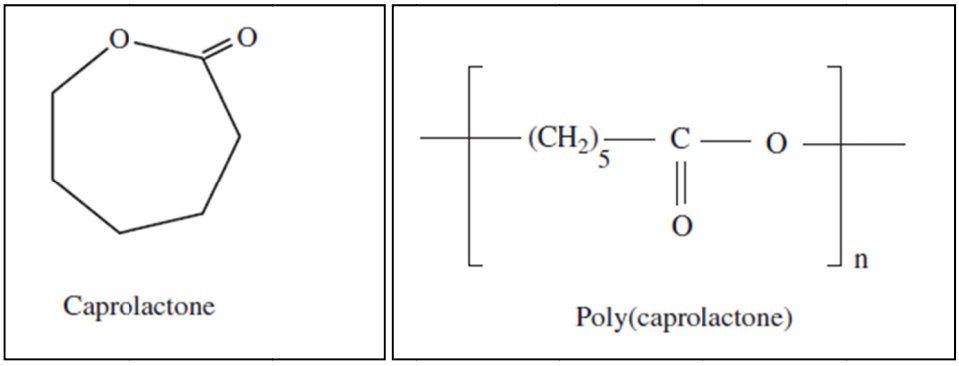


Figure 1.3. Chemical formula of cyclic caprolactone and corresponding homopolymer of PCL (Nair and Laurencin, 2007).

There are numerous researches on the design of PCL based delivery systems. For example, papaverine-loaded PCL microparticles were prepared for gastrointestinal tract delivery (Jeong et al., 2003). This system was an oral delivery form of microparticles for delaying the release of drugs at the colonic region. PCL was also

used as a perfect carrier for contraceptive hormone release as mentioned above (Dhanaraju et al., 2003; Dhanaraju et al., 2004). Levonorgestrel and ethinyl estradiol were encapsulated into PCL and this formulation was found suitable in contraceptive therapy (Dhanaraju, et al., 2005). PCL microspheres were also loaded with different anticancer drugs to minimize the severe side effects of the drugs like etoposide used for malignant lymphoma, brain stem gliomas, small cell lung carcinoma and ovarian cancer (Vivek et al., 2007), felodipine (Kim et al., 2005a) and disodium norcantharidate (DSNC) for primary hepatic carcinoma, breast cancer and abdominal caner (Wang et al., 2008a).

In long-term therapy, tamoxifen, a selective estrogen receptor modulator (SERM; Jordan, 1998), has some major side effects such as endometrial cancer (Johnston, 1997; Munster and Hudis, 1999; Osborne and Fuqua, 1994). Due to the side effects, targeted delivery to the site of tumor is needed. Tamoxifen was entrapped in PCL nanoparticles (Chawla and Amiji, 2002) and the ratio of absorbed tamoxifen by the tumor was improved by the selectivity of the treatment.

In recent years, protein-loaded polymeric structures have gained attraction. Cheng and his co-workers (2010) entrapped lysozyme inside PCL particles. With other polyester based systems, protein / peptide release usually continuous one to four months (Jiang et al., 2005). However, fast degradation of PLGA and acidic environment during degradation were reported as a disadvantage. Sustained release of proteins using PCL rather than PLGA is suitable due to these advantages (Sinha et al., 2004; Tang et al, 2007). Myoglobin was encapsulated into PCL as a model protein for evaluation of conformational changes upon encapsulation and to compare with BSA and lysozyme (Hnaien et al, 2011).

1.3. Doxycycline Hyclate

Doxycycline is a member of tetracycline group and it has been known as the most stable group member. It is a wide spectrum antibiotic for gram positive and negative bacteria, protozoa and anaerobes (Bokor-Bratic and Brkanic, 2000). It is available as both the monohydrate and the hyclate forms. It is a light yellow crystalline powder. It never melts, but chars at 201°C. While doxycycline hyclate is freely soluble in water, monohydrate is slightly soluble (Tamimi et al., 2007). This product is also reported to be soluble in methanol, sparingly soluble in ethanol, and insoluble in chloroform or ether. In Fig 1.4, the chemical structure of doxycycline hyclate is given (Scholar, 2007).

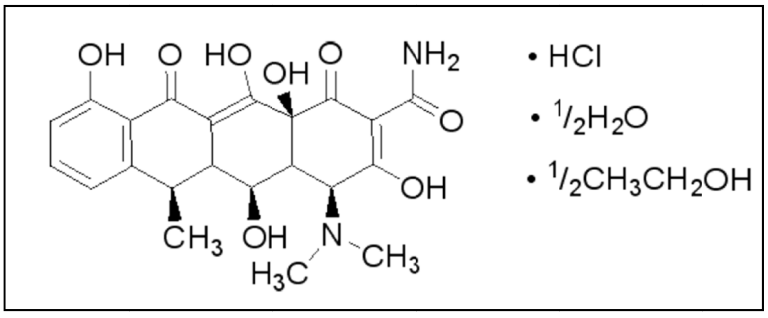


Figure 1.4. Chemical structure of doxycycline hyclate (www.sigmaaldrich.com)

It has an activity against periodontal pathogens such as *Actinobacillus actinomycetemcomitans (A.a.), Porphyromonas gingivalis (P.g.),* and *Bacteroides frosthytus (B.f.).* Tamimi, et al (2007) studied with doxycycline monohydrate and doxycycline hyclate sustained release systems for periodontal diseases. In clinical studies, doxycycline hyclate have shown the best results (Choi, et al., 2004).

Besides its antibacterial activity, doxycycline also inhibits the matrix metalloproteinase (MMP) activity (Nordstrom et al., 1998) and cytokines in periodontal diseases (Choi et al., 2004), reduce MMP activity in arthritis, periodontitis (Gapski et al., 2004), aortic aneurysms (Manning et al., 2003). It was

also shown that it acts as an inhibitor of MMPs in wound healing (Chin et al., 2003, Shanmuganathan et al., 2008).

For slow release formulations, the suitability of this drug was proven (Honnorat-Benabbou, et al., 2001). For septic arthritis therapy doxycycline hyclate was encapsulated into PLGA microspheres and slow release system was developed for infected joints of horses and cattle (Haerdi-Landerer, et al., 2008; Haerdi-Landerer et al., 2007). Another slow release treatment approach with doxycycline was applied for periodontitis (Patel et al., 2008). Chitosan microspheres were also loaded with this drug by ionic gelation method and KOH was used for crosslinking. In a recent study, Doxycycline was encapsulated into PCL microspheres and then coated with collagen for periodontal studies (Aishwarya et al., 2008). In another study for treatment of human periodontal pocket application PLGA/PCL blends was used in preparing microspheres for doxycycline release (Mundargi et al., 2007).

1.4. Chondroitin Sulfate

Chondroitin sulfate is a glycosaminoglycan, which is naturally found in the extracellular matrix of articular cartilage. It is composed of long unbranched polysaccharide chains with a repeating structure of glucuronic acid and N-acetylgalactosamine (Nerucci et al., 2000). High solubility in water is an important property of chondroitin sulfate. In clinical studies, CS is in use for treating osteoarthritis (OA). Benefits of CS treatment for OA can be listed in three main mechanisms, namely promoting the production of cartilage extracellular matrix, alleviating the inflamatory cytokines such as interleukin-1, and inhibition of cartilage matrix degradation (Jiang et al., 2011).

CS was encapsulated into PLGA with double emulsion method. The research reported that these microspheres were capable of treating osteoarthritis (OA) in vitro and in vivo (Jiang et al., 2011). In this study it was used to accelerate the diffusion of water into the microspheres thereby modifying the release properties of PCL microspheres as well as its disease modifying potential.

1.5. Theory of Mathematical Modelling

1.5.1. Mathematical Modeling of Release Kinetics of Drug Loaded Microspheres

For decades, polymeric systems have been used for pharmaceutical applications, especially to provide controlled release of drugs. Drug–polymer systems may also be useful in protecting the drug from biological degradation prior to its release. The development of this device starts with the use of non-biodegradable polymers and subsequently progresses to the use of biodegradable polymers. Based on the physical or chemical characteristics of the polymer, drug release mechanisms from a polymer matrix can be categorized in accordance with three main processes (systems):

1. Drug diffusion from the non-degraded polymer (diffusion-controlled system).

2. Enhanced drug diffusion due to polymer swelling (swelling-controlled system).

3. Drug release due to polymer degradation and erosion (erosion-controlled system).

In all three systems, diffusion is always involved. For a non-biodegradable polymer matrix, drug release is due to the concentration gradient by either diffusion or matrix swelling (enhanced diffusion). For biodegradable polymer matrix, release is normally controlled by the hydrolytic cleavage of polymer chains that lead to matrix erosion, even though diffusion may be still dominant when the erosion is slow. This categorization allows mathematical models to be developed in different ways for each type of system. In our system, the degradation of PCL was assumed to be negligible for the 3 months release period due to slow degredation property of PCL.

Mathematical modeling of drug release provides insights concerning mass transport and chemical processes involved in drug delivery system as well as the effect of design parameters, such as the device geometry and drug loading, on drug release mechanism. (Peppas et al, 1989)

The main mechanism determining the drug release profile from polymer matrix is diffusion. Drug dissolution or dispersion models are the other factors. Each of these models represents the release of a drug by a single mechanism that is either by dissolution or dispersion. Diffusion-dissolution or diffusion-dispersion combination

models can be possible. However, the verification of a complex model involving both mechanisms would be problematic; generally two simpler models are formulated (Crank 1956, Polakovic et al, 1999, Jo et al, 2004, Arifin et al, 2006).

1.5.2. Mathematical Models of Drug Release from Polymeric Systems

1.5.2.1. Diffusion Model

The release of the drug from the polymeric matrix generally follows Fick's second law of diffusion. The concentration gradient of the spherical particles follows the form

$$\frac{\partial c}{\partial t} = D\left(\frac{\partial^2 c}{\partial r^2} + \frac{2}{r}\frac{\partial c}{\partial r}\right) \tag{1.1}$$

where c is the local drug concentration at time t and at the distance, r, from the centre of the particle and D is the diffusion coefficient of the drug in the polymeric matrix.

The initial condition is

$$c = (r,0) = c_0 \tag{1.2}$$

The boundary conditions are

$$V\frac{\partial c_1}{\partial t} = \frac{3V_s}{R}D\left(\frac{\partial c}{\partial r}\right)_{r=R} \tag{1.3}$$

$$c = (r,t)_{r=0} = finite \tag{1.4}$$

V is the bulk liquid volume of the surrounding medium, V_s is the total volume of the particles.

After several steps of calculation, the resulting equation is (Crank, 1956):

$$\frac{c_1}{c_{1\infty}} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} e^{-\frac{n^2 \pi^2}{R^2} Dt} \qquad (1.5)$$

1.5.2.2. Dissolution Profiles

Drug release and/ dissolution from solid pharmaceutical dosage forms have been the subject of intense and beneficial scientific developments. Whenever a new solid dosage form is developed, it is important to ensure that drug dissolution occurs at an appropriate rate. The quantitative analysis of the values obtained in dissolution and/or release tests is easier when mathematical formulas which express the dissolution results as a function of some parameters of the dosage forms are used. In some cases, these mathematical models are derived from the theoretical analysis of the occurring process. In most of the cases the theoretical concept does not exist and some empirical equations have proved to be more suitable.

The kind of the drug, its polymorphic form, crystallinity, particle size, solubility and amount in the pharmaceutical dosage form can influence the release kinetic (Costa and Lobo, 2001). A water-soluble drug incorporated in a matrix is mainly released by diffusion, while for a low water-soluble drug the self-erosion of the matrix will be the principal release mechanism.

Some of the most relevant and more commonly used mathematical models describing the dissolution curves are shown in Table 1.1.

Table 1.1. Mathematical models used to describe drug dissolution curves (Model-dependent methods)

Zero Order	$Q_t = Q_o + K_o \cdot t$
First Order	$\ln Q_t = \ln Q_o + K_1 \cdot t$
Second Order	$Q_t / Q_\infty = K_2^2 \cdot t$
Hixson-Crowell	$Q_0^{1/3} - Q_t^{1/3} = K_s \cdot t$
Weibull	$\log\left[-\ln\left(1 - \left(Q_t / Q_\infty\right)\right)\right] = b \times \log t - \log a$
Higuchi	$Q_t / Q_\infty = K_H \cdot t^{0.5}$
Baker-Lonsdale	$\left(3/2\right)\left[1 - \left(-1\left(Q_t / Q_\infty\right)\right)^{2/3}\right] - \left(Q_t / Q_\infty\right) = K \cdot t$
Korsmeyer-Peppas	$Q_t / Q_\infty = K_k \cdot t^n$
Quadratic*	$Q_t = 100\left(K_1 \cdot t^2 + K_2 \cdot t\right)$
Logistic*	$Q_t = A / \left[1 + e^{-K \cdot (t-y)}\right]$
Gompertz*	$Q_t = A \cdot e^{-e-K \cdot (t-y)}$
Hopfenberg	$Q_t / Q_\infty = 1 - \left[1 - k_0 \cdot t / C_o \cdot a_o\right]^n$

*: less used models (Costa and Lobo, 2001)

1.6. Articular Cartilage

Articular cartilage is a specialized, avascular, aneural connective tissue that covers the osseous components of diarthroidal joints. It serves as a load-bearing material that absorbs impact and sustains shearing forces (Martel-Pelletier et al., 2008). In cartilage matrix, collagen and proteoglycans (aggrecan) are the main components. Cartilage has 4 different zones. These zones are layered from surface to the deepest layers of the cartilage as superficial, middle or transitional, deep or radial and calcified cartilage layers (Figure 1.5).

Superficial part is the thinnest part of the cartilage and also has lower proteoglycan content than the other layers. Synovial fluid is in contact with this layer. This layer also enables cartilage to resist shear, tension and compression (Martel-Pelletier et al., 2008). Middle part is the main part of the total cartilage in terms of size. This part is formed by proteoglycans and thicker collagen fibrils. The chondrocytes have round shape and have low density in this zone (Martel-Pelletier et al., 2008). In the deep zone of cartilage, chondrocytes have the same morphology and low density but they are aligned perpendicular to the articular surface in this layer. There is a radial arrangement of the thick collagen fibrils and this layer's aggrecan content is the highest in cartilage (Martel-Pelletier et al., 2008). The calcified cartilage is divided from the other parts by the "tide mark". This part separates the hyaline cartilage from the subchondral bone. In this zone, cell population is low and chondrocytes are hypertropic. The main function of this part is to attach the cartilage to the bone (Martel-Pelletier et al., 2008).

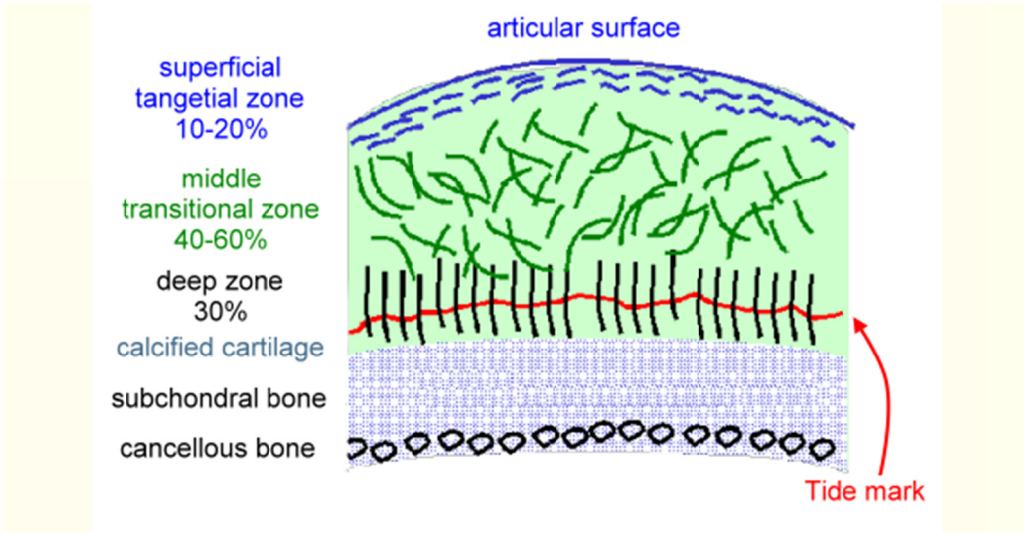


Figure 1.5. Illustration of layers of articular cartilage (Mow and Hung, 2001)

Collagen, proteoglycans and other proteins (glycoproteins) form only about 20% of the cartilage in wet weight. Water and inorganic salts corresponds to the most of the weight in the tissue. Water is an important tissue component. Inorganic salts such as sodium, calcium etc are dissolved in water. The diffusion of water through the cartilage helps in carrying the nutrients from the synovium to the cartilage tissue (Martel-Pelletier et al., 2008).

Collagen constitutes 50-60% of the cartilage in dry weight. Type II collagen is specific to cartilage tissue and forms 90-98% of the total collagen of the tissue (Martel-Pelletier et al., 2008).

Proteoglycans form the second largest component of the solid phase in cartilage. In wet weight, 5-10% of the tissue is proteoglycans. Glycosaminoglycan chains are attached to core protein of proteoglycans. The glycosaminoglycan molecules are formed from polydimeric saccharides such as chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate. Chondroitin sulfate occupies 60-90% of the whole structure (Martel-Pelletier et al., 2008).

1.7. Osteoarthritis

Joints are complex composites of connective tissue including bone, cartilage surfaces, ligaments and the joint capsule. These tissues come together to allow the motion of the body. Joint degeneration occurs after trauma like intensive sport activities or accidents, and also upon aging.

Osteoarthritis (OA) is a non-inflammatory, degenerative joint disease. Not only articular cartilage, but also subchondral bone is damaged during stages of OA. In Figure 6, healthy and osteoarthritic joints are compared. The tissue breakdown occurs with pain and joint stiffness, which are the main symptoms of OA. Loss of articular cartilage, subchondral bone sclerosis, osteophyte formation, changes in the synovial membrane and increased volume of synovial fluid with decrease in viscosity are the results of osteoarthritis of the joints (Gerwin, et al., 2006). The clinical stage of this disease is usually accompanied with chronic inflammation of the synovial membrane (Martel-Pelletier et al., 2008). In the United States, approximately 30 million people suffer from OA.

Osteoarthritic cartilage is weaker than the normal cartilage in terms of mechanical properties. Degenerated cartilage tissue has less stiffness and less pulling strength than normal cartilage. The initial symptoms of matrix degeneration in cartilage with arthritis are increased water permeability and water holding capacity of the tissue. The basic reason of this is the high osmotic pressure created by the interaction of water with negatively charged proteoglycans. Normally, the water holding capacity is controlled by collagens within the tissue. However, with degradation of collagens in osteoarthiritis, this control is lost.

Collagen network integrity also has important implications on the tribological functions of cartilage tissue. Cartilage samples with physical disruption of the collagen network in the superficial zone have been shown to exhibit higher friction levels compared to native cartilage. Enzymatic degradation of the collagen network has also been shown to increase the friction levels of cartilage. However, this effect occurs mostly through adversely affecting the proteoglycan and water contents of the cartilage tissue. A physical disruption of the collagen network can quickly exacerbate the wear of cartilage towards irreparable damage under *in vivo* conditions, due to the very high and repetitive loads involved. In OA and RA cartilages, proteoglycan loss results in a reduction of cartilage stiffness (Bonassar,

et al., 1995; Kempson, et al., 1979), whereas degradation and loss of type II collagen by collagenases (MMPs-1 and -13) result in an irreversible loss of tensile properties and structural integrity (Kempson, et al., 1979).

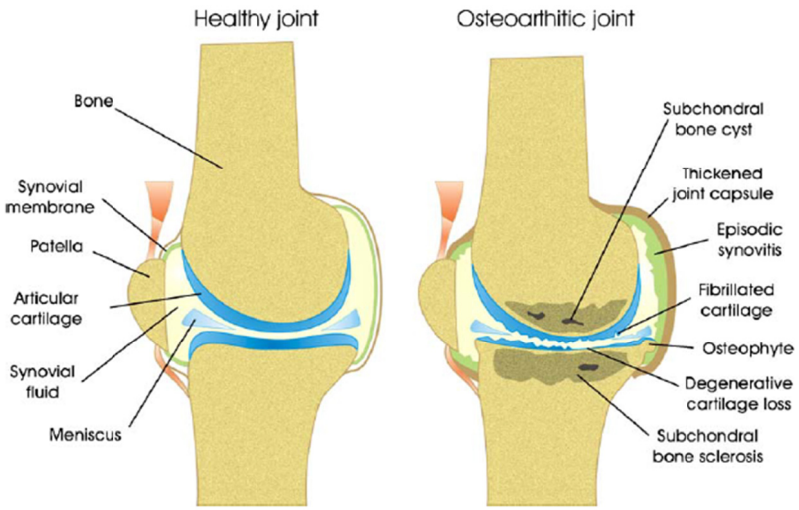


Figure 1.6. Comparison of healthy and osteoarthritic joints (Gerwin, et al, 2006).

1.7.1. Role of Cytokines in Osteoarthritis

Cytokine mediated anabolic and catabolic metabolisms are in balance in normal joints. Destructive processes outweigh the repair capacity with age and the loss of cartilage tissue increases with time. Age, genetic factors, gender (being woman), obesity, trauma, occupational factors, congenital or acquired changes in joint anatomy are risk factors of osteoarthritis (OA). With age, synovial fluid of the articular cartilage decreases and this reduces the ability to stretch. Hence, the loading on the joint surface increases.

Cytokines such as Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are known as proinflammatory cytokines. Interleukins are thought to be effective in the development of sepsis, diabetes, and osteoporosis, autoimmune and arthritic diseases. IL-1 was detected in degenerative cartilage (Towle et al., 1997), and was reported to be present in synovial fluid at concentrations ranging from tens of picograms to nanograms per milliliter (Fontana, et al, 1982; Hopkins et al., 1988).

Il-1, therefore, has been implicated in the progression of arthritis. In vitro stimulation of explanted cartilage with IL-1 has demonstrated utility as a model of the catabolic events leading to cartilage resorption (Wilson, et al, 2007).

In vitro studies have shown that IL-1 contributes to the accelerated damage of articular tissue and amplify the inflammatory response. The ability of IL-1 to promote tissue degradation appears to be intimately associated with its ability to stimulate the synthesis of matrix metalloproteinases (MMPs) such as collagenase and stromelysin. Thus, it was also reported that IL-1 not only induces tissue destruction, but also inhibits tissue repair (Blumenfeld, et al, 1999). It is well known that IL-1β is synthesized as an inactive precursor and must be activated by an enzyme to be released in the active form (Martel-Pelletier, 2004).

Matrix metalloproteinases (MMPs) are enzymes that degrade the cartilage and bone in an arthritic joint. They are divided into two groups: (1) aggrecanases (2) matrix metalloproteinases. They can cleave collagen type II, the major component of the cartilage. MMPs are prompted from inflammatory cytokines such as interlukin-1 (IL-1). The collagenases play role in collagen type II degradation includes collagenases 1, 2 and 3 (MMPs 1, 8, and 13, respectively) (Goldring, 2000). MMP-1 and MMP-13 are important in OA development (Kim, et al., 2005; Huh, et al., 2009). Among these enzymes, MMP-13, firstly found in human in breast cancer, is present in human bone and cartilage tissues. Collagen type II is the dominant type of collagen in cartilage tissue. MMP-13 breaks collagen type 2 more efficient than collagen type 1 (Fosang and Pelletier, 1996). Furthermore, the same collagenase breaks the basic matrix material called proteoglycan and a different form of collagen called gelatin (Greenwald, 1991, and Aigner, et.al., 2003).

The degradation steps are accompanied with chondrocyte death, tissue fibrillation and finally erosion (Huh, et al., 2009; Lark, MW. et al., 1997). At an early stage of the disease, aggrecanases start to degrade proteoglycans and at the late stage, MMPs finally degrade the aggrecan core protein, proteoglycan and collagen network (Huh, J.E., et al., 2009). Understanding the elements involved in the regulation of these two (IL-1 and IL-6) proinflammatory cytokines will therefore provide a better insight into therapeutic strategy. These cytokines are able (i) to increase the enzyme synthesis, (ii) to inhibit the synthesis of the major physiological inhibitors of these enzymes, and (iii) to inhibit the synthesis of the matrix constituents, such as collagen

and proteoglycans. These actions make these two cytokines prime targets for therapeutic approaches.

1.7.2. Treatments of OA

The clinical treatment regimes provide relief to the patient and may increase the quality of patients's lives nowadays. However, the effective cure of OA has not been found yet. Treatments with oral drugs have various side effects and shortcomings especially in long terms. In addition no blood circulation to the joint area makes it difficult to supply the drug to the joint efficiently. Therefore, intraarticular (IA) drug injection has developed as new treatment method in recent years. Localized nature of the disease makes it possible to apply glucocorticoid and hyaluronic acid (HA) formulations intraarticularly. However, these appoaches only make short-term pain relief to the patient.

In early phases, treatment regimes heal the inflammation of the joint (Kutsal G., 1999, 2003). For this purpose, anti-inflammatory drugs are used for decreasing prostaglandin production of the body. Most anti-inflammatory drugs are analgesic and painkiller when taken at low dosages. In general, for arthritis treatment, anti-inflammatory drugs should be used for long periods and at high dosages. Non-steroidal anti-inflammatory drugs (NSAIDs) can be used for pain and inflammation modulation. The most common anti-inflammatory drug is aspirin. However, the most powerful anti-inflammatory drugs are corticosteroids. Corticosteroids are generally used during blazing up period to stop the pain within short durations (Kutsal G., 1999, 2003). Most arthritis patients need not to undergo an operation. At later stages of the disease, operation might be needed to stop the pain and increase the mobility of the patient.

Tetracycline and its analogs have collagenase enzyme activity inhibition property as shown by many researches with in vitro studies. Doxycycline as one of these analogs is known for its negative effects on bone and teeth tissue. Besides that, this drug has antibacterial property restricting its systemic use. Researches on doxycycline analogs that inhibit MMPs in cartilage but not in other tissues or with non-antibiotic effect continue all over the world (Greenwald, 1998, Golub, 1991).

1.8. OA Models

1.8.1. *In Vitro* Models

There are several chondrocyte cell sources used for in vitro studies of OA (i.e., human, rabbit, rat, calf etc). Two-dimensional (2-D) or three dimensional (3-D) models can be established using these cell sources. In 3-D models, cells are either embedded in hydrogels like agarose, alginate, agar or imbibed into porous polymeric scaffolds in order to mimick the native cartilage environment. Another approach for constructing such models is the use of explant culture in which thin slices of cartilage are cultured in tissue culture media. By this approach, the integrity of 3D architecture of cartilage is maintained. However, the longevity of these explants is not very long and it is not easy to achieve.

Traumatic mechanical injury induces chondrocyte death, disrupts the collagen network, and causes glycosaminoglycan (GAG) release from articular cartilage. An in vitro study of the kinetics of GAG release found that one third of the GAG released within the first 24 h occurred during the first 4 h of culture, and remained significantly higher than controls at 24 h (DiMicco et al., 2004). The same study also found a 50–60% reduction in the incorporation of ^{35}S-sulfate and ^{3}H-proline, indicative of decreased GAG and collagen synthesis, in articular cartilage specimens subjected to injurious compression at a strain rate of 1/s. Furthermore, impact level has been found to correlate with tissue damage, demonstrating that there is a direct relationship between the degree of articular cartilage breakdown and the peak stress stress rate, and energy delivered by the impact (Ewers et al., 2001; Jeffrey et al., 1997; Milentijevic and Torzilli, 2005; Torzilli et al., 1999). A study by DiMicco et al. (2004) found that GAG release between 1 and 7 days post-injury was markedly reduced using an MMP inhibitor compared to no treatment case.

In Table 1.2, some of the *in vitro* OA Models are summarized. The model development of OA was done by using a chemical stimulator like IL-1. However, TNF-α, Oncostatin M and Azo-C are among the other widely used chemical stimulators (Kavas, et al., 2010; Little, et al., 2005; Cawston, et al., 1998).

The interaction between hyaluronan and CD44 has been shown to reduce IL-1β-induced MMP-1, MMP-3 and MMP-13 production in normal and OA explant cultures (Julovi SM, et al., 2002) and MMP-1 in chondrocyte monolayer cultures (Tanaka M. Et al., 2006).

Nerucci, F et al. (2000) have tested the effects of IL-1β and CS in the presence and absence of pressurization cycles on the morphology and metabolism of in vitro human chondrocytes. These tests confirmed the findings obtained with in vitro experimental models. It is currently accepted that IL-1 is an important mediator in cartilage destruction. IL-1 is a primary up-regulator of metalloproteinase (MMP) gene expression by chondrocytes, and it also down-regulates the production of the endogenous inhibitor of MMPs, the TIMPs (tissue inhibitor of metalloproteinases) as well as the synthesis of PG (proteoglycans) and collagens (Nerucci, F et al., 2000).

The experiments have shown that the addition of IL-1 causes a reduction in the concentration of the PG in the culture medium; this could be the result of the event that cytokine induces an inhibition of PG synthesis by the chondrocytes. When the cells were cultivated in the presence of IL-1β+CS there was a restoration of PG concentration in the culture medium. This fact confirms the protective role played by this substance which counteracts the IL-1β induced effects and might be used by the chondrocyte as a substratum for the synthesis of PG (Nerucci, F et al., 2000).

Model	Source	Reference
minced cartilage incubated with collagenase type II	rabbit	Kamarul, T. (2008); Kamarul, T. (2011)
collagenase (grade CLS)+ agarose embedding	chick embryo	Bruckner, P.(1989)
collagenase + coating with type I collagen	rat	Yorimitsu,M. (2008)
human cartilage explants culture + IL-1	human	Julovi, S.M. (2004);Ferna´ndez, P. (2004) ; Yudoh, K. (2007); Huh, J.E. (2009)
rabbit chondrocytes + IL 1beta	rabbit	Akasaki, Y. (2009)
cell culture system of chondrocytes+IL-1alpha	bovine	Zainal, Z. (2009)
cartilage explant+ IL 1 alpha	rabbit	Choi, J.H. (2002)
cell culture system chondrocytes in agarose+IL-1beta	dog	Kuroki, K. (2001);Dvorak, D.M. (2002); Kuroki, K (2003)
collagen scaffold+ IL 1 beta	calf	Cortial, D (2006)
collagen sponges +chondrocytes obtained byenzymatic digest	bovine	Roche, S (2001)
mechanic load+ IL 1 beta	human	Nerucci, F (2000)
cartilage explant+IL 1 alpha	calf	Wilson, C.G (2007)
cartilage explant + IL 1 beta	bovine	Palmer,A. W. (2009)

Table 1.2. *In Vitro* OA Models

1.8.2. In Vivo Models

In order to investigate the development and progression of OA, various animal models are used. These models can be divided into four groups. They are spontaneous OA models, mechanical OA models, chemical models and other joint models (except knee). For chemical models, trypsin, papain, collagenase (type II) are used to develop osteoarthritis in the knee (Table 3). There are several examples in literature that used chemical models to test the effectiveness of potential agents (glucosamine, chondroitin sulfate, adiponectin, etc.) (Dodge and Jimenez, 2003; Homandberget al., 2006, Chen et al., 2006).

In an experimental rabbit model, OA was induced by partial menisectomy and treated with NSAIDs, HA or with the combination of NSAIDs and HA over 2 weeks. Cartilage degeneration and the levels of matrix metalloproteases (MMP)-1, MMP-3 and MMP-13 in synovial lavage fluid were measured at the end of the experiment.

In another in vitro/in vivo study, human articular chondrocytes were cultured with NSAIDs and HA in the presence of interleukin (IL)-1β or IL-6+ sIL-6 for 24 h. After culture, the production of MMPs, IL-1β, and IL-6 was measured. The levels of MMP-1, MMP-3 and MMP-13 in synovial fluid from the NSAID-treated group were significantly higher than in controls. The increased production of MMPs induced by NSAIDs was counteracted by the concomitant administration of HA. In vitro studies showed that NSAIDs augmented IL-1β- and IL-6-induced production of MMPs from human chondrocytes, while completely inhibiting the IL-1β- and IL-6/sIL-6R-induced production of prostaglandin E2 (PGE_2). The addition of PGE_2 did not reduce the augmentation of MMP production. HA inhibited the promotion of cytokine-induced MMP and cytokine production by NSAIDs. Data clearly showed that HA inhibited NSAID-accelerated MMP production which was followed by inflammatory cytokine production from cytokine-activated chondrocytes. The results warrant further evaluation of the potential chondroprotective effects of co-administration of HA with NSAIDs (Hashizume, M. Et al, 2009).

Model	Source	Reference
mono-iodoacetate (MIA) injection OA	Rat	Guingamp, C (1997); Schuelert, N. (2006);Kalff, K.M. (2010); Takeshita, N.(2011)
anterior cruciate ligament and menisectomy transection (ACLMT)	Rabbit	Choi, S.I. (2007)
typically show moderate signs of osteoarthritis (OA) and joint pain	Hartley guinea pigs	Ciombor , D. McK.(2003); McDougall, J.J. (2010); Gurkan, I.(2010)
anterior cruciate ligament transection (ACLT)	rat	Galois, L. (2004)
cranial cruciate ligament (CCL)	Rabbit	Papaioannou, N.A.(2007)
anterior cruciate ligament transection (ACLT)	dog	Brandth, K.D.(1997)
rat meniscal tear (MT)	rat	Chockalingam, P.S. (2011)
anterior cruciate ligament transection (ACLT)	Rabbit	Batiste, D. L. (2004); Spriet, M.P. (2005)
defect creation	Rabbit	Kamarul, T. (2008); Kamarul, T. (2011)
anterior cruciate and medial collateral ligaments (ACL and MCL, respectively)	rat	Yorimitsu,M. (2008)
collagenase type II injection intraarticularly	rabbit	Kikuchi, T. (1998);Choi, J.H. (2002);Rudolphi, K. (2003); Huh, J.E. (2008);Huh, J.E. (2009)
bilateral anterior cruciate ligament transection (ACLT) using medial arthrotomy	rabbit	Akasaki, Y. (2009)
partial medial meniscectomy (MNX)	rat	Nielsen, R.H.(2011)
Cartilage surfaces of the metacarpal side of fetlock joint surgically damaged	sheep	Mastbergen, S.C. (2008)
Hulth-Telhag model of experimental OA	Rabbit	J. N. Rogart (1999)
partial meniscectomy	Rabbit	Mihara, M. (2007)

Table 1.3. *In Vivo* OA Models

1.9. Aim of the Study

Cartilage degenerations are widespread in our country like all over the world. Applied treatment methods are not able to solve the problem of joint degeneration completely. So, new methods are needed for treatment of the disease. In this study we aimed to prevent the progression of osteoarthritis occurring after trauma by developing a new approach for treating the disease using doxycycline, a clinically used antibiotic that also acts as a MMP inhibitor. For this end, we designed and optimized doxycycline and doxycycline/chondroitin sulfate PCL delivery systems that can be applied intraarticulary into the joint space to achieve local, long-term and more efficient treatment of OA. Diffusion-based mathematical models were used to fit the experimental results of in vitro release. Other benefits of this approach also involve reduction of the application frequency of the treatment and elimination of possible systemic site effects of drugs. In vitro cell culture studies were conducted with agarose-chondrocyte 3-D constructs to test the efficacy of the microsphere delivery sytems. Chondrocytes were isolated from the joints of ostoarthritic rabbits and were exposed to IL-1β for continuation of disease condition. In order to assess the treatment efficacy, the delivery systems were injected into the joints of collagen-induced OA rabbits and the results were compared with the conventional therapy (hyaluronan injection) and nontreated control OA groups in terms of histological, radiological and mechanical properties.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Doxycycline hyclate, chondroitin-4-sulfate, Poly-ε-caprolactone (PCL) (MW 14 kDa and 65kDa), Collagenase type II *(Clostridium histolyticum)* and Interleukin 1-β were supplied by Sigma-Aldrich USA. Polyvinyl alcohol (PVA) (MW 27,000) was provided from Fluka and gelatin (from porcine skin, type A) was purchased from Sigma, USA.

DMEM High Glucose, RPMI-1640 p red+ w/o phenol red, Trypsin-EDTA, Foetal Bovine Serum (FBS) was the products of Biochrom (Germany). Penicillin/ Streptomycin was purchased from PAA, Austria. L-glutamine was from Sigma Brazil. Folic acid was obtained from Sigma, China and sodium bicarbonate was purchased from Sigma (USA).

Papain and agarose, (low melting point) were purchased from Sigma Chemical Corporation (USA). L-cysteine, di-sodium hydrogen phosphate dihydrate and di-potassium hydrogen phosphate anhydrous, calcium carbonate were purchased from Fluka Chemical GmbH (Switzerland). Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate were purchased from Merck, Germany. Glycine was te product of Aldrich (Germany). Sodium acetate was taken from Sigma Aldrich, USA. Acetic acid sodium salt, citric acid (anhydrous) and ethylene diamine tetra acetic acid disodium salt dihydrate (~99%) was purchased from Sigma, USA. Chloramine-T hydrate (98%) was purchased from Aldrich, France. Trans-4-Hydro

L-proline was purchased from Sigma, France and 4-(Dimethylamino benzaldehyde was purchased from Fluka, India. Sodium hydroxide, potassium hydroxide, potassium chloride were obtained from Riedel-de Haen Germany (Sigma-Aldrich Company). Sodium chloride, sodium hydroxide, sodium azide and potassium hydroxide were purchased from Sigma Chemical Corporation (USA).

Trypan blue, Thiazolyl blue tetrazolium bromide (97.5% TLC cell culture tested) (MTT) and Deoxyribonucleic acid from calf thymus (genomic, unsheared) were purchased from Sigma (USA). 1,9 dimethyl methylene blue (DMMB) were obtained from Aldrich (Germany). Dimethyl sulfoxide (DMSO) (cell culture grade) was from AppliChem, Germany. Hoechst 33258 was purchased from Invitrogen, USA. MMP-13 Elisa kit was purchased from Cusabio, USA.

Organic solvents; Chloroform, tetrahydrofuran, methanol, acetonitrile, ethanol, 2-propanol were (all HPLC grade) obtained from Merck. Perchloric acid was from Aldrich, USA.

Alfazyne (2%, injectable) (xylazin hydrochloride) and Alfamine (10%, injectable) (ketamine hydrochloride) and purchased from Alfasan, Holland. Adant (25 mg/2.5 ml, intraarticularly) (Hyaluronan) was purchased from Tedec-Meiji Farma, S.A., Spain.

Haematoxylin Eosin, Massons' trichrome, Safranin O dyes, fixative 10 percent formaldehyde solution in phosphate buffer, fixative 2.5 % gluteraldehyde solution in phosphate buffer, decalcifying Decastro solution (Chloral hydrate, ethanol, distilled water), paraffin embedding equipment for light microscopy, plastic embedding equipment for electron microscopy, medium exchange were purchased for histology experiments.

2.2. Methods

2.2.1. Preparation of Empty Poly-ε-caprolactone (PCL) Microspheres

Poly-ε-caprolactone (PCL) microspheres were first prepared empty to optimize the preparation conditions for desired properties such as morphology and size. Among various concentrations and formulations of emulsifiers and stabilizers (Pluronic F 68, PVA, Gelatin, etc.) polyvinyl alcohol, (PVA) was selected for use in drug loaded microsphere preparation studies. Single emulsion was obtained by adding the polymer (PCL) solution (7% in chloroform) into PVA solution while stirring with a magnetic stirrer (Schott Instruments, Germany). Organic phase was then evaporated under hood with continued stirring at 25°C. Polymeric microspheres were collected by centrifugation of aqueous medium at 6000 rpm for 10 minutes (Hettich Zentrifugen, EBA 20, Germany). They were then washed with distilled water and centrifuged again. Upon overnight drying in vacuum oven (Nüve- EV060, Turkey), microspheres were kept in desiccator at 4°C till use.

In order to optimize properties of PCL microspheres, such as surface properties, average size and shape, different concentrations and formulations of PVA and PVA-gelatin combinations were tried in aqueous phase. The optimum temperature for evaporating organic phase was also adjusted considering gelatin and doxycycline presence in the medium.

2.2.2. Preparation of Doxycycline Loaded Poly-ε-caprolactone (PCL) Microspheres

Doxycycline encapsulated microspheres were prepared by single emulsion-solvent evaporation method as described in part 2.2.1. Doxycycline powder was added into PCL solution at the drug:polymer ratio of 1:2 (w/w) and mixed homogeneously. This mixture was then added drop-wise into aqueous phase and the following preparation steps were the same as in the previous section. Different sets of microspheres were prepared using two different molecular weight (MW) PCL sources: 14 kDa and 65 kDa.

2.2.3. Preparation and Optimization of Doxycycline and Chondroitin Sulfate (D-CS) Co-Loaded Poly-ε-caprolactone (PCL) Microspheres

In order to provide a regenerative agent to the damaged cartilage tissue while modifying doxycycline release profile, chondroitin sulfate (CS) co-loading to doxycycline-PCL microspheres was also studied. For this purpose, preparation conditions for CS encapsulation into PCL microspheres were firstly optimized in the absence of doxycycline. Single emulsion method was applied in the microsphere preparation using two different aqueous phase composition; PVA (4 %) and PVA-gelatin (1%, each). In the organic phase chloroform was used to dissolve PCL (MW of 14 kDa or 65 kDa). 4-chondroitin sulfate (2 mg powder) was added to the organic phase and mixed homogeneously with vortex for about five minutes. This phase was then added drop-wise to aqueous phase and stirred at 3400 rpm with a homogenizer (IKA-Ultraturrax T-25, Germany) for three hours. The particle size of microspheres was reduced by using homogenizer. Chloroform was evaporated under hood at 1100 rpm overnight. To remove surfactant from microspheres's surfaces, they were washed with distilled water twice and centrifuged at 6000 rpm. After washing steps, the microspheres were left in a vacuum oven (Nüve- EV060) overnight for drying.

Considering the morphology (i.e. shape and surface properties) of chondroitin sulfate (CS) microspheres, 4% PVA, as the aqueous phase and low molecular weight polymer (PCL, 14 kDa) were selected for the preparation of D-CS co-loaded microspheres. Shortly, doxycycline (D) (100 mg) was added into the CS containing polymer phase (PCL, 7% in chloroform) and vortexed to disperse both CS and D homogeneously. This phase was then added drop-wise into 40 ml of aqueous solution and stirred at 3400 rpm with homogenizer (IKA-Ultraturrax T-25) for half an hour. In the following step, the volume of aqueous solution was raised to 75 ml and stirring was continued at 1100 rpm under hood until chloroform was evaporated. The microspheres were washed and dried as described before. They were stored at 4°C in desiccator until use.

2.2.4. Determination of Encapsulation Efficiency

2.2.4.1. Determination of Encapsulation Efficiency of Doxycycline

2.2.4.1.1. Extraction Procedure

In order to determine the amount of doxycycline loaded into microspheres, 15 mg of microspheres were dissolved in tetrahydrofuran (THF)-methanol solution (1/1, v/v). For complete extraction of drug the mixture was stirred at 37°C for 4-5 days and then centrifuged for 20 min at 6000 rpm. Collected supernatant was analyzed by High Performance Liquid Chromatography (HPLC) for determining the doxycycline content of microspheres.

2.2.4.1.2. HPLC Instrumentation and Conditions

All samples were filtered through 0.20 μm filters (PALL, USA) before analysis with a Shimadzu Prominence Model (Japan) HPLC System. The system consisted of a C_{18} column (Inertsil ODS-3, 5 μm, 250x4.6 mm; GL Sciences, Japan), degasser (Shimadzu model DGU-20A3), UV-Vis absorbance detector (Shimadzu model SPD-20A) set at 275 nm, HPLC pump (Shimadzu model LC-20AT), column oven (Shimadzu model CTO-10ASvp), system controller (Shimadzu model CBM-20Alite) and an auto injector (Shimadzu model SIL-10ADvp). The injection volume was 20 μl. The mobile phase was composed of methanol, acetonitrile and THF (50:40:10, v/v). The flow rate was set at 1.0 ml/min.

After determination of the doxycycline contents of extracted samples they were converted to drug amounts using the calibration curve of free drug in same HPLC system/procedure (Appendix A). Percent encapsulation efficiency and drug loading of microspheres were then calculated from the following equations:

$$\%\ Drug\ Loading = \frac{Weight\ of\ Drug\ in\ Microspheres}{Weight\ of\ Microspheres} x100 \quad (2.1)$$

$$\%\ Entrapment\ Efficiency = \frac{\%\ Drug\ Loading}{\%\ Theoretical\ Loading} x100 \quad (2.2)$$

2.2.5. Determination of Drug Release Profiles of Microspheres

The release profiles of D and D-CS from microspheres were determined by incubating 50 mg of microsphere placed in dialysis bags (Mw cutoff: 12 kDa) in phosphate buffered solution (PBS, 0.01 M,pH 7.4) (80 ml for D-CS and 100 ml for D loaded MS). The microspheres were placed in dialysis bags with 2 ml of the release medium. The two ends of dialysis bags were closed with clamps to prevent any microsphere loss to the external medium. The whole setup was placed into shaking water bath (Nüve-ST 402, Turkey) and kept at 37°C throughout the release experiments. Aliquots from the release medium were taken at certain time intervals. Absorbance of the aliquots was measured at 274 nm by using spectrophotometer (Hitachi, U2800A, Japan) and released doxycycline (D) amounts were determined from the calibration curve constructed with different concentrations of the drug (Appendix B). Release studies were done in triplicates and averages for released amounts with standard deviation were represented in the graphs. The PBS solution was refreshed at least once a week.

2.2.6. Particle Size Measurements

Particle size measurements were done with Malvern Mastersizer 2000 at Central Laboratory, METU.

2.2.7. Scanning Electron Microscopy (SEM) Studies

SEM analyses were carried out at SEM Laboratory of Department of Metallurgical and Materials Engineering, METU. In order to provide conductive surfaces suitable for SEM, microspheres were sprinkled onto the copper stub and then coated with gold before examination.

2.2.8. Differential Scanning Calorimetry (DSC) Measurements

The thermal characteristics of PCL microspheres (empty and D loaded) and commercial PCL were measured by using Perkin Elmer Diamond DSC in N_2 atmosphere at Central Laboratory, METU. Two temperature cycles were applied for commercial PCL. First run from -65°C to 100°C (40°C/min) was followed by a cooling step (-10°C/min) allowing to see the crystallization (T_c) and melting (T_m) temperatures.

A second run from -65°C to 100°C (40°C/min) was applied to see the glass transition temperature (T_g). One temperature cycle was applied for microspheres for observing T_m shifts and to calculate crystallinities. The measurements were carried out at a scan rate of 10°C/min between –40°C and 100°C. The melting point was determined at the maximum of the melting endotherm. Crystallinity was calculated assuming proportionality to the experimental heat of fusion using the reported heat of fusion of 139.5 J/g for the 100% crystalline PCL (Estelle´s et al, 2008).

$$X_c(\%) = \frac{\Delta H_m}{136.5} \times 100 \qquad (2.3)$$

X_C = Crystallinity

ΔH_m = Experimental heat of fusion

2.2.9. Fourier Transform Infrared (FTIR) Spectroscopy Measurements

FTIR Spectroscopy was performed using a Bruker FTIR IFS 66/s (Germany) and Alpha with ATR accessories at Central Laboratory, METU. Collection and primary analysis of data, as well as control of the instrument were achieved using OPUS 6.5 software. A total of 50 scans at a resolution of 4 cm^{-1} were averaged for each sample. A straight baseline was obtained in the region of 400-4000 cm^{-1}. Atmospheric compensation was used in measurements. Background correction was done with KBr.

2.2.10. Gel Permeation Chromatography (GPC) Measurements

Gel permeation chromatography was performed to determine the molecular weight distributions of PCL by using Polymer Laboratories PL-GPC 220 at Central Laboratory, METU. GPC Columns were calibrated with polystyrene narrow standards. Tetrahydrofuran (THF) was used as the eluent. Column temperature was maintained at 30°C.

2.2.11. Cell Culture Studies

2.2.11.1. In Vitro Cytotoxicity Studies

The toxicity of the doxycycline and the biocompatibility of microspheres were evaluated with in vitro cytotoxicity tests using 3T3 Swiss albino mouse fibroblast cell line. The 3T3 fibroblast cell line was obtained from Foot-and-Mouth Disease Institute of Ministry of Agriculture and Rural Affairs of Turkey. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM high glucose-glutamine) supplemented with fetal bovine serum (FBS, 10 %, v/v) and penicillin/streptomycin (10 U/ml) at 37°C under humidified atmosphere of 5 % CO_2- 95 % air in incubator (5215, SHEL LAB, USA). The medium was refreshed 2 times a week. When the cells reached 80-90 % confluency, they were passaged in 1:3 ratio after detachment with trypsin-EDTA (0.1 % in PBS)

5, 10, 15 and 20 μg/ml dosage Doxycycline hyclate (D) formulations were prepared in Dulbecco's modified Eagle's medium (DMEM high glucose-glutamine). 4.4 $x10^4$ cells/well 3T3 fibroblasts cells were incubated in the presence of different concentrations of D (0, 5, 10, 15 and 20 μg/ml) for 2 days at 37°C.

Doxycycline loaded and D-CS combination loaded PCL microspheres were sterilized with gamma irradiation at a dose of 25 kGy (Dorati, R. et al, 2005). Irradiation process was performed at Sarayköy Nuclear Research and Education Center (SANAEM) with ISSLEDOVATELJ (Gamma-cell) Co60- γ irradiation source in air at room temperature. The dose rate of the gamma cell was 1.74 KGy/h. Empty and loaded, PCL (14 and 65 kDa) (5mg/well) microspheres were placed in 24 well plates. These wells were then seeded with cells at a density of 4.4 $x10^4$ cells/well and incubated for 2 days at 37°C.

2.2.11.1.1. Microscopic Examinations

After two days of incubations, 3T3 cells were evaluated in terms of morphological changes and degree of attachment by light microscopical examination (Nikon Eclipse TS100, China) and photographed.

2.2.11.1.2. MTT Cell Viability Assay

At the end of 2-days incubation, the density of viable cells was determined with MTT assay. This method is based on reduction of a tetrazolium salt by mitochondrial dehydrogenases to a dark blue formazan product (Mossmann, 1983). The extent to which MTT was reduced to a formazan product has been correlated with the cell viability.

Accordingly, the medium was removed and 500 µl MTT solutions (5 mg/ml in DMEM low glucose) was added to each well and incubated for 4 hours at 37 ^{o}C in dark. After removing MTT solution and washing with PBS, 500 µl dimethyl sulfoxide was (DMSO) added to solubilize the formazan crystals formed inside the cells. The absorbance was measured at 550 nm wavelength using µQuant^{TM} Microplate Spectrophotometer (Biotek Instruments Inc. USA).

Results were demonstrated in terms of percent relative viability, which was normalized by control (no treatment).

2.2.12. Determination of Effectiveness of Microspheres for Treatment of Osteoarthritis in a Three Dimensional Osteoarthritis Model

2.2.12.1 Isolation of Articular Chondrocytes from Rabbits

Newborn and OA developed knee joints of adult male rabbits were used for the isolation of articular chondrocytes For OA development, collagenase type II (Clostridium histolyticum) was injected into two hind leg knee joints of rabbits. Injections were repeated at day 4 and the animals were awaited for 6 weeks under standard conditions for OA development at the injected joints (Kikuchi, 1998). Prior to intraarticular injection, collagenase was dissolved in saline and filtered through a 0.22 µm membrane. For all procedures, rabbits were anaesthetized by using Ketamine hydrochloride (10 %) (35 mg/kg) and Xylazin hydrochloride (2 %) (5 mg/kg) combination.

After terminating the animals, the joints were removed and immersed in ethanol (96 %) for 3 minutes. They were then transferred into RPMI-1640 medium and incubated in carbon dioxide incubator for 4-5 hours. The soft tissues surrounding the joints were scraped off aseptically and cartilage pieces were immersed in 70 % ethanol solution for 1 minute. After treating with collagenase type II (100 Units/ml)

for 16-17 hours at 37°C, the obtained cell suspension was filtered through a sterile 100 µm cell strainer. The filtrate was centrifuged at 2000 rpm for 5 min by centrifuge (HettichZentrifugen, EBA 20, Germany). Viable cell counting was done with trypan blue staining. Chondrocytes were cultured in RPMI-1640 medium supplemented with 10 % foetal calf serum (FCS), 10 Units antibiotic/ml, at 37°C under 5 % CO_2 in an incubator (SL SHEL LAB, Faster, Italy) (Salvat et al, 2005). The growth medium was changed every third day and the chondrocytes were passaged with trypsin-EDTA solution (0.05 %) in a 1:3 ratio. 1st passage chondrocytes were used for cell culture studies.

2.2.12.2. Establishment of Three-Dimensional In Vitro Osteoarthritis Model

For in vitro experiments, a three dimensional (3-D) OA model was developed using chondrocytes of OA induced rabbits. Chondrocytes were embedded in agarose for mimicking the cartilaginous matrix structure. 2% low-melting agarose (gelling temperature 25 ± 5 °C) (Sigma, USA) was prepared in phosphate buffer solution and sterilized by autoclaving (ALP CL-40M, Labomar, Turkey) at 121°C for 20 min. Then, equal volumes of double strength RPMI-1640 containing 20% foetal bovine serum (FBS) and chondrocyte suspension were mixed with 2% agarose to produce a chondrocyte concentration of 10^6 cells/ml in each well of a 24-well tissue culture plate. The plates were awaited at 4°C for 5-10 minutes for gelling of the agarose in wells. 1 ml of RPMI-1640 media with 10 % FBS and 0.1 % streptomycin was then added into each well. The plates were incubated at 37 °C with 5% CO_2 and 95% humidity during the experiment. Medium was refreshed every 3 days. On the third day of the experiment, 20 ng/ml interleukin-1β was added into the medium to mimic the osteoarthritic conditions of synovial fluid. Same amount of IL-1β was added during media changes (Kuroki, K, et al., 2003).

In the in vitro study, four experimental groups were used. First group was positive control; treatment agents were not added into the medium of 3-D construct. Second group, named as negative control; included only 20 ng/ml interleukin-1β. Third and fourth groups were 5 mg D-MS and 5 mg D-CS MS treatment groups, respectively. These groups were also given 20 ng/ml interleukin-1β throughout the experiments. D MS encodes microspheres of PCL (M_W=14kDa) that encapsulated only doxycycline (D). D-CS MS encodes microspheres of same polymer that encapsulated the combination of doxycycline- chondroitin sulfate (D-CS) (Table 2.1).

Microspheres were placed in cell culture inserts during cultivation with the cells to prevent their loss during medium changes (Figure 2.1).

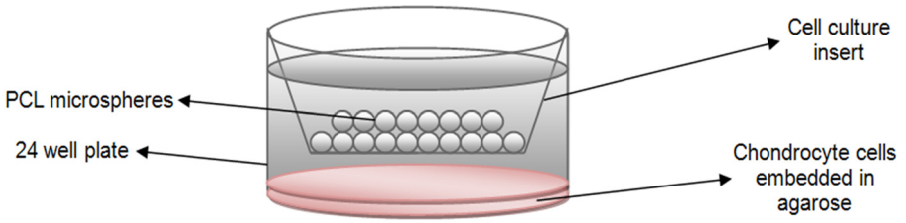


Figure 2.1. Schematic illustration of the system used for evaluation of efficacy of microspheres in 3D in vitro model

Release system effectiveness and establishment of the in vitro disease model were evaluated with GAG and collagen (hydroxyproline) amounts, collagen type II and MMP-13 measurements after incubations for 9, 15 and 24 days. The media was collected and stored at -80°C for MMP-13 analysis and -20°C for other tests. Agarose discs with chondrocytes were digested with papain for GAG and collagen (hydroxyproline) quantitation analyses. DNA amounts of chondrocytes in agarose were determined in papain digests to normalize the results of GAG and collagen amounts of discs. In addition, the amount of drug released from microspheres into the media was determined spectrophotometrically at 341 nm and converted into drug amounts using calibration curve of the drug in same media (Appendix C).

Table 2.1. The treatment groups of microspheres used for *in vitro* experiments

Treatment groups	**Encapsulated Bioactive agent**
D MS	Doxycycline (D)
D-CS MS	Doxycycline – Chondroitin Sulfate (D-CS)

2.2.12.3 Glycosaminoglycan (GAG) Analyses

Total sulfated glycosaminoglycan (sGAG) amounts both in agarose-chondrocyte discs and in mediums were determined by using dimethylmethylene blue (DMMB) assay (Farndalle. et al., 1986). Discs were digested with papain at a concentration of 0.5 mg/ml in 100 mM sodium phosphate buffer/10mM PBE Buffer (pH 6.5 with 10 mM L-cysteine) at 60°C for 20 hours (Ceuninck, F. et al., 2004). 50 µl of the supernatant of the media and papain digests of discs were mixed with 0.25 ml DMMB dye solution (16 µg DMMB/ml in Glycine/NaCl solution, pH 3) in a 48-well plate and the optical densities were determined at 525 nm with a microplate spectrophotometer (BioTek µQuant, USA). sGAG content of the discs was quantified by the calibration curve constructed with chondroitin sulfate from bovine trachea sodium salt type A (0-5 µg) in PBE buffer solution (Appendix D). Total sGAG amounts of discs were normalized with DNA amounts by using fluorimetric measurements as described in Part 2.2.12.5 and reported as GAG/DNA (µg/µg). GAG concentrations of liquid media were reported as µg/ml (Kuroki, K. et al., 2003).

2.2.12.4. Collagen Amount Determination

Collagen amounts in both in agarose-chondrocyte discs and in media were determined by quantitating hydroxyproline (HYP) amounts. The papain digests used in DMMB assay and media were used for hydroxyproline assay (Kuroki, K. et al., 2003). A 25 µl aliquot of medium and papain digest of discs was mixed gently with equal volumes sodium hydroxide (2 N final concentration) and then hydrolyzed by autoclaving. After then, 450 µl Chloramine-T reagent (0.056 M, pH 6.5), which was prepared by 50 % n-propanol and acetate-citrate was added to the hydrolyzate. At room temperature, this hydrolyzate was awaited for 25 minutes. 500 µl Ehrlich's Reagent (1 M) was added to each sample and incubated for 20 minutes at 65°C for color development. Optical densities were read at 550 nm with a spectrophotometer. Calibration curve was done with 4-hydroxyproline standard (Appendix E). (Reddy and Enwemeka, 1996). HYP content of discs were normalized with DNA amounts by using fluorimetric measurements as described in Part 2.2.12.5 and reported as HP/DNA (µg/µg). HP concentrations of liquid media were reported as µg/ml (Kuroki, K. et al., 2003). Collagen content was calculated from HYP:collagen converting factor of 1:8 (Reddy and Enwemeka,. 1996).

2.2.12.5. Fluorimetric Assay for DNA Quantitation

Fluorescent dyes (e.g. Hoechst dye, DAPI, propidium iodide) can bind to DNA to visualize the chromatin of the cells. DNA amount of papain digested discs were determined by Hoechst 33258 dye (Invitrogen) with Modulus Fluorometer. Hoechst 33258 dye excites at 350 nm and emits at 450 nm (Masuoka, et al., 2005). Calf thymus was used as standard to calibrate the Modulus Fluorimeter and determine DNA amounts quantitatively. For determining the DNA amounts, the protocol of Modulus Fluorimeter was used. Hoechst 33258 stock dye (1 mg/ml) was prepared for experiments. TNE buffer stock solution (10X) was prepared with Tris base [Tris (hydroxymethyl) aminomethane], EDTA disodium salt dehydrate and sodium chloride and pH was adjusted to 7.4. For the experiments, (1X) TNE buffer was used for diluting samples. Dye solution (2X) was prepared from stock solution and dilute with TNE (1X) buffer to obtain the concentration of 200 ng/ml. The dye solution and diluted sample was mixed at a 1:1 ratio in methacrylate cuvettes (10 x 10 mm). For calibration, same procedure was followed. The calibration curve constructed with bovine DNA is presented in Appendix F. Diluted standard and dye solution were added at a ratio of 1:1 in cuvettes. The samples were measured according to selected unit from the fluorimetry before calibration.

2.2.12.6. Matrix Metalooproteinase-13 (MMP -13)

MMP-13 concentrations were quantified by enzyme linked immunosorbent assay (ELISA) (Cusabio, USA). The media collected and stored at -80°C were used in MMP-13 assay. The protocols of the kits were used in experiments. All samples were assayed in duplicates. MMP-13 concentrations were measured as ng/ml. Calibration curve was presented in Appendix G.

2.2.13. Animal Studies

2.2.13.1. In Vivo Tests and Establishment of Experimental Osteoarthritis.

In vivo OA was established by collagenase type II (Clostridium histolyticum) (885 units/ mg solid collagen, Sigma) intra-articular injectioṇ into hind knee joints of local albino adult male rabbits (weighing average: 2.5 ± 3.5 kg). Animals were housed individually and maintained in accordance with the Helsinki Animal Rights and Turkish Veterinary Medicine Deontology Regulations (6343/2). The approval of

studying on the animals was obtained from the Animal Care and Ethics Committee of the Middle East Technical University.

Rabbits were anesthetized with intramuscular injection of 10 % Ketamine hydrochloride (35 mg/kg) and 2 % Xylazin hydrochloride (5 mg/kg). Collagenase type II was dissolved in saline (4 mg/ml) and filtered through a 0.22 mm membrane for sterilization before the intraarticular injections. After the knee joint was shaved, 0.5 ml collagenase solution was injected into each joint transcutaneously by an orthopedic surgeon experienced in intraarticular injections. The injection was performed at day 1 and repeated at day 4 as suggested in the literature (Kikuchi, et al., 1998) for establishment of OA. Cartilage degeneration was determined at X-ray radiographs obtained on weeks 3 and 6 after collagenase injections. The knee joints of rabbits were dissected at week 6 after the initiation of the injections to evaluate the development of OA. The evaluations were done by radiography, hardness (indentation testing) and histology.

After establishment of OA, rabbits were divided into groups as presented in Table 2.2. At 7^{th} week, rabbits were divided into groups. As the first group, healthy rabbits in which OA was not developed and no medication was given were used for comparison of the results. The second group presented in the table had osteoarthritic rabbit. This rabbit did not receive any treatment and established as the negative control group. These rabbits were terminated at 8^{th} week. Third and fouth groups were hyaluronan (25mg/ml, Adant, Tedej Meiji, Spain) injection groups. At 7^{th} week after OA establishment, 0.5 ml hyaluronan was injected into joints and these rabbits were terminated at 4^{th} and 8^{th} weeks. The other groups of rabbits were injected with microspheres (mentioned in Table 2.1) together with hyaluronan (0.5 ml) at 7^{th} week after OA establishment. Microsphere treatments were used in two different dosage forms; 5 mg and 7.5 mg. The animals were terminated at 8^{th} week after the initiation of the treatments and the joints were harvested for radiography, hardness (indentation testing) and histological evaluations.

Table 2.2. In vivo experiment groups

Group	Groups	Time (week)
1	Healthy joint (positive control)	-
2	OA no treatment	8
3	OA HYL treatment	4
4	OA HYL treatment	8
5	OA 5 mg D MS injection	8
6	OA 7.5 mg D MS injection	8
7	OA 5 mg D-CS MS injection	8
8	OA 7.5 mg D-CS MS injection	8

2.2.13.2. Histological Evaluations

2.2.13.2.1. Light microscopy

Histological analysis was performed in Hacettepe University Faculty of Medicine Department of Histology and Embryology. The joint specimens were removed and immersed in 10 % neutral formalin solution (pH 7.0) at room temperature. All specimens were decalcified in De Castro solution (chloral hydrate, nitric acid, distilled water) and embedded in paraffin by using an automated tissue processor with vacuum. Five to six micrometer thick sections were stained with hematoxylin & eosin (HE), Masson's trichrome (MT) and safranin O (SO). Photomicrographs of each sample were generated by a light microscope (Leica, DMR) attached computerized digital camera (Model DFC 480, Leica Westlar Germany). Both Mankin and the OARSI OA cartilage histopathology scoring systems were used (1, 2). The subcategories of the Mankin score are structure, tidemark integrity, proteoglycan staining, and the cellularity. The OARSI score is the multiplication of the grade and the stage of each sample (Tables 2.3, 2.4, 2.5). Grade is an index for the OA depth progression (severity). Stage is defined as the horizontal extent of cartilage involvement within one side of a joint compartment irrespective of the underlying grade. Score is defined as assessment of combined OA grade and OA stage. All the samples were scored by 2 blinded observers (PK and EB) and the averages were used.

Table 2.3. Mankin's Cartilage Degeneration Histology Scale (Farshid, et al, 2009)

Structure	**Score**
Normal	0
Surface irregularities	1
Pannus	2
Cleft to transitional zone	3
Cleft to radial zone	4
Cleft to calcified zone	5
Complete disorganization	6
Tidemark integrity	
Intact	0
Crossed by blood vessels	1
Proteoglycan staining	
Normal	0
Slight reduction	1
Moderate reduction	2
Severe reduction	3
No dye noted	4
Cellularity	
Normal	0
Diffuse hypercellularity	1
Cloning	2
Hypocellularity	3

Table 2.4. Pritzker's OARSI Cartilage Degeneration Histology Scale (Pritzker, et al, 2006; Farshid, et al, 2009)

Grade 0:
surface intact,
Cartilage morphology intact
matrix structure is normal
intact, uninvolved cartilage
Grade 1:
surface intact,
Matrix:superficial zone intact, edema and/or fibrillation
Cells: hypertrophy death, proliferation (clusters)
Reaction must be more than superficial fibrillation only
Grade 2:
surface discontinuity
Matrix: Discontinuity at superficial zone
Cationic stain matrix depletion (Safranin O or Toluidine Blue) upper 1/3 of
Medium zone increased focal pericondrial staining
Disorientation of chondron columns
Cells: hypertrophy death, proliferation (clusters)
Grade 3:
vertical fissures
Vertical clefts on matrix through midzone, branced fissures
Cationic stain depletion (Safranin O or Toluidine Blue) into lower 2/3 of cartilage
New collagen formation (polarized light microscopy, Picro Sirius Red stain)
Cells: hypertrophy death, proliferation (clusters)
Grade 4:
erosion
Superficial zone delamination, Cartilage matrix loss, cyst formation within cartilage
Mid zone excavation
Grade 5:
denudation
Surface is sclerotic bone or reparative tissue including fibrocartilage
Limited repair of microbrokes on bone surface
Grade 6:
deformation
Bone remodelling Deformation of articular surface contour (more than osteophyte
Joint margin and central osteophytes ; Includes: microfracture and repair

Table 2.5. OA cartilage histopathology-stage assessment (Stage=extent of joint movement) (Pritzker, et al, 2006)

Stage	% involvement (surface, area, volume)
0	No OA activity seen
1	<10 %
2	10-25 %
3	25-50 %
4	> 50 %

Table 2.6. OA score-semi quantitative method (score=grade x stage) (Pritzker, et al, 2006)

Grade	Stage			
	S1	S2	S3	S4
G1	1	2	3	4
G2	2	4	6	8
G3	3	6	9	12
G4	4	8	12	16
G5	5	10	15	20
G6	6	12	18	24

2.2.13.3. Radiological Evaluations

The animals were dissected at predetermined period after the initiation of the treatments and the same joints were harvested for radiographic and histological evaluations. X-ray pictures of knee joints were obtained at METU Medical Center with the Siemens Multix-C X-Ray Machine. The Agfa Cruix films were used in this application. The distance between X-Ray source and knee joints was 100 cm. The X-Ray machine was calibrated into 3.2 mA/s and 42KW. Radiological scoring was applied to see the levels of radiological values of OA. These joints were scored by two blinded orthopedic surgeons by using method of Kellgren-Lawrence according to scale given in Table 2.7.

Table 2.7. Kellgren-Lawrence radiographic grading scale of osteoarthritis of the tibiofemoral joint (Kijowski et al, 2006).

Grade of OA	Description
0	No radiographic findings of OA
1	Minute osteophytes of doubtful clinical significance
2	Definite osteophytes with umimpaired joint space
3	Definite osteophytes with moderate joint space narrowing
4	Definite osteophytes with severe joint space narrowing and subchondral sclerosis

2.2.13.4. Hardness Testing (Indentation)

Hardness of the medial and lateral femoral condyle was measured using a hand-held, self-contained material testing device, called durometer (Kori Seiki, Japan) immediately after termination at room temperature according to ASTM D2240 standards (Niederauer, et al., 2004). A scale from 0 to 99 was used. On this scale low values represented softened cartilage. Three measurements were made at each condyle and the average was calculated.

The hardness tester is known as durometer, and its measuring principle is mainly based on Rockwell Hardness. For rubbery and plastic materials, the used instrument has been applied according to various different standards such as ASTM D-2240, JIS K-7312. In relation to the measuring stiffness, the probe geometry was changed from conical to round and flat. The measured hardness is given names as Shored A Hardness, Shored D Hardness, etc. For harder samples, the used probe should be like in Figure 2.2.

Figure 2.2. Hardness tester (Durometer)

The measured points of the cartilage of the joint is demonstrated in Figure 2.3. Lancet points the medial and lateral points of the cartilage where the indentor measured hardness.

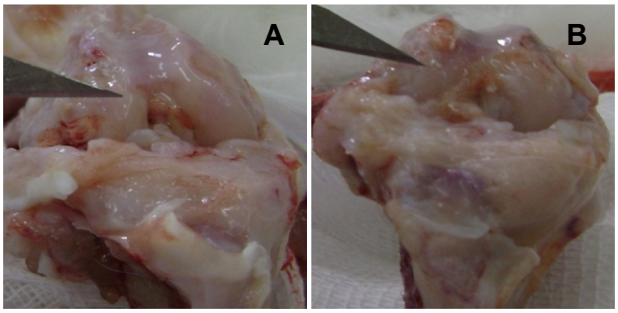


Figure 2.3. Location of the indentation test zones. Lancet points the a) medial b) lateral view of the articular joint

In Figure 2.4., measuring cartilage stiffness with the indentation method is shown. The probe of the durometer comes in contact with the measured area of the cartilage.

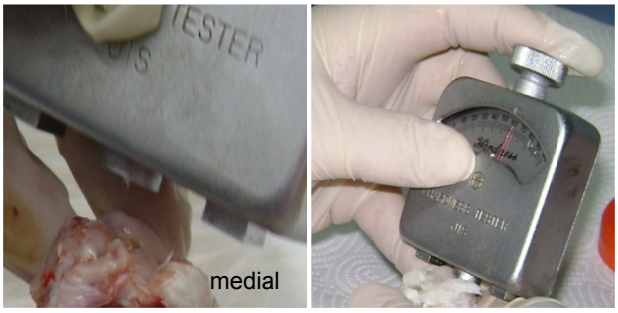


Figure 2.4.Measuring of indentation value by durometer

2.2.14. Statistical Analysis

2.2.14.1 Evaluation of In Vitro Experiments

Statistical comparison was performed among groups at each collection time by One-Way ANOVA and Tukey's Multiple Comparison Tests. Within each groups, Nonparametric Mann-Whitney U test was applied using SPSS-9 Statistical Software (SPSS Inc, USA). Differences between groups were considered to be significant at $p<0.05$ level.

2.2.14.2. Evaluation of In Vivo Experiments

Independent variables were the groups and the dependent variables were the histology and radiology test parameters. The normality of distribution and the homogeneity of variances of the sample were established using the Shapiro-Wilk test. All parameters were analyzed by nonoparametric tests Kruskal-Wallis was used for multiple comparison and Dunn as post-hoc test. Correlation between the histology and radiology measurements was assessed by using Spearman test. Friedman test and its posthoc test were used to assess the difference in time. Descriptive statistical values were expressed as median, minimum and maximum. The difference was considered significant if $p<0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Optimization of Empty Poly-ε-caprolactone (PCL) Microspheres Prepared with Low Molecular Weight PCL (Mw=14 kDa)

In optimization studies, empty microspheres were prepared using PCL with 14 kDa molecular weight. Different concentrations of PVA solutions (0.5, 1, 2, 4 and 6 %, w/v) were used. Scanning electron microscopy was used to examine the morphology of the particles formed. In Figure 3.1.a and b, SEM images of microspheres prepared in 0.5 % PVA solution and 1 % PVA solutions are shown. Accordingly, 0.5 % PVA solution did not provide a suitable aqueous environment for preparing microspheres, but spherical structures were obtained using 1 % PVA solution. However, the particle size distribution of these microspheres (Fig 3.1.a and b) was not homogeneous and their surfaces were quite rough. These properties could further be improved by increasing the PVA concentration. As shown in SEM images in Figure 3.1.c and d, smoother spherical structures that have diameters ranging between 5-100 μm could be obtained by 4 % and 6 % w/v PVA solutions. The former group of microspheres was more homogeneous and better in terms of contours of the spheres than the other. SEM analysis of the microspheres also showed that by using low concentrations of emulsifiers (2 % PVA solution) larger sized particles were obtained than the high concentration cases.

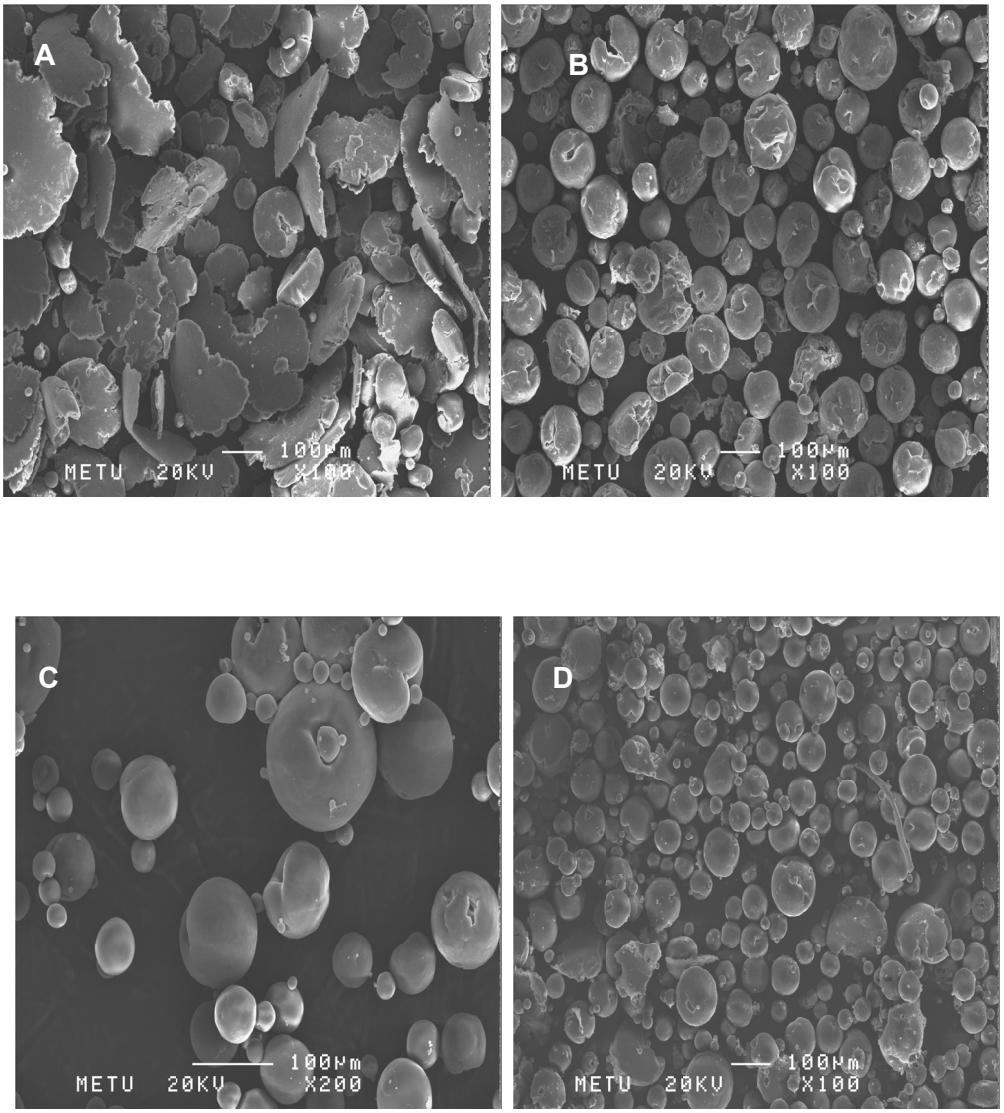


Figure 3.1. SEM images of empty PCL (14 kDa) microspheres prepared with different PVA concentrations a) 0.5 % w/v PVA b) 1 % w/v PVA c) 4 % w/v PV d) 6 % w/v PVA

The effect of emulsion stabilizer on size of microspheres was also demonstrated in the literature by other researchers. Smaller microspheres were obtained when PVA was used in preparation of microspheres with the same polymer (PCL) (Kim, et al, 2005). The effect of PVA on particle size was mainly due to the amphiphilic behavior of the surfactant (PVA). The molecules of PVA alienated at the droplet surface to stabilize the droplets by decreasing the free energy at the interface of two phases and preventing the coalescence and flocculation of microspheres. This results in

stabilization of microsphere droplets at smaller sizes. Besides that, when the concentration of PVA increases the viscosity of the aqueous phase increases which also prevents coalesce and forms a stable emulsion with uniform droplets. On the contrary, it was shown that low viscosity of PVA allowed microspheres flocculate and form larger particles (Shukla, et al, 2007). In other words, external phase would prevent coalescence of droplets at high concentrations of PVA (Aishwarya, et al. 2008).

Aishwarya (2008) used different PVA concentrations from 1 to 5 %, w/v for preparing doxycycline loaded PCL microspheres and observed similar effect of PVA concentration with the present study. In another study, when the concentration of PVA was changed from 0.25 to 2.0 %, microsphere sizes also decreased (Vivek, et al., 2007).

It is known that during microparticle formation, PVA stabilizes the particle surface by penetrating between polymer molecules. PVA is a hydrophilic polymer and high PVA concentration might have caused high amounts of residual PVA. Even if PVA is removed from the system via washing microspheres, at least 8-12 % of PVA still remains on the microspheres (Vivek, et al., 2007). This property limits the usage of PVA. Therefore, during optimization studies, the concentration of PVA was not further increased but gelatin was added to the aqueous phase to obtain more viscous emulsion.

In order to analyze the effect of gelatin, PVA concentration was kept same and gelatin concentration was increased from 1 to 3 % w/v. When gelatin concentration was increased, evaporating organic phase became much more difficult at low temperatures due to gelation below 35-40°C. Considering the possible adverse effects of temperature on doxycycline bioactivity, the optimum temperature was selected as 37°C. So, it was not possible to use high gelatin concentration for aqueous phase.

The properties of aqueous solution were not the only parameter in controlling microsphere size distribution and morphology for further optimization of microspheres. Stirring speed was changed and formed to have effect on particle size as it gives energy to disperse the organic phase more in water. To avoid excessive foaming at high stirring rates, the stirring rate was reduced from 1100 rpm to 500 rpm in preparing microspheres. Microsphere sizes decreased with increasing

stirring rate since increased stirring results in the formation of finer emulsions by breaking up into smaller droplets. (Aishwarya, et al., 2008; Wang, et al, 2008a).

SEM images of PCL microspheres prepared in PVA-gelatin media at different evaporation temperatures are shown In Figure 3.2. Effect of evaporation temperatures was also evaluated; at 37°C, the shape and size distribution was more homogeneous (Figure 3.2.a) than those observed at 45°C (Fig. 3.2.b) Increase in gelatin concentration decreased the homogeneity and particle size of the microspheres (Fig. 3.2.c). Stable, uniform and spherical microspheres were obtained when gelatin concentration was 1 % w/v (Figure 3.2.a).

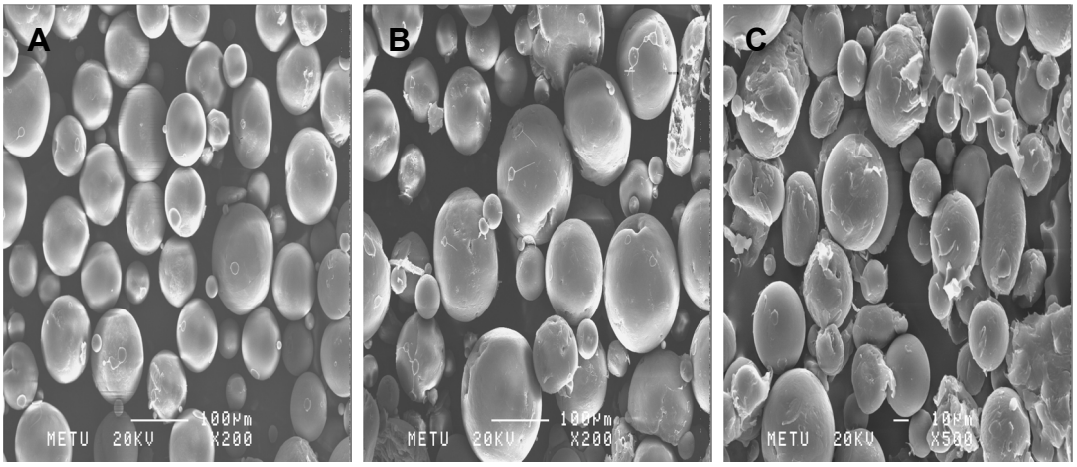


Figure 3.2. SEM images of PCL (14 kDa) microspheres prepared in different aqueous media and at different evaporation temperatures a) at 37 °C; % PVA/ 1 % gelatin b) at 45 °C; 1 % PVA / 1 % gelatin c) at 37 °C; 1 % PVA / 2 % gelatin

It can be clearly seen that PCL microspheres prepared with different concentrations of PVA-gelatin combination appeared to have more spherical contour and smooth, less porous surface. The effect of gelatin concentration was investigated by various gelatin concentrations with stable PVA (1 %) concentration. The increased gelatin concentration decreased the particle size of the spheres as seen in Figure 3.2. Although increased concentration of gelatin produced more stable microspheres, the rough microspheres and aggregated gelatin around the microspheres were noticed (Park, et al, 2006). On the contrary, the use of gelatin protected microspheres from agglomeration during microsphere preparation. Stable and undistorted microspheres were prepared when the gelatin concentration did not exceed 1 % w/v (Park, et al, 2006).

The particle size measurement results supported the SEM outcomes. The higher the concentration of PVA solution, the lower the mean particle size of the microspheres were obtained (Wei, et al, 2004; Bolourrtchian et al, 2005; Kemala, et al., 2010). The microspheres prepared with 2 % PVA solution were found to have mean particle size of 100 μm (Figure 3.3.a) and 90 % of microspheres were smaller than 250 μm. Those prepared with 4% PVA solution; however had mean particle size distribution of 57 μm, while 90 % of this group was smaller than 157 μm (Figure 3.3.b). At the highest concentration (6 % PVA solution), microspheres had particle size distribution with a mean of 32 μm and 79.6 μm size of 90^{th} percentile (Figure 3.3.c).

Particle size distribution of microspheres prepared by PVA-gelatin (1 %, each) solution (Figure 3.3.d) was demonstrated to have a mean of 84 μm and 90^{th} percentile value of 140 μm. Additionally in 1 % (PVA-gelatin) media, gelatin was shown to improve the uniformity and decreased the width of the peak .

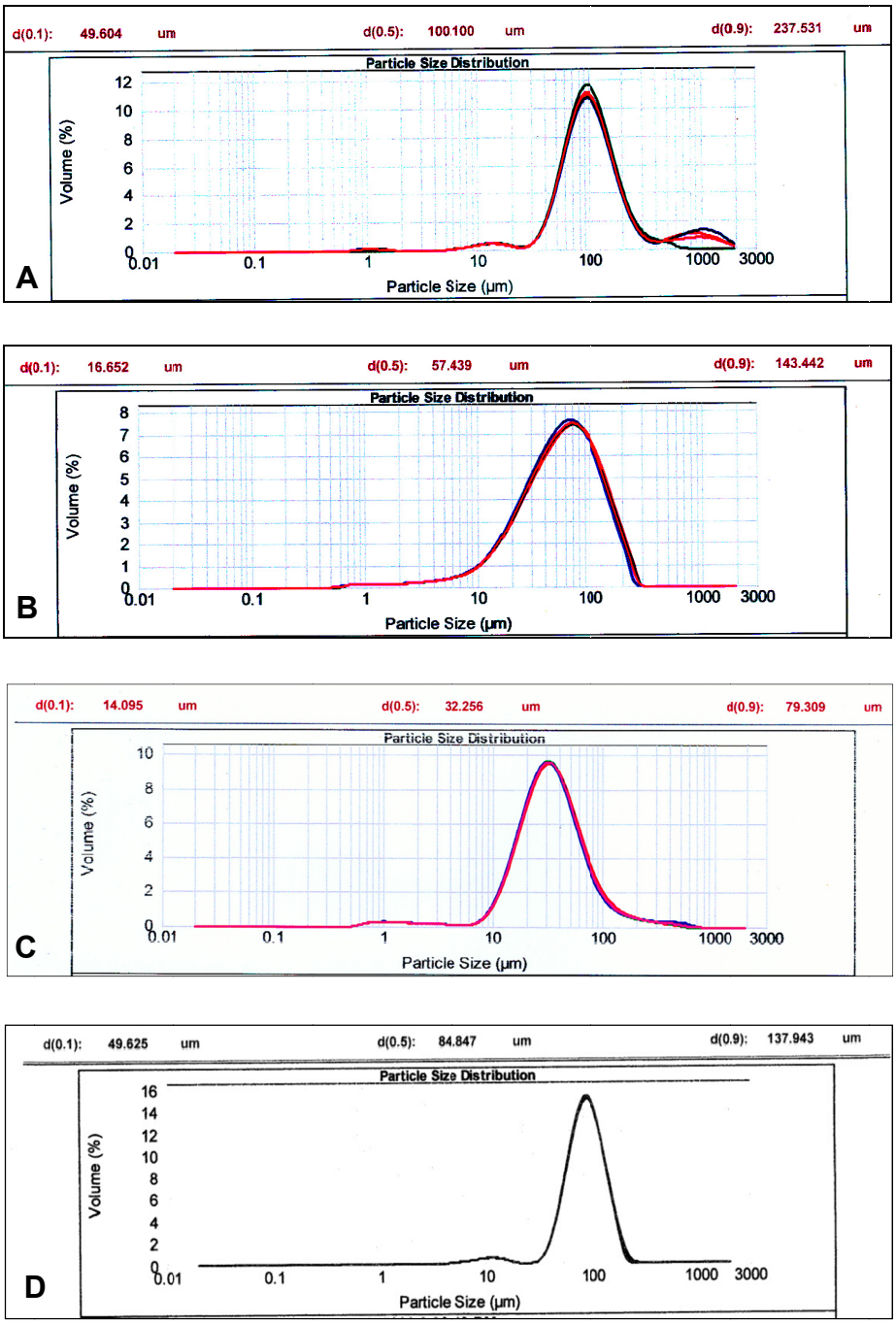


Figure 3.3. Particle size distribution of PCL microspheres prepared by A) 2 % PVA B) 4 % PVA C) 6 % PVA D) PVA-gelatin (1 %, each) solution

According to particle size and SEM results, use of 4 % w/v PVA solution in microsphere preparation was decided to be appropriate in terms of size, shape and morphology of microspheres for intraarticular applications. Below 4 % w/v, the microparticles were too fragile and had tendency to aggregate to each other. Above 4 % w/v, some of the spheres disintegrated into polymer particulates. The uniform size distribution and homogeneity of microparticles were only seen in 4 % w/v of PVA solution. It was also indicated that 4 % PVA concentration enhanced the microparticle recovery when preparing doxycycline loaded PCL microspheres (Aishwarya, et al. 2008).

SEM images confirmed the uniformity of microspheres prepared with 4 % w/v of PVA solution. According to particle size distribution analysis, microspheres prepared at 4 % w/v PVA solution was found to be the first appropriate group for release studies as they have more uniform and better spherical contour. Second appropriate group was PVA-gelatin (1% w/v, each) combination. Indomethacin loaded PCL microspheres were also prepared with PVA-gelatin combination to obtain the desired spherical form (Park, et al, 2006).

The doxycycline loaded microspheres were prepared with these selected groups and the release studies of these microspheres were evaluated.

3.2. Results of Doxycycline Loaded PCL (Mw=14 kDa)

In Figure 3.4, the particle sizes of doxycycline loaded microspheres are shown. Microspheres prepared with 4% PVA solution were found to have mean particle size of approximately 74 μm and the 90 % of microspheres were smaller than 152 μm. Those prepared with 1% PVA-gelatin solution; however had mean particle size distribution of approximately 90 μm, with 90 % of the group having sizes smaller than 149 μm.

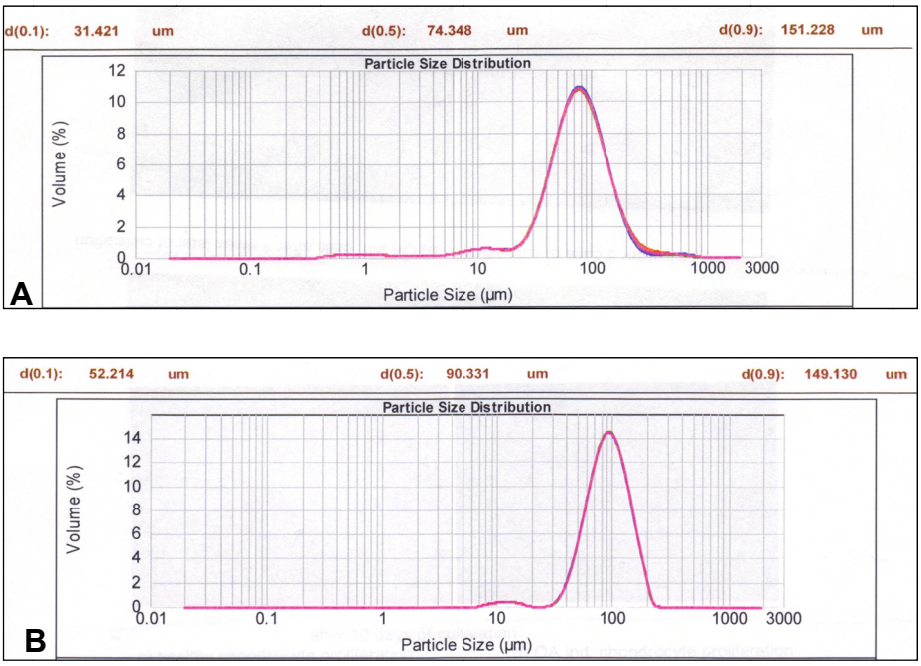


Figure 3.4. Particle size distribution of doxycyline loaded microspheres prepared by A) 4 % PVA B) PVA-gelatin(1 %, each) solution

The surface morphology of doxycycline loaded microspheres was evaluated by SEM studies (Figures 3.5 and 3.6). Doxycycline lodeded PCL (14 kDa) microspheres prepared by PVA-gelatin (1 %, each) was more spherical than microspheres prepared by PVA (4 %). However, size distribution of microspheres prepared by PVA (4 %) was more homogeneous than microspheres prepared by PVA-gelatin (1 %, each). Microspheres prepared by PVA-gelatin (1 %, each) had less porous and smoother surface than those prepared by PVA (4 %) only.

The doxycycline loaded microspheres were also less porous and coarser than empty ones. The coarseness and porosity were suggested to be due to solvent removal during processing (Dash, 1997; Wang, et al, 2008a). The cross-section of disodium norcantharide (DSNC) loaded PCL microspheres were highly porous when compared with empty ones. The differences in morphology were considered as an outcome of physicochemical structure of the drugs. It has been reported that if the drug is in salt form, it can cause osmotic pressure inside the microsphere and a phase separation could be obtained during polymer coacervation (Weidenauer et al, 2003; Wang, et al, 2008a). The best proof of this hypothesis was obtained when

encapsulating NaCl rather than drug. NaCl encapsulated microspheres had coarse surface and porous structure. However, when microspheres were encapsulated with 5-fluorouracil or other model drugs in non-salt form such as norcantharidin, (the acid anhydride form of DSNC), they had smooth surface and less porous structure (Wang, et al, 2008a). In this study, the main reason of obtaining less-porous doxycycline loaded microspheres was the chemical structure of the doxycycline. The other factor that contributes to smooth structure of microspheres was the evaporation rate of the organic solvent. Researchers indicated that low evaporation rates resulted in low porosity on the surface of the spheres (Izumikawa et al, 1991; Chung et al, 2001; Freiberg, and Zhu, 2004).

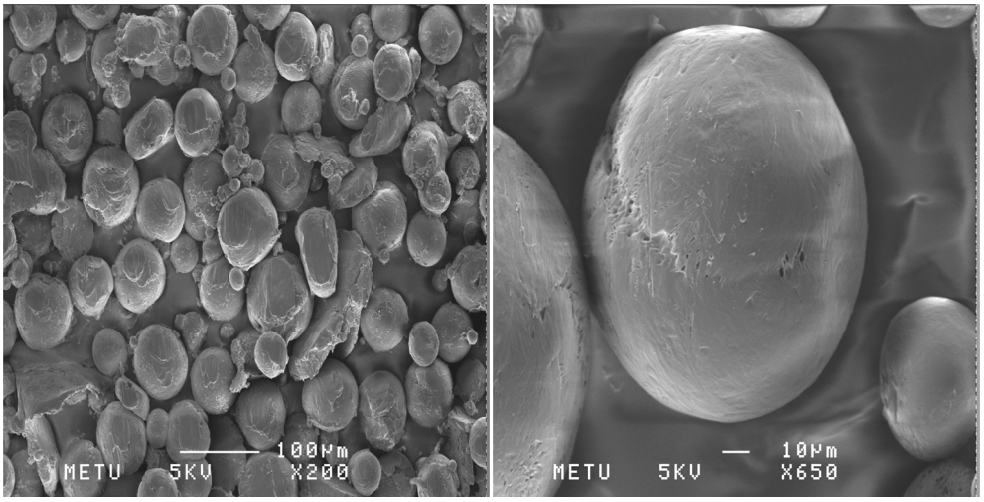


Figure 3.5. SEM images of doxycycline loaded PCL (14 kDa) microspheres prepared by PVA (4 %)

When examined at a magnification of x650, the surface of the microspheres was smooth (Figs 3.5 and 3.6).

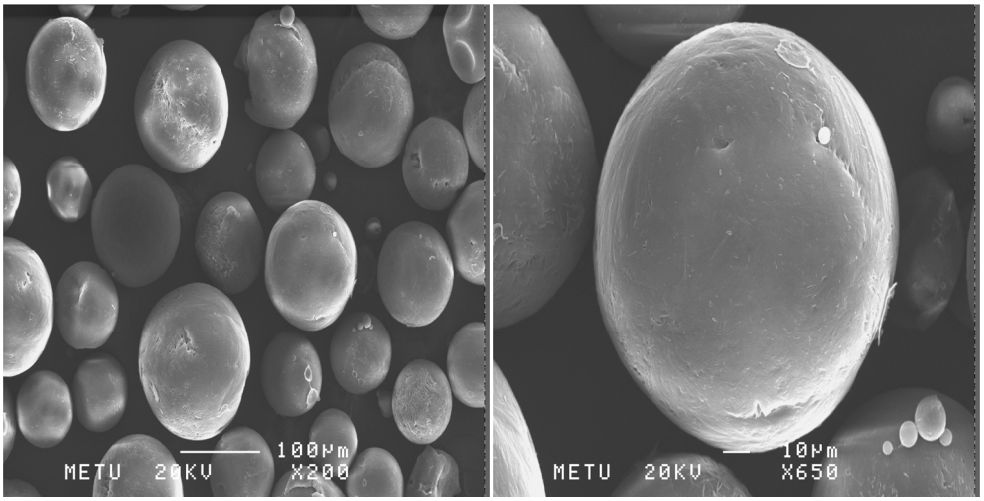


Figure 3.6. SEM images of doxycyline loaded PCL (14 kDa) microspheres prepared by PVA-gelatin (1 %, each)

Doxycycline loaded PCL microspheres were larger than empty PCL microspheres. Zalfen, A.M. et al (2008) compared the empty and levonorgestrel loaded PCL microspheres and observed that empty microspheres were smaller than levonorgestrel loaded microspheres. The mean particle size of PCL microspheres prepared by PVA (4 %) was slightly smaller than microspheres prepared by PVA-gelatin (1 %, each).

3.3. Optimization of Empty Poly-ε-caprolactone (PCL) Microspheres Prepared with High Molecular Weight PCL (Mw=65 kDa)

Several emulsifying agents and concentrations were applied as done in previous parts.The selected emulsifying agents and concentrations for PCL (14 kDa) were used in preparing microspheres of high molecular weight PCL (65 kDa).

SEM images of PCL (65 kDa) microspheres are presented in Figure 2.7. In this figure, microsphere surfaces were rough and irregular. Also, their size distribution was quite non-homogeneous. Some of the microspheres did not have perfect spherical shapes as observed in previous microspheres prepared with low molecular weight PCL (14 kDa).

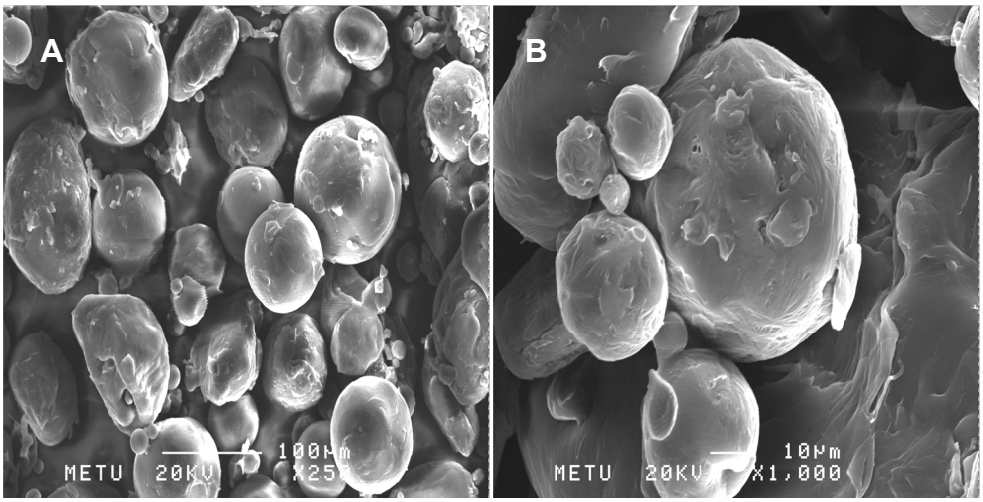


Figure 3.7. SEM images of PCL (65 kDa) empty microspheres a) Microspheres PVA-gelatin (1 %, each) at 37°C b) Microspheres PVA (4 %)

Particle size distribution of these microspheres was shown in Figure 3.8. Microspheres prepared with 4% PVA solution were found to have mean particle size of 104 µm and 90 % of these microspheres were smaller than 168 µm. Those prepared with 1% PVA-gelatin aqueous media; had similar mean particle size value (106 µm). However, 90th percentile value was greater (224 µm).

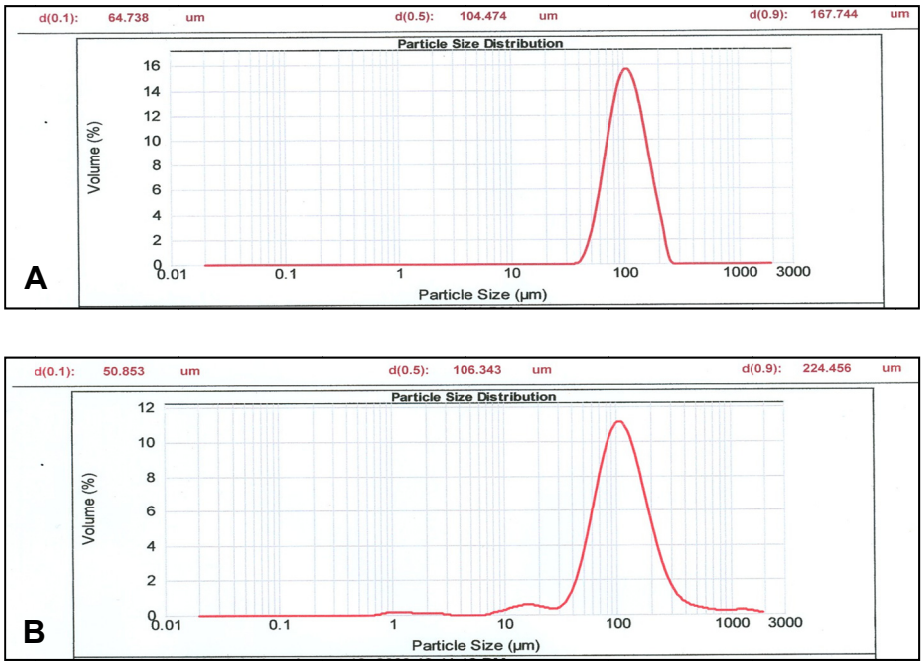


Figure 3.8. Particle size distribution of PCL (65 kDa) microspheres prepared by A) 4 % PVA B) PVA-gelatin (1 %, each) solution

3.4. Results of Doxycycline Loaded PCL (Mw=65 kDa)

The particle sizes of doxycycline loaded PCL (65 kDa) microspheres are shown in Figure 3.9. Microspheres prepared with 4% PVA solution were found to have mean particle size of 94 µm and the 90 % of microspheres were smaller than 370 µm. Those prepared with 1% PVA-gelatin solution; however had mean particle size distribution of 122 µm, with 90 % of the group having sizes smaller than 341µm. Also the later group had a wider distribution indicating a large range of particle size.

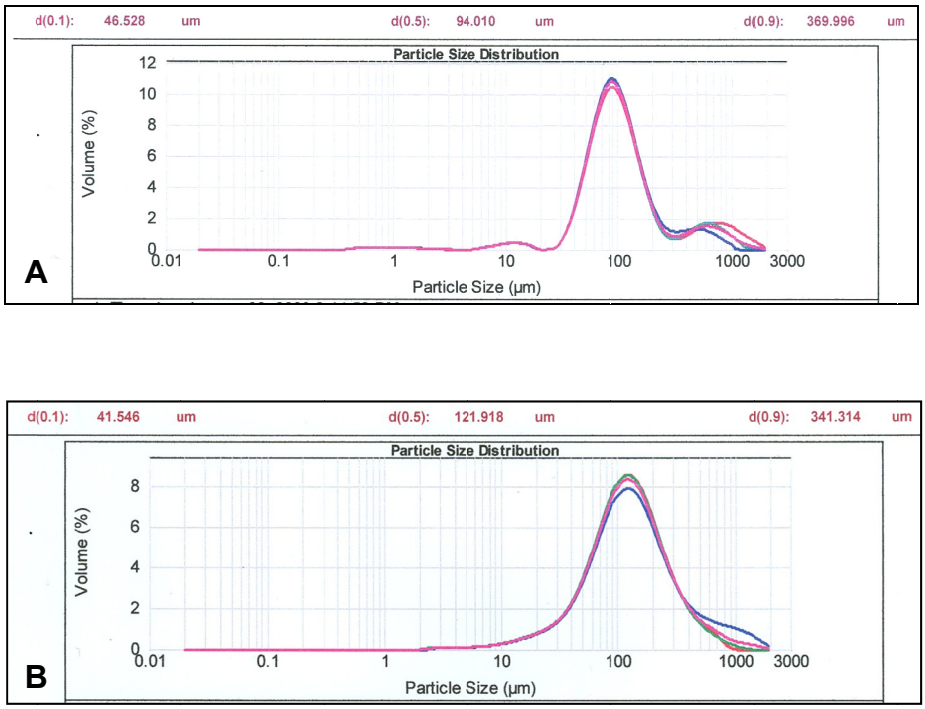


Figure 3.9. Particle size distribution of doxycycline loaded PCL (65 kDa) microspheres prepared by A) 4 % PVA solution B) PVA-gelatin(1 %, each) solution

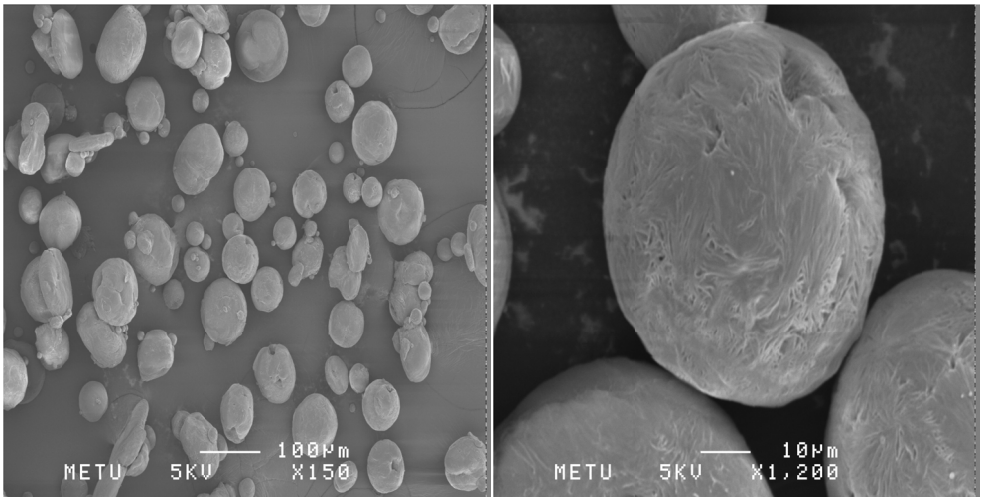


Figure 3.10. SEM images of doxycyline loaded PCL (65 kDa microspheres prepared by PVA (4 %)

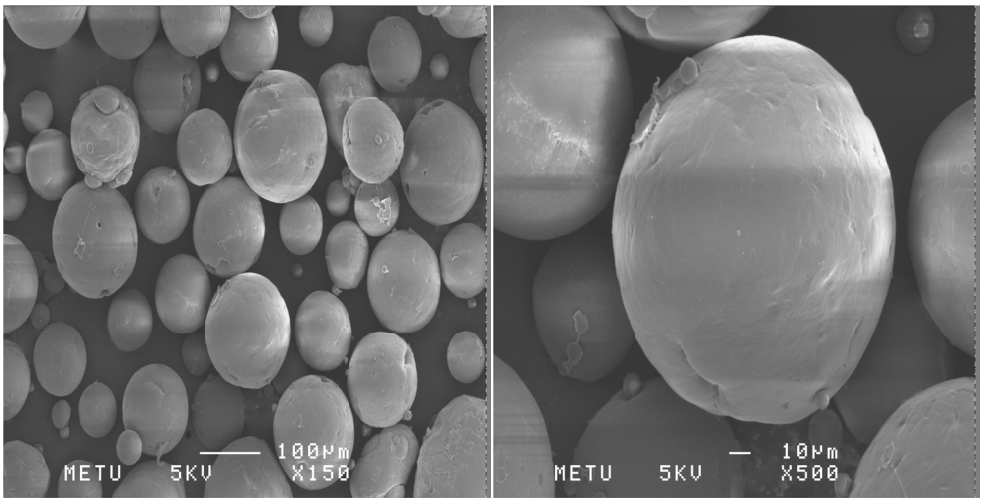


Figure 3.11. SEM images of doxycyline loaded PCL (65 kDa) microspheres prepared by PVA-gelatin (1 %, each)

In Figures 3.10 and 11, SEM images of doxycycline loaded microspheres prepared with PCL (65 kDa) are shown. Microspheres prepared by PVA-gelatin (1 %, each) were smoother and their distribution was more homogeneous than those prepared

by PVA (4 %). The size of PCL (65 kDa) microspheres prepared by PVA (4 %) was smaller than microspheres prepared by PVA-gelatin (1 %, each).

As seen before in doxycycline loaded and empty PCL (14 kDa) microspheres in Parts 3.1 and 3.2, empty microspheres were smaller than doxycycline loaded microspheres which were prepared with PCL (65 kDa).

The microspheres prepared with high Mw PCL (65 kDa) was larger than those of low Mw PCL (14 kDa). It was indicated that the organic phase viscosity of PCL (65 kDa) was higher than PCL (14 kDa) (Kim, et al 2005). Due to high viscosity of organic phase of PCL (65 kDa), particle size was increased (Hnaien, et al., 2011). The increase in molecular weight increased the particle size of the microspheres as expected (Jeong, et al., 2003).

3.5. Optimization of Doxycycline and Chondroitin Sulfate Co-Loaded Poly-ε-caprolactone (PCL) Microspheres

3.5.1. Preparation of Chondroitin Sulfate Loaded Microspheres

Chondroitin sulfate loaded PCL (Mw of 14 kDa and 65 kDa) microspheres were prepared using PVA (4 %) and PVA-gelatin (1%, each) and two different molecular weight PCLs. Homogenization was used to set the o/w emulsion before CS diffusion into the aqueous phase. This step was needed due to very high reactivity of CS with water. Since high shear was created during homogenization, the resultant emulsion droplets, thus, microspheres had much smaller particle sizes than those produced by magnetic agitation (O'Donnell, and McGinity, 1997).

The micrographs of the resulting microspheres demonstrated that their structure was not perfectly spherical but particles had quite homogenous size distribution (Figure 3.12a and b). SEM examination also showed that polymer particles formed smooth layers underneath the surface (Figure 14 c and d). There was no pores observable on their surfaces at 3000-4000 magnifications. The high stirring rate during homogenization decreased the size of the droplets and pores. The mean diameter of this set of microspheres was approximately 10 μm (Figure 3.12.b).

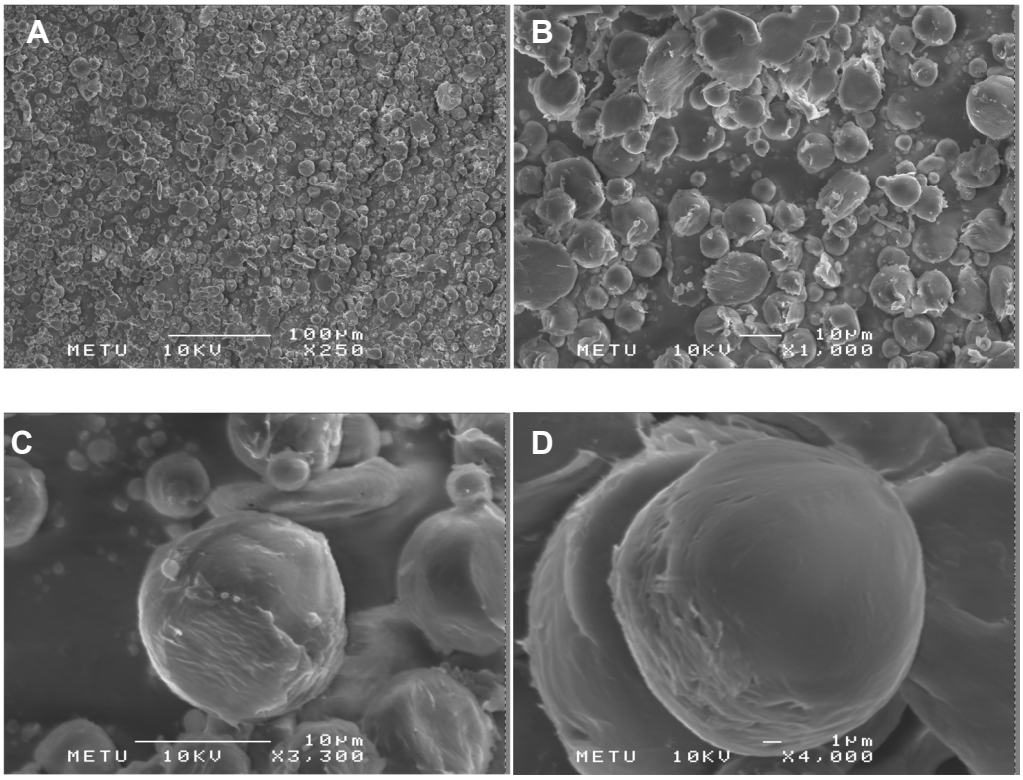


Figure 3.12. SEM micrographs of chondroitin sulfate loaded PCL (14 kDa) microspheres prepared by PVA (4 %)

As the second set of CS added microspheres, PVA-gelatin (1%, each) was used in the aqueous phase. The morphological characterization of these microspheres with SEM showed that they had denser microsphere population with more amount of microspheres formed (Figure 3.13.a) than the previous set (Figure 3.12.a). However, these spheres were also observed to adhere to each other and form aggregated masses of microspheres (Figure 3.13.b) despite extensive washing steps after preparation process. These results indicated that, 1% PVA-gelatin combination was not suitable for in vivo experiments as they cannot be injected in this form.

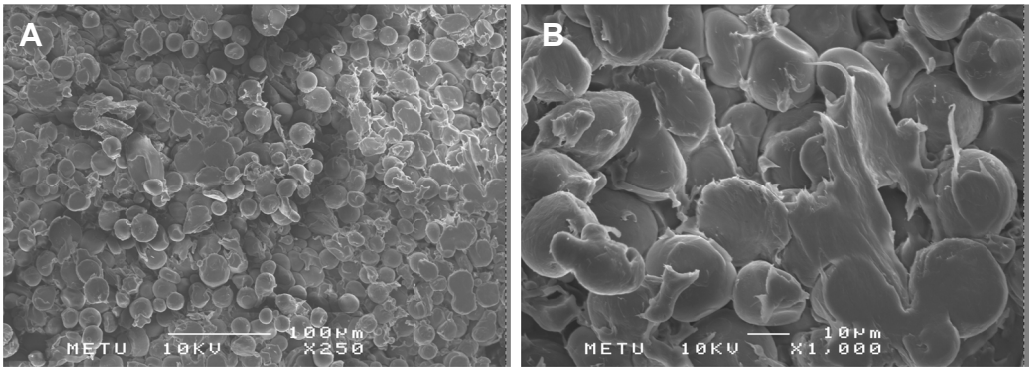


Figure 3.13. SEM micrographs of chondroitin sulfate loaded PCL (14 kDa) microspheres prepared by PVA- gelatin (1 %, each)

Preparation of micropheres was also done with high molecular weight PCL (65 kDa). When 4 % PVA was used in the aqueous phase, a homogenous particle distribution was observed (Figure 3.14.a) with scanning electron microscopy (SEM) images. However, there was a membrane-like layer underneath the microspheres as observed in the prevous sets of chondroitin sulfate loaded microspheres (Figure 16 b). Microspheres were either fused or attached to each other within this layer. Finally, it was concluded that high molecular weight PCL (65 kDa) was found not suitable for preparing chondroitin sulfate loaded PCL microspheres.

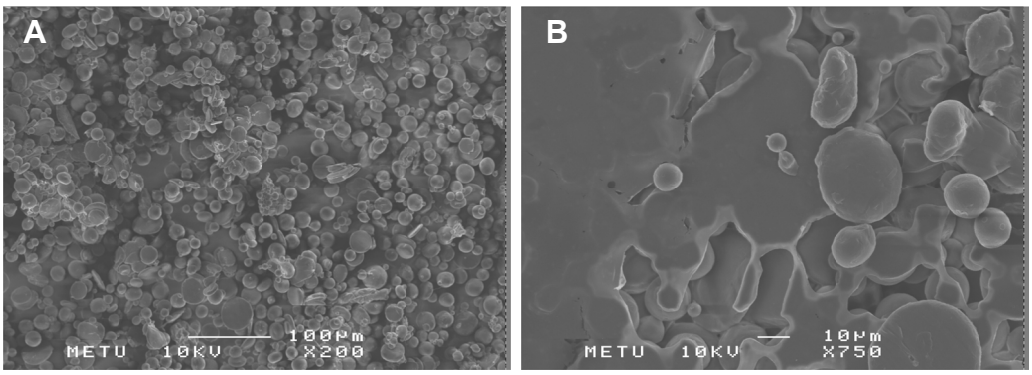


Figure 3.14. SEM micrographs of chondroitin sulfate loaded PCL (65 kDa) microspheres prepared by 4 % PVA

As a result, 4 % PVA as surfactant and low molecular weight of PCL (14 kDa) were selected for the preparation of doxycycline-chondroitin sulfate co-loaded microspheres.

3.5.2. Preparation of Doxycyline and Chondroitin Sulfate Co-Loaded Microspheres

From the results of D and CS-PCL microsphere optimization studies, 4 % of PVA concentration, low molecular weight (14 kDa) PCL and stirring at 3400 rpm with a homogenizer (IKA-Ultraturrax T-25) for three hours were selected for formation of doxycycline-chondroitin sulfate (D-CS) co-loaded microspheres.

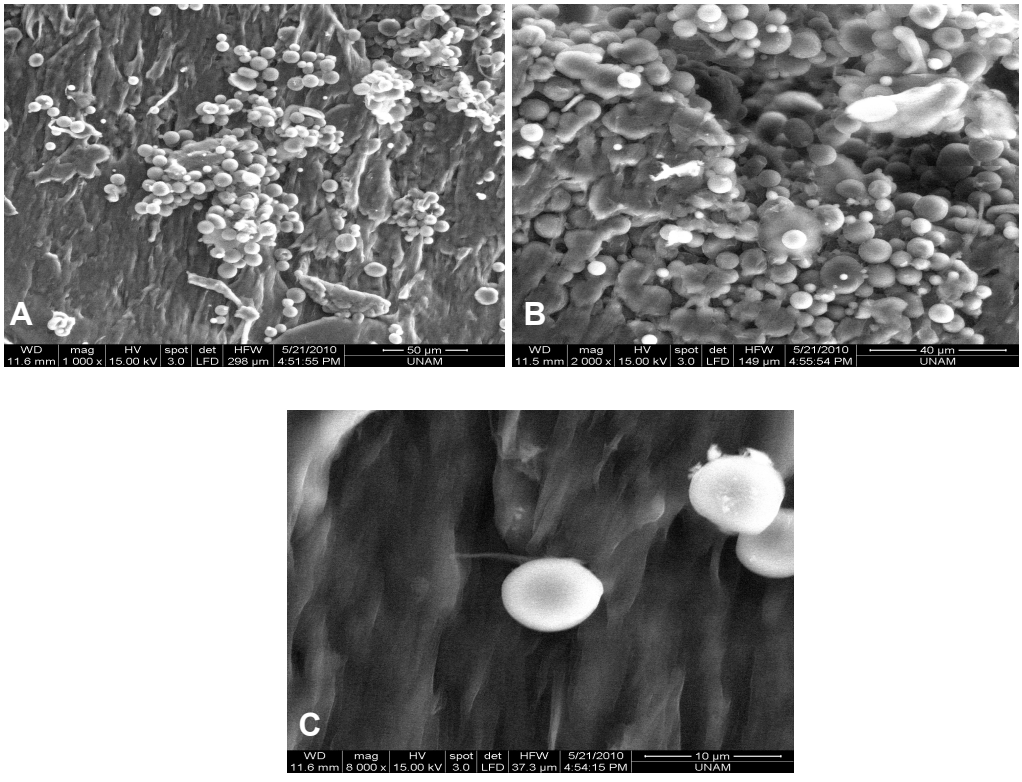


Figure 3.15. SEM micrographs of D-CS loaded PCL (14 kDa) microspheres prepared by 4 % PVA

As shown in Figure 3.15 a and b, microspheres could be formed with good spherical contours. However, the membrane-like formation was again present as in previous two sets. The individual microspheres were adhered to this structure. Further optimizations on doxycycline-chondroitin sulfate loaded microspheres were needed.

For the optimum shape and surface properties of chondroitin sulfate (CS) microspheres, all chemical conditions were kept the same but stirring time with homogenizer was changed from three hours to half an hour. In the following step, volume of aqueous solution (using same concentration PVA solution) was raised from 40 ml to 75 ml after half an hour stirring at high speed 3400 rpm and then stirring was continued at 1100 rpm under hood until chloroform evaporated. Due to decrease in stirring time, the size of the microspheres increased.

The microsphere stability was also influenced from the volume of emulsifying agent (aqueous phase). Excessive amounts of PVA were reported to decrease the stability of microspheres (Kemala, et al, 2010). Due to this reason, the initial volume of PVA solution was selected as 40 ml. However, when CS was added to process, polymers had turned into membrane-like forms rather than spheres. This was thought to be related with high reactivity of CS with aqueous phase thus decreasing the emulsifying effect of the PVA solution. Thus, to increase the emulsion stability increasing the volume of the PVA solution was also applied. Moreover, in the first group of microspheres prepared without homogenizer, the aqueous phase volume was set at 75 ml. In Figure 3.16., the SEM micrographs of microspheres were presented.

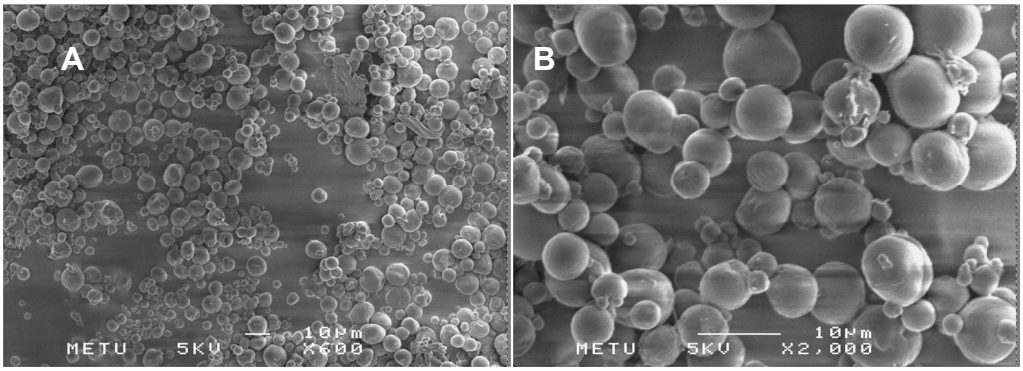


Figure 3.16. SEM micrographs of D-CS Coloaded PCL Microspheres at (a) 600x and (b) 2000x magnifications

According to SEM results, (Figure 3.16), microspheres had a homogeneous particle size distribution. The surface of microspheres was smooth and they had good spherical contour. The majority of microspheres had diameter around 15 µm. They did not form any irregular fusion products.

Particle size distribution of doxycyline-chondroitin sulfate (D-CS) combination loaded PCL microspheres (Figure 3.17) showed a mean diameter of 12 µm. Besides that, 90 % of them had diameter under approx. 85 µm values. Particle size distribution of microspheres was homogeneous and they were suitable for injecting intra-articularly. A small portion of the spheres were observed to have very small sizes with diameter being around 1 µm and some had larger size being around 90 µm.

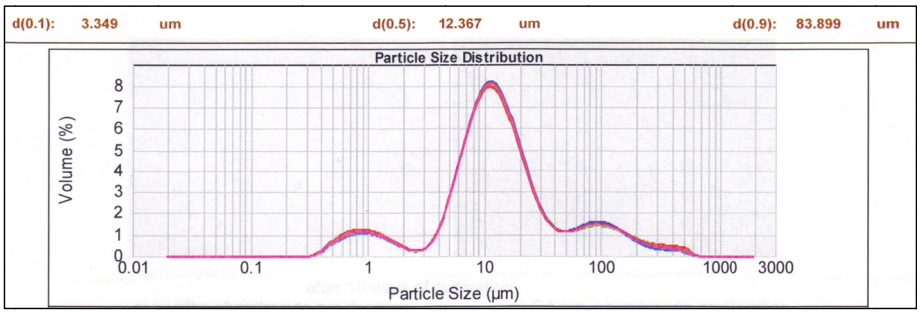


Figure 3.17. Particle size distribution curve of D-CS combination loaded PCL microspheres.

3.6. Extraction Studies

3.6.1. Determination of Encapsulated Doxycycline Amount of PCL Microspheres

A single step extraction procedure was suitable for determining encapsulation efficiency of the microspheres. Doxycycline is unstable in most of the solvents. It was reported that with single step extraction procedures acidic solvents usage should be avoided. If used, reversible epimers and lower extraction ratios of doxycycline were obtained.These solvents interfere with doxycycline peak in the chromatographic separation (Ruz, et al., 2004).

The wavelength spectrum of doxycycline (D) in water was obtained with UV-Vis spectrophotometer as given in Figure 3.18. According to optical density scan result, D had two wavelengths 274 and 341 nm at which the absorbances (optical densities) had a maximum. Other researches also indicated that D had two λ_{max} values; 267 and 351 nm (Scholar, E., 2007).

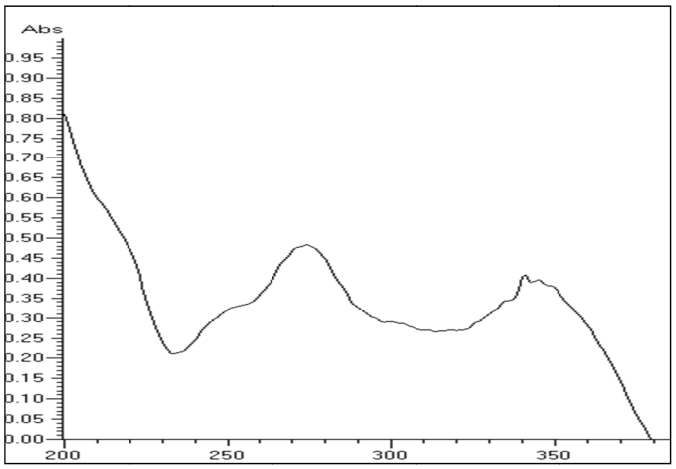


Figure 3.18. Light absorption spectrum of doxycycline (D) in water

Doxycycline is an amphoteric compound (pKa 3.4, 7.7 and 9.7) which has a strong influence in chromatographic separation. In some studies, extreme tailing in the chromatograms of doxycycline for reverse phase HPLC columns was observed. (Ruz, et al., 2004).

Acetonitrile which was a component of the mobile phase is also being used for extraction of drugs from polymeric microspheres. Wang et al used this solvent for disodium norcantharidate extraction from PCL microspheres (Wang, et al., 2008b). Although acetonitrile is a deproteinising agent that disrupts the strong interaction between doxycycline and plasma proteins, it was suitable in HPLC as mobile phase solvent in pharmaceutical samples (Mitic, et al, 2008; Ruz, et al., 2004). Buffers were mostly used in mobile phases for determining doxycycline and the use of high ratio of buffers in this phase made better resolution. However, retention time was delayed and the peaks were broadened. pH of the mobile phase was also important in separation of doxycycline and its degradation products (Skulason, et al., 2003). In this study, since degradation of the drug was not expected for the time period and conditions of the experiment, only the loaded doxycycline amount was detected. Selection of the mobile phase mainly aimed at dissolving the PCL matrix and separating drug from it by solubilizing the drug in the same solvent. Since acetonitrile is a poor solvent for PCL and tetrahydrofuran was added to the extraction solution to improve the solubilization with acetonitrile.

Calibration curve for doxycycline (D) was obtained by HPLC. The linearity of response was verified over the concentration range of 4–20µg/ml (r^2 = 0.9731). According to calibration curve, area under the peak was firstly converted to drug concentration and then used to estimate total drug amount encapsulated in microspheres (Appendix A).

A representative HPLC chromatogram for D is given in Figure 3.19. On this figure, the retention time and peak area were seen respectively. Known concentrations of the drug were used to calculate the total area of the second peak and then, calibration curve was obtained. The retention time of the drug was approximately 4 minutes.

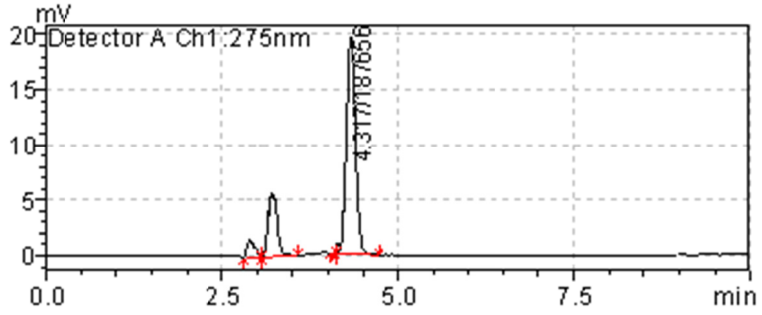


Figure 3.19. Representative chromatogram for Doxycycline hyclate standard peaks (5 µg/ml)

The drug peak obtained from extraction of D loaded PCL (14 kDa) microspheres (prepared in 4 % PVA solution) is shown in Fig.3.20. Areas under these two peaks were used in calculation of the D content of this microsphere group. Hence, similar chromatogram with doxycycline standart was seen with the HPLC analysis of the extraction samples

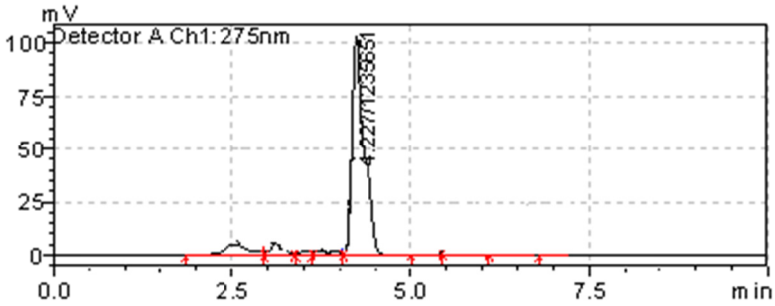


Figure 3.20. The peak of drug extracted from PCL (14 kDa) microspheres prepared with 4% PVA as emulsifier.

The percent encapsulated drug and entrapment efficiency were calculated from loaded drug amount. In Table 3.1, loading (%) and encapsulation efficiency (%) of microspheres are given.

Table 3.1. Loading and encapsulation efficiency (%) of doxycycline loaded PCL microspheres

MS	Emulsifier	PCL (kDa)	Drug Loading (%)	Entrapment Eff. (%)
1	4 % PVA	14	17.58	52.79
2	1 % PVA-gel	14	10.80	32.43
3	4 % PVA	65	24.48	73.48
4	1 % PVA-gel	65	29.40	88.28

3.6.2. Determination of Encapsulated Drug Amount of Doxycycline-Chondroitin Sulfate Combination Loaded Microspheres

For determining the amount of D in D-CS coloaded microspheres, extraction method with tetrahydrofuran-methanol solution and HPLC analysis were applied. The peak for drug from extracted microspheres was observed to arrive at approximately 4th minute (Figure 3.21).

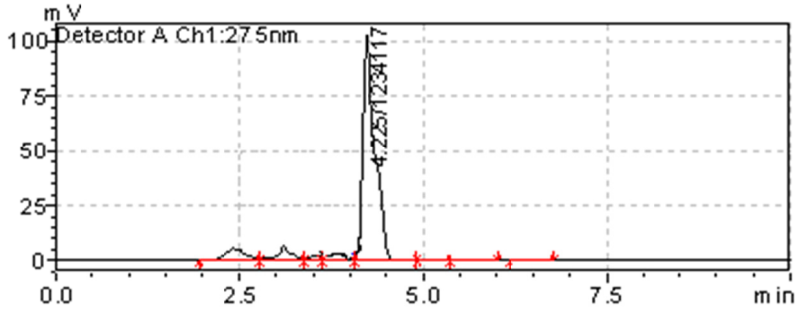


Figure 3.21. The peak of drug in D-CS PCL microsphere extracts.

The standard peak obtained for D in HPLC system (Figure 3.19) was also observed to arrive at about 4th minute. Calibration curve for doxycycline (D) obtained by HPLC method is shown in Appendix A.

Percent encapsulated drug and entrapment efficiency were calculated from loaded drug amount. Percent loading was calculated as 10.1 % and entrapment efficiency was calculated as 30.4 % for this microspheres.

3.6.3. Effects of Emulsifying Agents, Stirring Rate, Molecular Weight on Particle Size and Doxycycline Encapsulation of Microspheres

In Table 3.2, the particle size distributions of microspheres prepared with PCL (14 kDa) are summarized. Mean particle size of microspheres prepared with PVA solution were smaller than those prepared with PVA-gelatin solution either in the empty or loaded case. However, loading with doxycycline affected the size such that doxycycline (D) loaded microspheres were larger than empty microspheres when prepared with both PVA (4%) and PVA-gelatin (1%, each) solutions.

Table 3.2. Comparison of particle sizes of microspheres prepared with PCL (14 kDa)

MS type	d(0.1) (µm)	d(0.5)(µm)	d(0.9)(µm)
PVA (4%) (empty)	16.652	57.439	143.442
PVA (4%) (D loaded)	31.421	74.348	151.228
PVA-Gel (1%) (empty)	49.625	84.847	137.943
PVA-Gel (1%) (D loaded)	52.214	90.331	149.130

Table 3.3. Comparison of particle sizes of microspheres prepared with PCL (65 kDa)

MS type	d(0.1) (µm)	d(0.5)(µm)	d(0.9)(µm)
PVA (4%) (empty)	64.738	104.474	167.744
PVA (4%) (D loaded)	46.258	94.010	369.996
PVA-Gel (1%) (empty)	50.853	106.343	194.456
PVA-Gel (1%) (D loaded)	41.546	121.918	341.314

In Table 3.3, the particle size distributions of microspheres with PCL (65 kDa) are presented. Mean particle size of microspheres prepared with PVA solution were smaller than those prepared with PVA-gelatin (1%, each) solution either in the empty or loaded case. Mean size of Doxycycline (D) loaded microspheres were bigger than empty microspheres when prepared with PVA-gelatin (1%, each) solution but smaller in PVA (4%) case. However, the expected incease in size with drug loading was obvious for both conditions in size range that covers the 90 % of all particles.

Besides composition, the increase in speed of stirring is also an important factor that results with decrease in size of the microspheres (Wang, et al, 2008a; Radin, et al, 2009). Jalil and Nixon (1990) also studied the variation of sphere size with respect to the stirring rate. Increase in stirring rate decreases the particle size. In our study, the 4% PVA solution aqueous phases of MS was stirred at 1100 rpm, however, when 1% PVA-gelatin solution was used the rate was 500 rpm. The small differences in

terms of sizes might also be caused by stirring rate difference besides difference in aqueous phase.

It was reported that, when molecular weight of PCL was increased, encapsulation efficiency increased. This was mainly due to the viscosity of the PCL. The viscosity of organic phase of PCL (65 kDa) was higher than PCL (14 kDa). Viscous organic phase prevented the doxycycline diffusion into the external aqueous phase before hardening of microspheres. In another study, felodipine loaded microspheres were prepared with molecular weights of 10, 65 and 80 kDa PCLs. Microspheres prepared with highest molecular weight PCL (80 kDa) had the most viscous organic phase, so the encapsulation efficiency was the highest. The second highest encapsulation efficiency was obtained in microspheres prepared with PCL (65 kDa) and the lowest encapsulation efficiency was measured for microspheres prepared with PCL (10 kDa) (Kim, et al 2005). In our study, when such comparison was made, microspheres of 14 kDa PCL encapsulated less doxycycline than those prepared using 65 kDa PCL.

Dimercaptosuccinic acid (DMSA) loaded PLGA microspheres were prepared by using several PVA concentrations with several stirring rates. Smaller microspheres had higher encapsulation efficiency than larger microspheres. (Shukla, et al., 2007). In our study highest loading and encapsulation efficiency was obtained with PCL 65 kDa microspheres prepared in PVA-gelatin aqueous phase, although they were formed at slow stirring rate and had the largest in mean particle size. Hence we may conclude that the effect of molecular weight overweighted the effect of other factors but when polymer had low Mw the composition of aqueous conditions were more effective on modifying encapsulation and loading results. The increase in molecular weight increased the particle size of the microspheres (Jeong, et al., 2003). As mentioned before, high molecular weight PCL increased the viscosity of the organic phase, thus the emulsion droplet size increased which, increased the size of microspheres. In addition, viscous organic phase tended to reduce the amount of unencapsulated drug in the external aqueous phase (Jeong, et al., 2003).

3.7. Drug Release Results of Microspheres

3.7.1. Drug Release Profiles of Doxycycline Encapsulated Microspheres Prepared with Low Molecular Weight PCL

In order to calculate the drug amounts of release samples optical densities were measured at 274 nm by using spectrophotometer (Hitachi, Japan) and they were converted to drug amounts using calibration curve of the drug at the same wavelength (Appendix B).

Next and major step of optimization studies for a drug delivery system involves the characterization of drug release behavior. Drug encapsulated in the polymer would be released from the system depending on many parameters of the system and the environmental conditions. Doxycycline is a water soluble drug that makes its dissolution and diffusion easier. However, PCL is a hydrophobic polymer that mostly retains its content for extended time period. Thus, release of the drug into surrounding medium is a result of combination of many factors such as drug dissolution and diffusion, size of microspheres, polymer to drug ratio, entrapment efficiency, etc.

In order to optimize the release experiments, doxycycline encapsulated PCL microspheres prepared by 4% PVA were used. Figure 3.22 demonstrates the results of release experiments of these microspheres. At the initial stage, there was fast release period (burst effect) related to the drug entrapped at or close to the surface of the microspheres. Drug release continued more slowly. This release profile was caused by the delay of water penetration, thus the diffusion of the drug through the amorphous region into the release medium was retarded (Zalfen, et al, 2008). At the later stage, drug was released slowly due to low permeability of water into hydrophobic polymer; PCL (Figure 3.22). The mechanism of drug release from PCL matrix is different from PLGA matrix. PCL degrades more slowly than PLGA which is less hydrophobic than PCL. Drug release of PLGA matrices may depend on both degradation and diffusion (Vivek, et al., 2007, Jeong, et al., 2003).

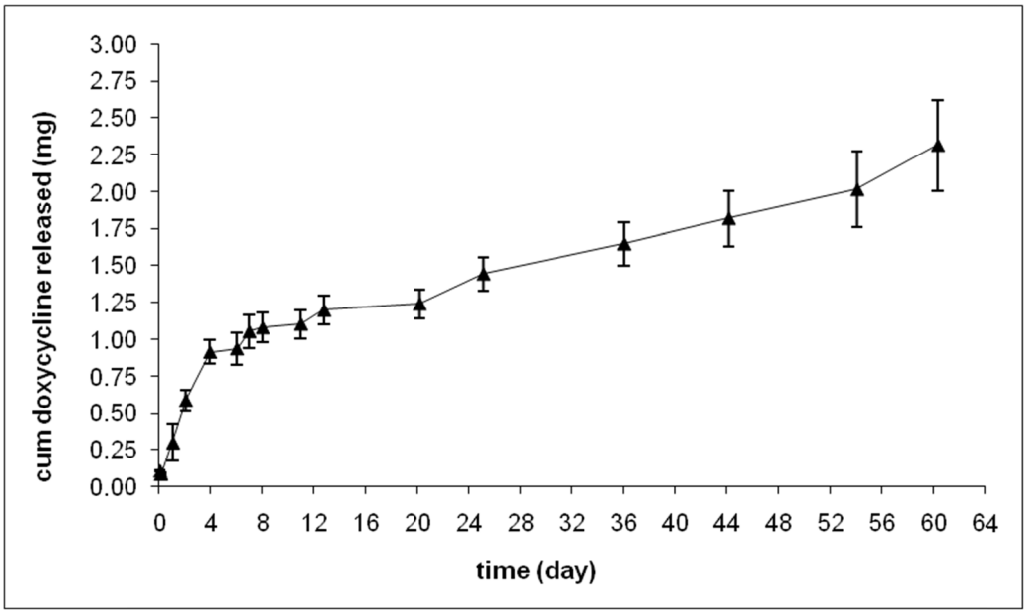


Figure 3.22. Release profile of PVA (4 %) used doxycycline loaded PCL microspheres (n=3).

Besides polymer characteristics, matrix porosity may also play an important role on release of encapsulated drug. Freiberg and Zhu (2004) indicated that highly porous matrix released drug at a higher rate than less porous matrix. Increasing the number of pores was shown to increase the release rate In accordance with these results the PCL microspheres, were observed to have a less-porous structure. In Figure 3.23, changes on surface structure of these microspheres were shown after release studies by SEM. Surfaces of microspheres were rougher and were more porous than before release.

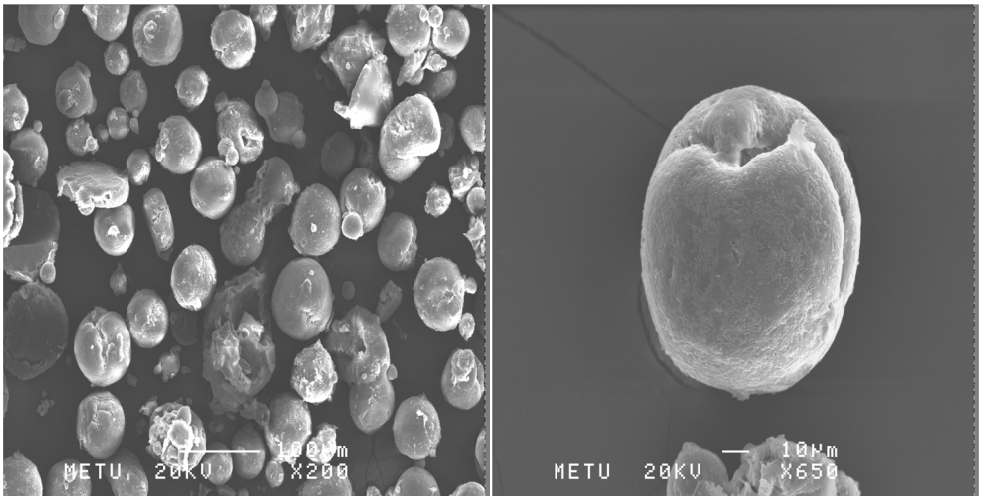


Figure 3.23 SEM images of doxycycline loaded PCL microspheres (PVA, 4 %) after release experiments

In the 2nd set of release experiment, microspheres prepared in PVA-gelatin media were used. Gelatin improved the surface characteristics of microspheres; however, by increasing the viscosity of aqueous phase, it also delayed the evaporation time of the organic solvent. So, this situation also changed the drug release kinetics. In this group, evaporation time effect was also evaluated. The viscous structure of PVA-gelatin delayed the evaporation time of the microspheres. Yet, the residual chloroform could cause unwanted outcomes both In terms of microsphere formation and biological safety.Therefore, in order to investigate the effect of extended evaporation time (for removal of all organic solvent in the high viscosity aqueous environment conditions), the evaporation time was changed and all other steps were applied the same. The effect of evaporation time on release of D from the microspheres was investigated and the results are presented in Figure 3.24.

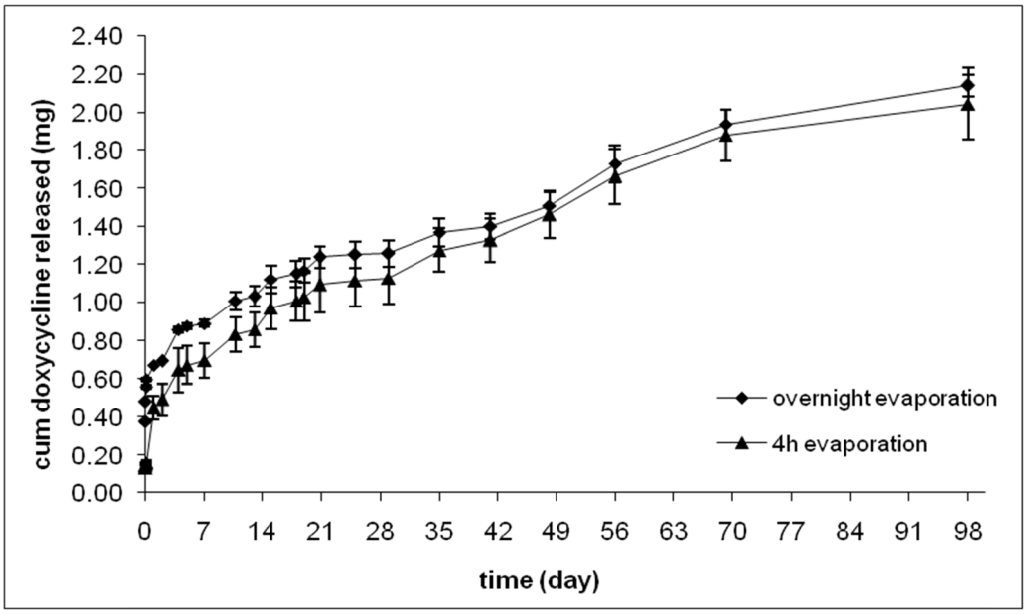


Figure 3.24. Release profiles of PVA-gelatin (1 %, each) used doxycycline loaded PCL (14 kDa) microspheres; evaporation time effect (n=2)

There was no significant difference in terms of release behavior and amounts when the evaporation period of organic phase was extended. The burst effect was slightly smaller in 24 h evaporation case indicating some loss of drug molecules onto near the surface of the microspheres. At the seventh day, approximately, 0.75-0.90 mg of drug was released. This amount and the release behavior were also very similar with release results of D-microspheres prepared with PVA (4%) solution. In Figure 3.24, the released total drug amount at 7th day was approximately, 0.75-1.0 mg.

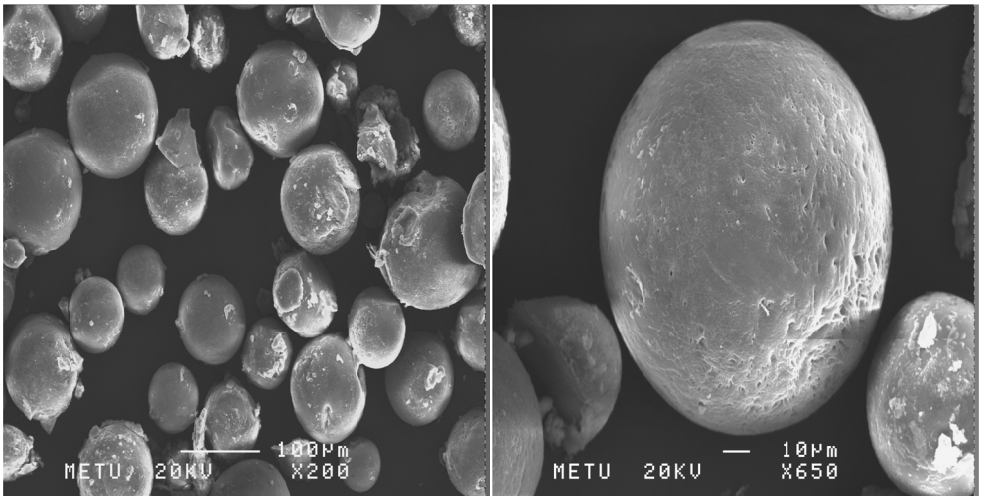


Figure 3.25. SEM images of doxycycline loaded PCL microspheres (PVA-gelatin; 1 %, each) after release experiments

In Figure 3.25, morphologies and surface characterizations after release study of these microspheres are shown with SEM images. Surfaces of microspheres were rougher and much more porous than before release as in the case of 1st set of microspheres. The surface morphology did not change like PVA (4%) used doxycycline loaded microspheres. Surfaces of microspheres were almost alike to those of before release.

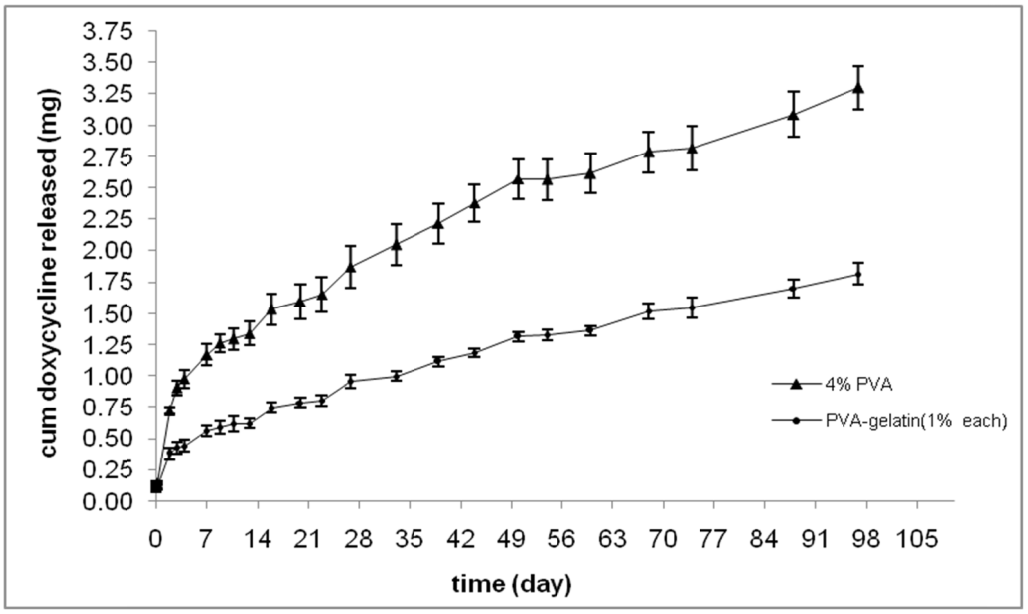


Figure 3.26. Drug release profiles of doxycycline loaded PCL (14 kDa) microspheres prepared with two different aqueous environment (n=3)

Upon completing the optimization studies of preparation conditions for PCL (14 kDa) microspheres the release behavior of the two were compared. In Figure 3.26, the related release study results are shown. According to these curves, microspheres prepared by using PVA (4 %) released much more drug than microspheres prepared by using PVA-gelatin (1 %, each) for the same time points. This difference might be caused by the difference in particle sizes of the microspheres. Microspheres prepared by using PVA-gelatin (1 %, each) were larger than those prepared by using PVA (4 %) which caused a decrease in total surface area of microspheres. Decrease in the total surface area directly reduces the amount of contact with water (Jeong, et al., 2003) besides that increase in area/volume ratio increases the amount of released drug by enforcing the diffusion of the drug molecules from the micrsopheres to the release medium.

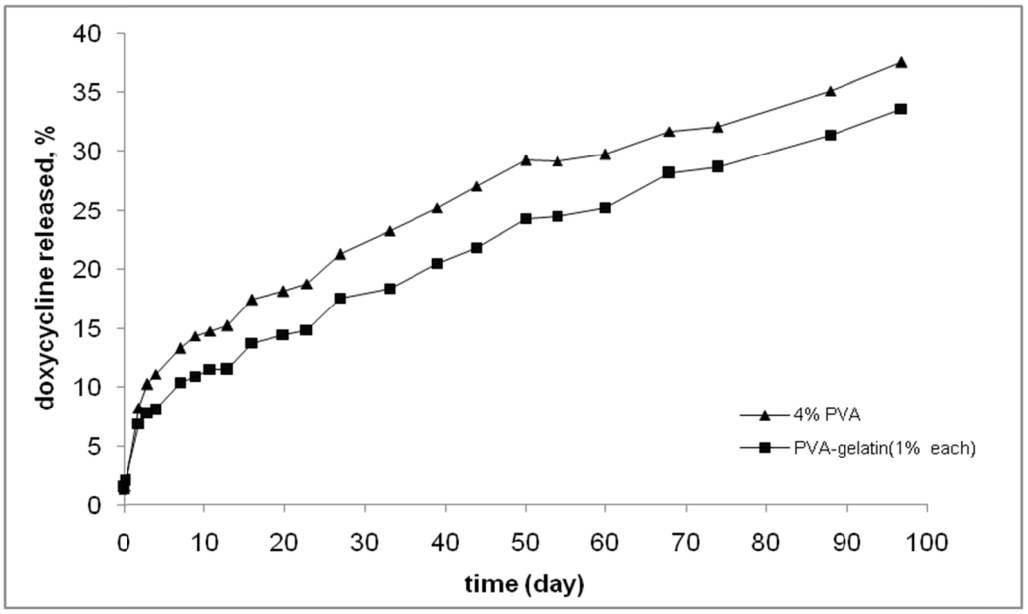


Figure 3.27. Comparison of doxycycline release (%) profiles of PCL (14 kDa) microspheres prepared with two different aqueous environment (n=3)

When the percent release behavior of these two sets of microspheres was compared they provided similar results except for the amount observed for burst effect (Fig.3.27). During the first few days of the release, a faster release due to burst effect was seen. Then release of drug progressively slowed down and followed a continuous regime. Rapid release phase was thought to be caused by high water solubility of doxycycline molecules at the superficial regions of the microspheres. Release profile of disodium norcantharidate (DSNC) loaded PCL microspheres were mainly influenced by high solubility of DSNC in water (Wang, et al, 2008a). Despite this high water solubility of the drug, in one month period, microspheres prepared in PVA (4 %) and microspheres prepared in PVA-gelatin (1 %, each) aqueous media could release only approximately 23 % and 18 % of drug, respectively.

Since the surface adsorbed drug generated the burst release, the drug entrapped near the surface of microspheres was estimated to be approximately 7-10 % of the loaded drug according to the released amounts at day 3. Hydrophobic polymers had the lowest burst release (Vivek, et al., 2007). Figure 3.27 shows that PCL microspheres prepared with PVA (4 %) released approximately 37 % of doxycycline, whereas PCL microspheres prepared with PVA-gelatin (1 %, each) released

approximately 33 % of doxycycline at the end of three months. PCL microspheres prepared in PVA-gelatin (1 %, each) solution, encapsulated less (10.80 %) doxycycline than microspheres prepared in PVA (4 %)(17.58 %). Therefore, the significant difference in the amount of doxycyline released, despite similar % release results was thought to be related with the initial amnounts of drug deposition within the microspheres. Besides that PCL microspheres prepared with PVA-gelatin (1 %, each) were larger in average diameter (90 μm) than microspheres prepared with PVA (4 %). This might have also contributed to faster release of the drug by first group.

The solubility properties of drugs are known to influence drug loading and also the release profiles. p-Nitroaniline has low water solubility than rhodamine B and these two model drugs were encapsulated in PCL (10 kDa) in a recent study. Poorly water soluble drugs have higher solubility within organic phase and thus have higher loading percent than others. Water solubility of p-Nitroaniline and rhodamine B were approximately 1 and 10 mg/ml respectively. Drug loading of p-Nitroaniline was 47.14 %, whereas that of rhodamine B was 7.84 %. Thus, p-Nitroaniline which was more hydrophobic can be distributed more regularly in the polymer matrix whereas rhodamine B being more hydrophilic was mostly distributed close to the surface of microspheres (Wang, et al, 2009). Wang and coresearchers (2009) reported that decrease in drug solubility in water increase in encapsulation efficiency and the initial burst amount decreased. Doxycycline is also a highly water soluble drug (50 mg/ml) and is insoluble in organic solvents such as chloroform and ether. Thus, initial burst release of doxycycline was also related with drug entrapment near the surface due to its solubility properties.

In another study, the drug, etoposide, which is hydrophobic in nature, was entrapped in different polymers such as PLGA (50:50), PLGA (75:25) and PCL. The highest entrapment efficiency of this drug was obtained with the most hydrophobic polymer (PCL) among the group. The entrapment efficiency was low as in etoposide loaded PLGA (50:50) microspheres. Highest initial burst release was observed when the most hydrophilic PLGA (50:50) microspheres were used (Vivek, et al., 2007) Jain et al (2000) observed a relatively high burst release of cytochrome c from microsphere preparations with the more hydrophilic polymer as Vivek and coworkers (2007) observed similar etoposide release. Hydrophobic polymers have low amounts of surface adsorbed drugs compared with hydrophilic polymers.

Burst release is a critical problem of injectable microparticles if slow release for large periods of time is required (Hassan, et al, 2007). Release stage following burst effect was very slow due to low permeability of water into. However, burst release might bring some advantages like establishing a certain concentration of the drug at the site in the initial stages of treatment where disease has established to some degree. This would probably provide good impact on causes of the disease before longer term sustained provision of the drug. Water penetration and degradation was low in PCL and drug release was only based on diffusion from amorphous regions of polymer matrix (Jeong, et al., 2003). In this study, despite the high solubility of doxycycline in water, hydrophobic characteristics of PCL decreased the burst effect compared to those observed with hydrophilic polymers such as PLGA and its copolymers in literature (Hassan, et al, 2007).

3.7.2. Drug Release Profiles of Doxycycline Encapsulated Microspheres Prepared with High Molecular Weight PCL (Mw=65 kDa)

The selected two emulsification conditions were used in the preparation of microspheres with high molecular weight polymer (PCL). Their release profiles were compared in Figure 3.28. Drug release was continuous throughout the release period for both set. Unlike low molecular weight polymer microspheres, they had constant release rate with similar amounts of drug being released in each time interval. In one month period, microspheres prepared in PVA (4 %) and microspheres prepared in PVA-gelatin (1 %, each) aqueous phases, released approximately 9 and 11 % of the drug, respectively. Hence release profile has changed owing to use of high molecular weight polymer (as compared with previous release experiments in Section 3.7.1).

Microspheres prepared with 4% PVA released approximately 3.1 mg drug which was 25.39 % of the loaded amount (Figure 3.28 and 3.29). However, samples prepared with 1 % PVA-gelatin solution released approximately 4.3 mg drug which was 29.46 % of the loading. This difference was thought to be related with most of the particles (given in Figure 3.9) of the second preparation method being smaller than those prepared by the first one (appox. 341 vs. 370 μm, for 90 % of the distribution).

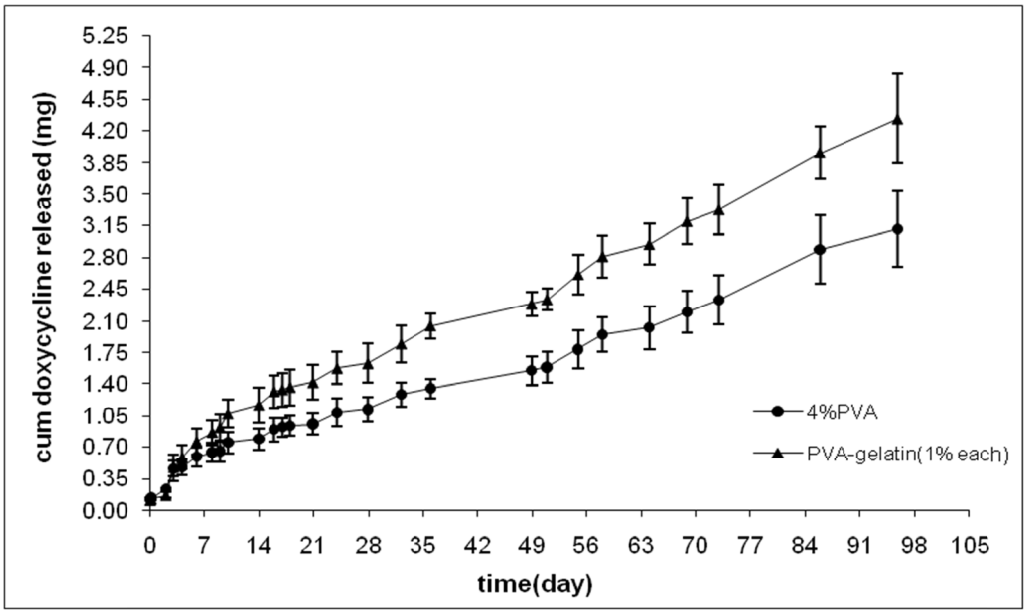


Figure 3.28. Drug release profiles of doxycycline loaded PCL (65 kDa) microspheres prepared with two different aqueous environment (n=3)

At the first stage of release where the burst effect was seen the behaviour of high molecular weight PCL microsperes were different than those of low Mw PCL. In the high mwt case burst was not as appearant as as high Mw case. On the third day of the experiment, the released doxycycline amount was only the 3 % of the total amount. This was probably due to the lower water permeability of PCL (65 kDa). Zalfen et al (2008) studied with PCL (50 kDa) and the burst release was small due to high molecular weight similar to the present study.

In general, drug release rate may be inflenced from polymer properties such as polymer type, molecular weight etc (Vivek, et. al, 2007). Here, released drug percentage of microspheres prepared from PCL (65 kDa) was lower than PCL (14 kDa). It was observed that the higher molecular weight polymers resulted in rapid solidification of the system during preparation. Therefore, drug diffusion and release rates of drugs decreased (Vivek, et al, 2007). On the other hand, particle size of microspheres prepared from PCL (65 kDa) was higher than PCL (14 kDa).

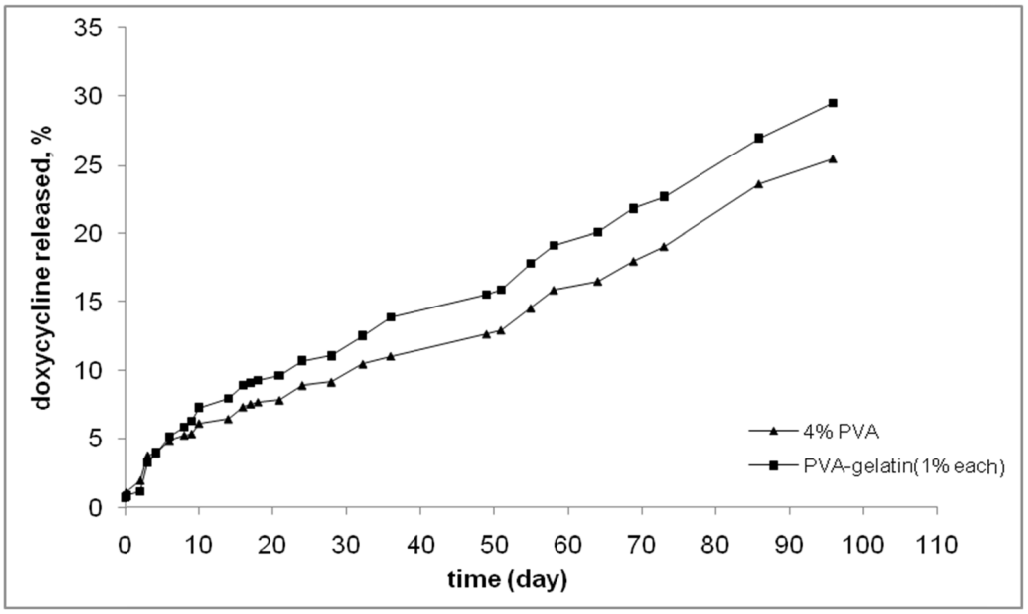


Figure 3.29. Comparison of doxycycline release (%) profiles of PCL (65 kDa) microspheres prepared with two different aqueous environment (n=3)

In Figure 3.30, SEM images of high molecular weight PCL microspheres after release experiment are shown. Microspheres prepared in PVA-gelatin (1%, each) aqueous media were more homogeneous than PVA (4%) microspheres. Also, the surface of the PVA-gelatin (1%, each) microspheres were smoother than PVA (4%) microspheres. After release studies, former microspheres's surface roughness increased and they appeared more porous. Surfaces of PVA-gelatin (1%, each) microspheres were almost the same as before release. The insignificant differences were mainly due to slow degradation of PCL. Gelatin usage increased the smoothness of the surface of the microsphere. So, after release, changes in the smoothness of microspheres prepared with PVA-gelatin (1%, each) were less than those prepared with 4%.

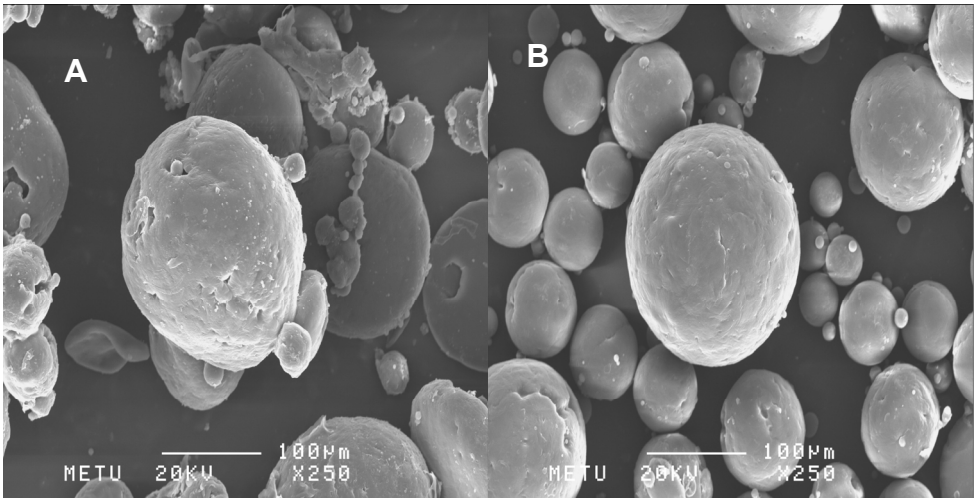


Figure 3.30. SEM images of doxycycline loaded PCL (65 kDa) microspheres a) PVA; 4 % b) PVA-gelatin; 1 %, each) after release experiments

3.7.3. Mathematical Modelling of D Release from Low and High MW PCL Microspheres

The release kinetics of doxycycline loaded PCL (14 kDa) and PCL (65 kDa) microspheres prepared by PVA (4 %) and PVA-gelatin (1 %) are described by Higuchi and Korsmeyer-Peppas models. Higuchi and Korsmeyer-Peppas model equations are

$$Q_t/Q_\infty = K_H.t^{0.5}, \qquad (3.1)$$

$$Q_t/Q_\infty = K_{KP}.t^n, \qquad (3.2)$$

as already given in Table 1.1 in Section 1.5, respectively. Here, Q_t/Q_∞ Is fractional release of drug in time t, K_H and K_{KP}, are Higuchi and Korsmeyer-Peppas constant characteristics of drug-polymer system, and *n* is diffusion exponent characteristic of release mechanism.

It was reported that suitability of some equations mainly depended on the cumulative released drug amount (Avachat and Kotwal, 2007). Korsmeyer-Peppas which is called diffusion model equation can be applied to the first 60 % of fractional release (Avachat and Kotwal, 2007).

Briefly, drug release from a polymer matrix depends on properties of polymer and drug. For degradable systems, diffusion of drug and erosion of polymer matrix can be the main mechanisms of release. The importance of these mechanisms change for systems due to composition, molecular weight of polymer and drug, crystallinity percent, hydrophobicity, degradation rate, particle size, pore size and shape of the drug delivery system.

The geometric shape of the system influences the diffusion exponent *n*. In Fickian diffusion, *n* is 0.50 for slab, 0.45 for cylinder and 0.43 for sphere. In non-Fickian (anomalous) diffusion, *n* is between the Fickian diffusion value and zero-order value (*n*=1) for non-swellable systems. For swellable systems, *n* is between the above and case-II transport (*n* = 1 for slab, 0.89 for cylinder and 0.85 for sphere) (Miao, et al., 2006; Chang, et al., 2006).

Curve fittings were performed on the data presented in Figures 3.26 and 3.28 in section 3.7.1 and 3.7.2 and results are presented in Tables 3.4 and 3.5 indicated that the kinetics of doxycycline release from PCL (14 kDa) microspheres are described effectively by the Higuchi Model (linear relationship of release amount with the square root of time), i.e., governed by Fickian diffusion. Thus a decrease in doxycycline (D) release over time is expected as drug is depleted in the matrix and the diffusion path length increases. The release rate was boosted by raising the drug loading in the matrix, correlating with the response of diffusing species to a higher concentration gradient. The values of the release exponent (*n*) in the Korsmeyer-Peppas model for doxycycline loaded PCL microspheres was found below 0.43 for PCL (14 kDa) microspheres, providing further support for a release mechanism governed by Fickian diffusion of doxycycline from PCL (14 kDa) matrices.

Table 3.4.Higuchi Model of doxycycline release from PCL microspheres

Aqueous phase	PCL (kDa)	K_H	R^2
4% PVA	14	3.968	0.970
1% PVA-gelatin	14	3.360	0.988
4% PVA	65	2.081	0.936
1% PVA-gelatin	65	2.484	0.951

Table 3.5. Korsmeyer-Peppas model of doxycycline release from PCL microspheres

Aqueous phase	PCL (kDa)	K_{KP}	*n*	R^2
4% PVA	14	7.508	0.374	0.986
1% PVA-gelatin	14	5.003	0.432	0.989
4% PVA	65	1.652	0.550	0.965
1% PVA-gelatin	65	1.652	0.600	0.988

The verification of complex release mechanisms would be problematic. Here, experimental results were compared with mathematical model called diffusion model. Diffusion model was applied for the description of the experimental data of doxycycline release and for the identification of the release mechanisms for the PCL (14 kDa) and PCL (65 kDa) microspheres. Equation (1.5) from Section 1.5.2.1 was used for graphical administration of the model.

Firstly, the estimation of diffusion coefficient was calculated from release data. Ritger and Peppas, (1987) simplified equation (1.5) and obtained equation (3.3) to calculate the diffusion coefficient for all microsphere types as

$$C_1/C_\infty = 6.\sqrt{(D|\pi.R^2)} \qquad (3.3)$$

The experimental data for burst release and after transition to a different release pattern were omitted to obtain linear release rate (Kurnik and Potts, 1997).Then, diffusion coefficients were calculated from equation (3.3) (Romero-Cano and Vincent, 2002).Calculated diffusion coefficients for the experiments presented in Figures 3.26 and 3.28 from equation (3.3) are given in Table 3.6.

Table 3.6.Diffusion coefficients calculated using experimental data presented in Figures 3.26 and 3.28

Aqueous phase	PCL (kDa)	D (cm^2/sec)
4% PVA	14	1.00×10^{-9}
1%PVA-gelatin	14	1.25×10^{-9}
4 % PVA	65	0.60×10^{-9}
1%PVA-gelatin	65	0.90×10^{-9}

Fan, et al. (2004) measured the diffusion coefficient of doxycycline in water through PVDF membrane as 6.59×10^{-6} cm^2/sec from Franz Diffusion Cell. Diffusion coefficients of drug from microspheres mainly depend on crystallinity of polymer, shape, molecular weight, pore size, and properties of microspheres. These factors affect the doxycycline diffusion from polymer matrix. Lidocaine loaded PLA nanoparticles had smaller diffusion coefficient than diffusion coefficient of lidocaine through PLA films (Polakovic, 1999). The estimated diffusion coefficients for lidocaine loaded PLA nanoparticles were in the range of 5-7x 10^{-20} m^2/s. The diffusivity of an antibiotic, gentamicin sulphate in the porous PCL matrix (8.3 % loading) calculated as 1.5×10^{-9} cm^2/s. For comparison, the diffusion coefficient of solutes in water is of the order 2×10^{-5} to 1×10^{-6} cm^2/s (Chang, et al., 2006)

In Figures 3.31 and 3.32, experimental release profiles were compared with diffusion model equation (1.5) in which the calculated diffusion coefficients were used. The diffusion coefficients for PCL (14 kDa) microspheres were larger than the ones for PCL (65 kDa) microspheres resulting the higher release rate for PCL (14 kDa) microspheres than PCL (65 kDa) microspheres. Increase in diffusion coefficient accelerated the release rate (Romero-Cano and Vincent, 2002).

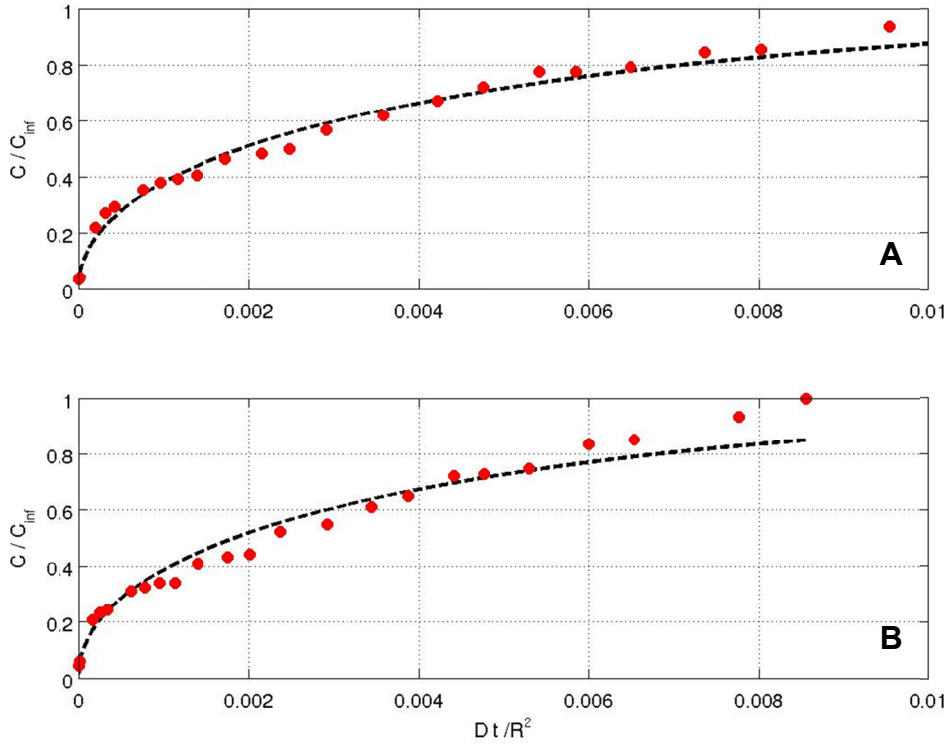


Figure 3.31. Mathematical modeling of in vitro release of doxycycline-loaded PCL (14 kDa) microspheres prepared by a) PVA (4 %) b) PVA-gelatin (1 %, each)

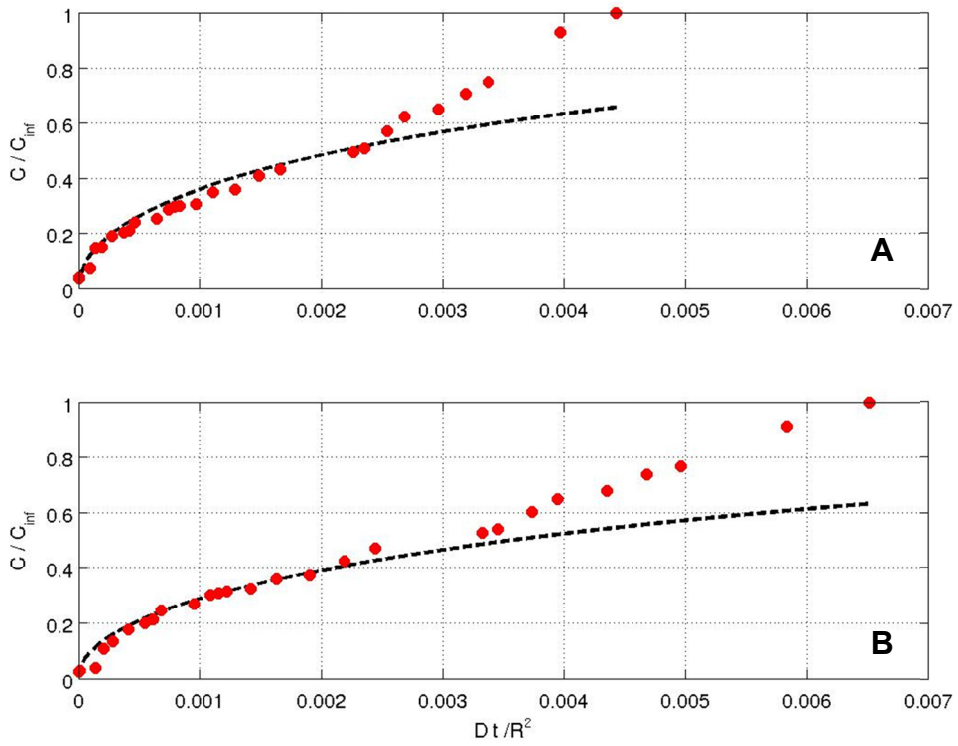


Figure 3.32. Mathematical modeling of in vitro release of doxycycline-loaded PCL (65 kDa) microspheres prepared by a) PVA (4 %) b) PVA-gelatin (1 %, each)

Release of microspheres prepared by PCL (14 kDa) can be explained by diffusion model as shown in Figure 3.31. However, release from microspheres prepared by PCL (65 kDa) cannot be explained by the diffusion model completely because the release profiles have two distinct behavior (Figure 3.32). At the beginning of the release, the release results fitted with diffusion model. When considering all of the data, the release model of the microspheres was mainly like zero order release. Korsmeyer-Peppas model indicated that, this release profile was non-Fickian diffusion. Also, it was not zero-order release (n=0.55 and n=0.60 for PVA; 4 % and PVA-gelatin; 1%, respectively). Lao (2008) indicated that, the difference of the behavior was due to water penetration, degradation of polymer and drug solubility and drug removal to the surrounding medium usually diffusion process. So, it can be concluded that the rate limiting property of microspheres determined the release profile.

Small diffusion coefficients were mainly due to dense polymer matrix (Romero-Cano and Vincent, 2002). Small but significant differences were encountered while release period was long. Mechanism of drug release was Fickian for PCL (14 kDa) microspheres and non-Fickian for PCL (65 kDa) microspheres.

The modeling results showed that in the case of high drug loadings (about 30%), where the whole drug or a large part of it was in the crystallized form. On the other hand, the drug release was diffusion-controlled at low loadings (about 10 %) where the solid drug was randomly dispersed in the matrix (Polakovic, et al., 1999). The doxycycline loaded PCL (14 kDa) microspheres had low loadings than doxycycline loaded PCL (65 kDa) microspheres presented at Table 3.1 in Section 3.6.1. In the case of doxycycline release from PCL microspheres, diffusion was found to be the rate controlling mechanism at low loadings. As a result, diffusion model could be used for description of PCL (14 kDa) microspheres due to their low loading ratio.

3.7.4. Drug Release Profile of Doxycycline-Chondroitin Sulfate (D-CS) Co-Encapsulated Microspheres Prepared with Low Molecular Weight PCL (Mw=14 kDa)

Release profile of the D-CS combination loaded microspheres was given in Figure 3.33. According to this profile, burst release was seen at first 5-8 days. The burst release might be related to the surface adsorbed drug particles dispersing rapidly from microsphere into buffer in the first few days (Huang, et al., 2009). After the initial burst phase, the release pattern followed gradual increase. This release profile is commonly observed for PCL microsphere systems and is described as “PCL microspheres gave an initial burst phase and then cumulative amount release increases over time” (Chang, et al, 2006). At day of 3, the released drug amount was 9.69 % of total release. The same degree of burst effect was also seen at only D loaded PCL (14kDa) microspheres.

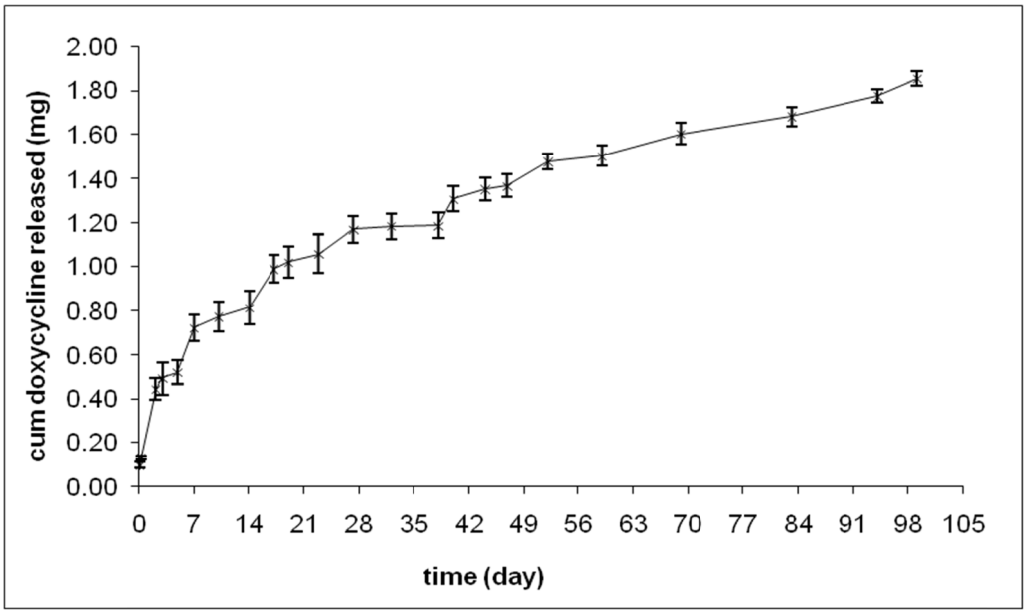


Figure 3.33. Doxycycline release profile of D-CS coloaded PCL (14 kDa) microspheres (n=3)

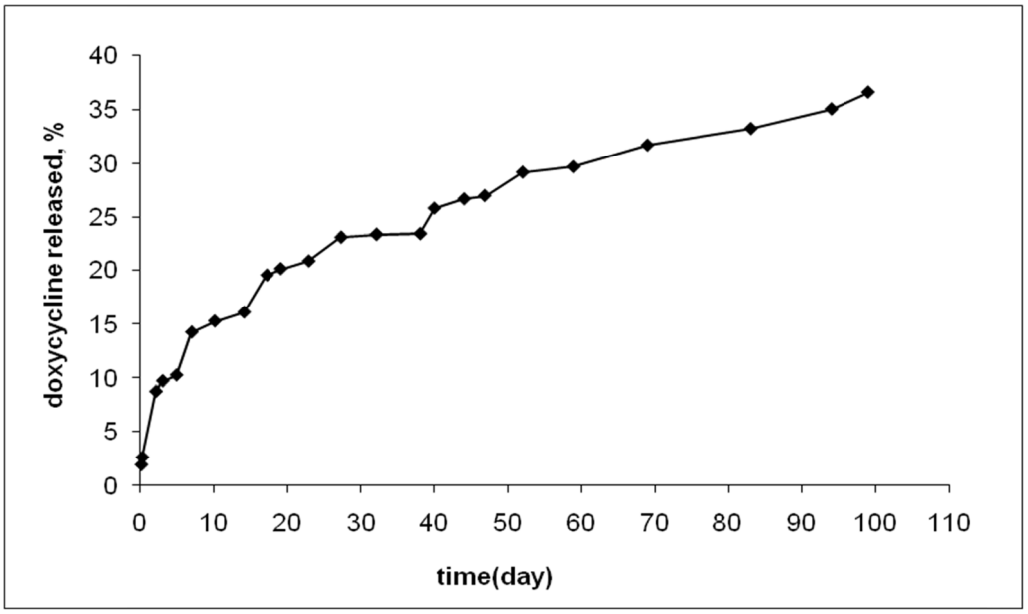


Figure 3.34. Doxycycline release (%) profile of D-CS coloaded PCL (14 kDa) microspheres (n=3)

In one month period, approximately 20 % of the loaded drug was released. After 3 months, 36.65 % of doxycycline was released. This proved that these microspheres had potential to release doxycycline at least for 6 months. These results showed that CS addition to the microspheres did not cause any significant change on release profile and amounts of D from PCL microspheres. This was thought to be due to the fact that low amount of CS could be added into the system and smaller particle sizes were obtained with dual loeded microspheres. Overall results suggested that the D-CS combination loaded PCL microspheres were suitable for in vitro cell culture and in vivo applications.

The ideal release kinetic was constant release rate over the time named as zero order. However, due to various parameters such as polymer crystallinity, size and shape of microspheres, and diffusion from hydrophobic matrices, obtaining zero order release is difficult (Baker, 1987). Despite all, according to Figure 3.28, there seemed to be zero order release kinetic for D released from high molecular weight PCL microspheres.

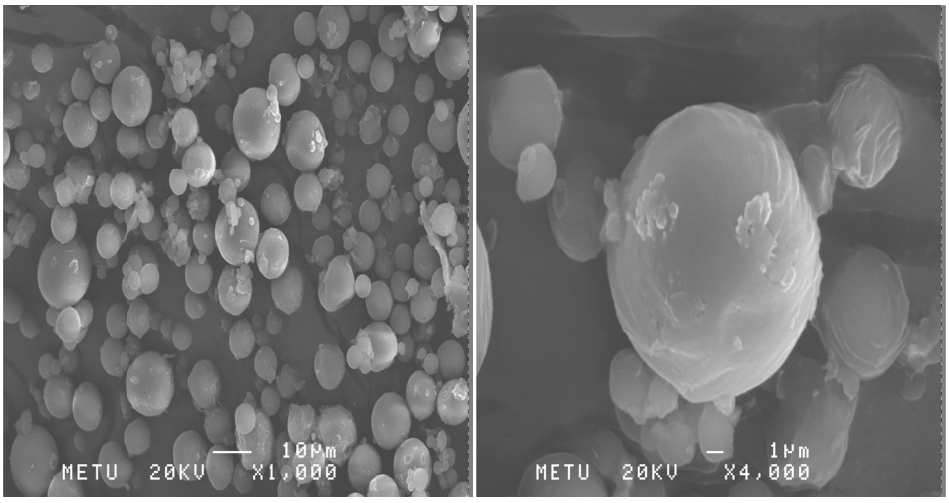


Figure 3.35. SEM images of D-CS coloaded PCL microspheres after release

SEM images of D-CS coloaded PCL microspheres are shown in Figure 3.35. The surface structure of microspheres was not notably different from those of microspheres before release. Hence, it was thought that release mainly depended on diffusion from the small pores and routes through the matrix.

3.7.5. Gel Permeation Chromatography (GPC) Measurements

In Appendix H, the results of Gel Permeation Chromatography (GPC) measurements were shown. The molecular weight changes before and after release experiments and the polymer (PCL) itself were measured.

3.8. Fourier Transform Infrared (FTIR) Spectroscopy

In this study, FTIR spectroscopy was employed to examine if there occurred any change in the chemistry of polymer and drug upon microsphere processing procedures and whether they formed a new type of observable interaction or not. In order to evaluate the characteristic peaks for the polymer and drug were first determined by their single spectra and then compared with that of microcapsules. FTIR spectra of commercial PCL (14 kDa, Figure 3.36) and doxycycline (Figure 3.37 are presented.

PCL showed its characteristic peak at about 1730 cm^{-1} due to C=O of ester carbonyl groups. The stretching band from the CL was strong and clearly detected (Aishwarya, et.al, 2008). PCL (80 kDa) had the C=C stretching vibration peak and C-O stretching peak at 1468 and 1191 cm^{-1}, respectively (Park, et al, 2006).

Other characteristic absorption bands for PCl are at about 2943 cm^{-1} (attributed to the stretching of C–H) and at 1721 cm^{-1} (C=O stretching band). The bands at 1163 cm^{-1} were attributed to the C–O stretch (Zheng et al, 2008; Ha et al, 1999).

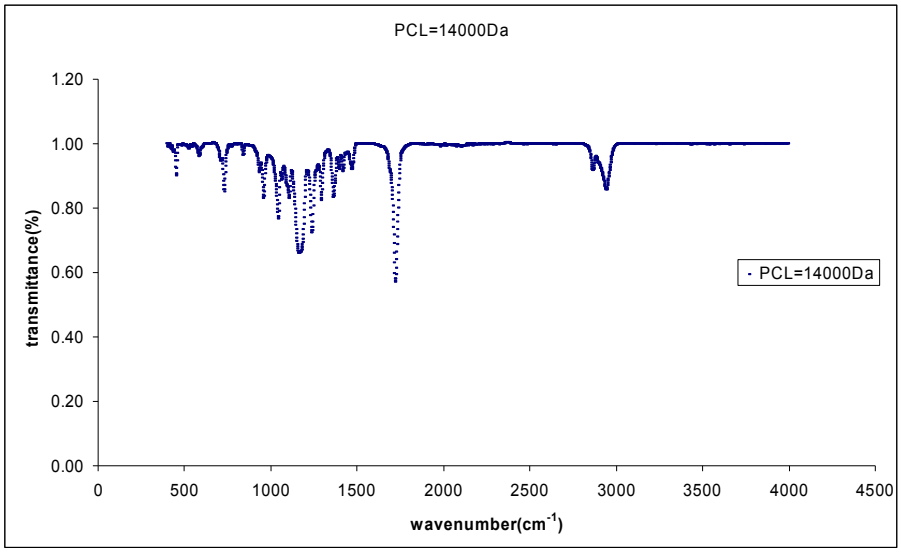


Figure 3.36. FTIR spectrum of PCL (14 kDa) used in preparation of microspheres

Doxycycline had characteristic bands of different functional groups. Band appearing at 3358 cm^{-1} was due to O-H / N-H stretching vibrations and those at 2924 and 2854 cm^{-1} were due to C-H stretching vibrations. Primary amide (N-H) bending was at 1666 cm^{-1} whereas aromatic N-H bending was at 1581 cm^{-1}. Carbonyl (C-O) stretching vibrations were seen at 1615 cm^{-1}. $-CH_2$ and C-H bending vibrations were measured at bands of 1460 and 1329 cm^{-1}, respectively. C-N stretching vibrations were seen at 1220 and 1173 cm^{-1} (Patel, et al, 2008). Tamimi, et al. (2007) compared FTIR spectra of doxycycline hyclate and doxycycline base. In the 1650 cm^{-1} region, related to NH_2 group, there seemed a main difference between the drugs. This group interacted with HCl in doxycycline hyclate and therefore the intensity of the 1650 cm^{-1} transmittance decreased in doxycycline hyclate samples. Conversely, doxycycline monohydrate (base) samples had a more apparent transmittance peak at 1650 cm^{-1} because of the HCl. That confirmed the fact that HCl has been removed from the molecular complex and doxycycline hyclate was converted into doxycycline monohydrate.

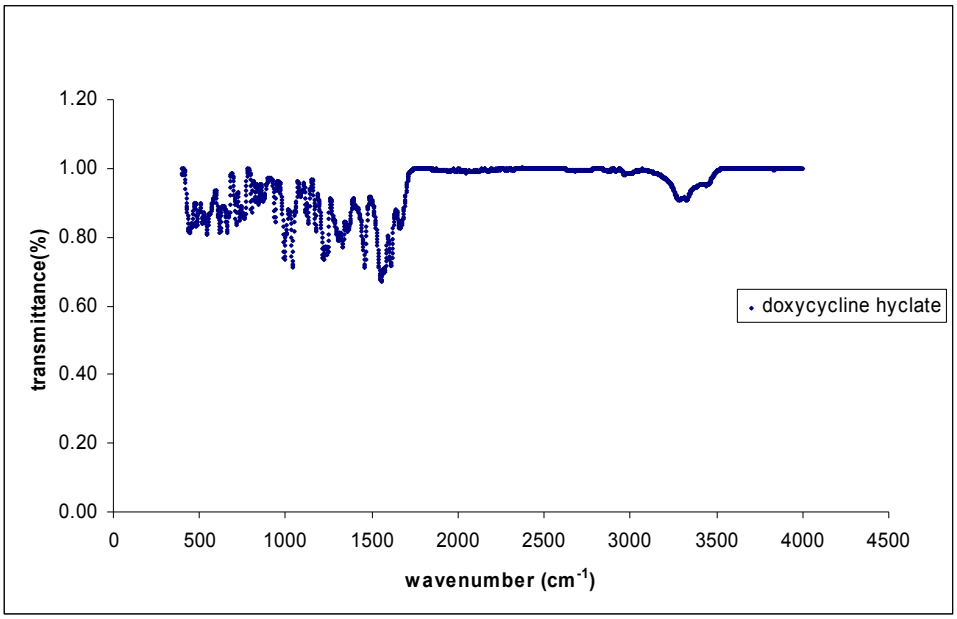


Figure 3.37. FTIR spectrum of doxycycline.

In Figures 3.38 and 39, FTIR spectra of empty and D loaded PCL (14 kDa) microspheres prepared by 4% PVA and 1% PVA-gelatin were presented, respectively. Accordingly, there was no difference between FTIR spectra in the presence or absence of doxycycline. Similarly, doxycycline loaded PLGA microspheres and empty PLGA microspheres were compared in literature. There was no difference in spectra of those microspheres as in the case of this study (Patel, et al, 2008). Some bands of doxycycline were not distinct due to identical stretching of empty PCL microspheres and doxycycline loaded microspheres at the same bands. This indicated that doxycycline structure did not change during the production of microsphere (Patel, et al, 2008). This might also be due to lower amount of doxycycline in microspheres after loading compared to the amounts used in single drug FTIR analysis.

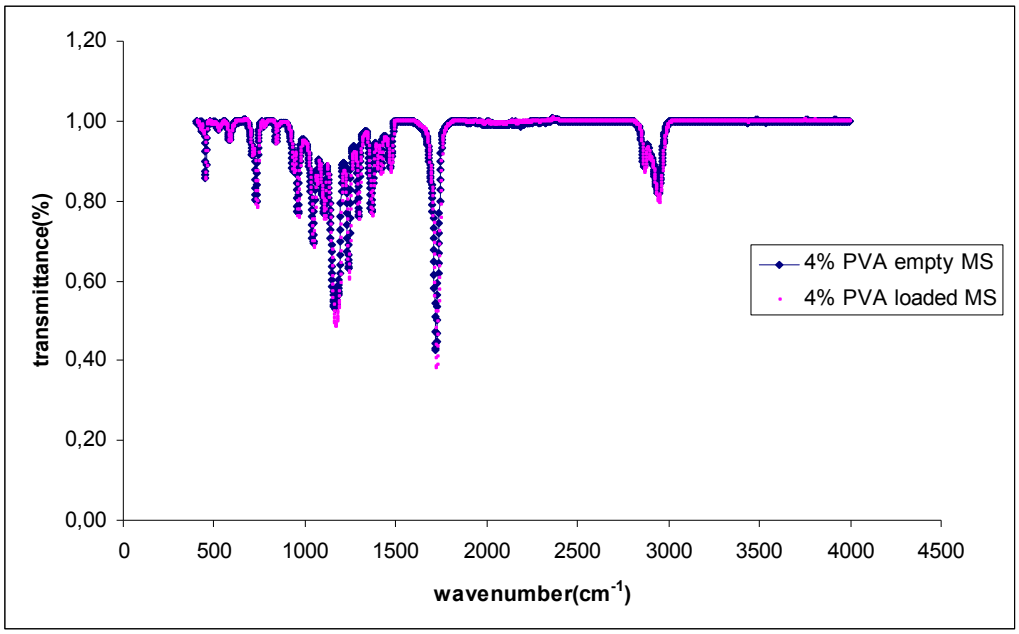


Figure 3.38. FTIR spectra of empty and loaded microspheres prepared by PVA (4%).

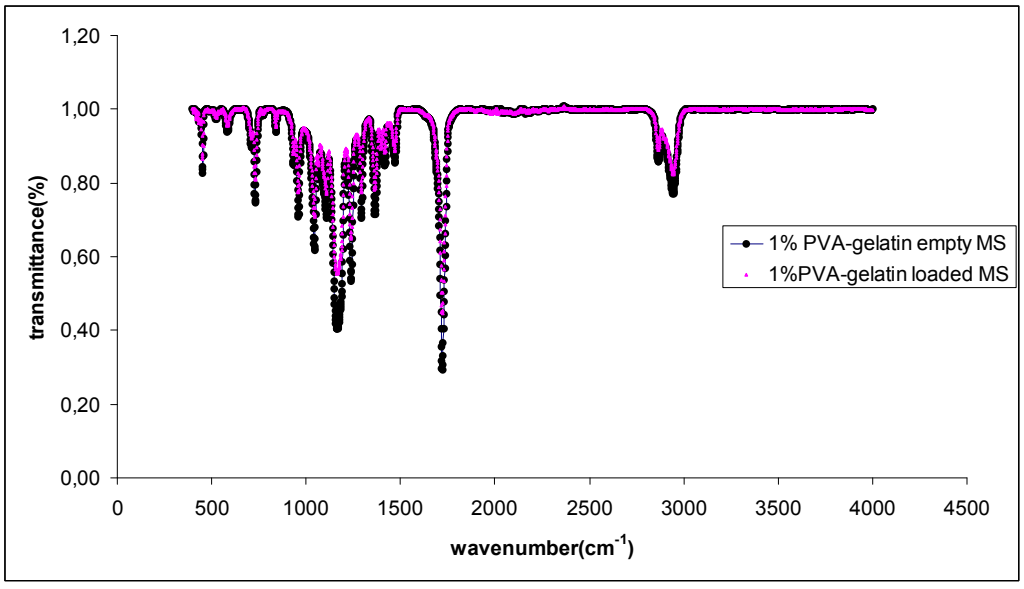


Figure 3.39. FTIR spectra of empty and loaded microspheres prepared by PVA-gelatin (1%, each).

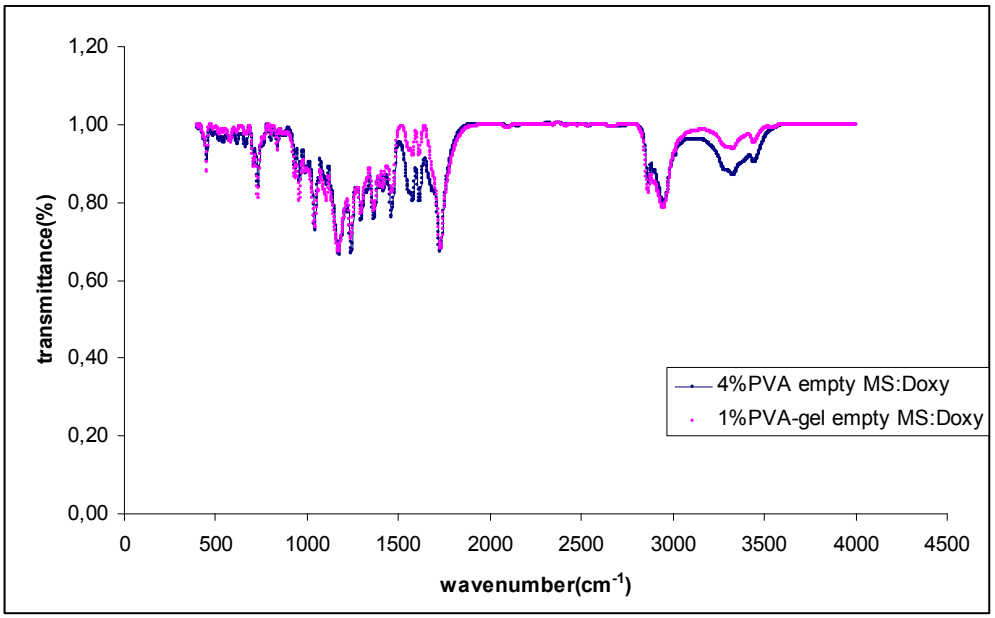


Figure 3.40. FTIR spectra of empty microspheres prepared by PVA (4%) and PVA-gelatin (1%, each): Doxycycline hyclate mixture (1:5)

In order to ensure the results, empty PCL (14 kDa) microspheres were mixed with doxycycline hyclate (1:5) to show the doxycycline hyclate presence (Figure 3.40). It was observed that the structure of doxycycline hyclate was hindered by the peaks of the PCL structure.

3.9. Differential Scanning Calorimetry (DSC) Measurements

DSC experiments were carried out to determine the physical state of the polymer and the drug in the formulation.It was also conducted to study whether the interactions between the drug and polymer changed their thermal properties in microspheres before and after release experiments. The effects of different preparation conditions (such as PVA) in terms of DSC results were also evaluated. Chawla and coworkers (2002) indicated that Pluronic F-68 absence increased the melting point of tamoxifen loaded PCL nanoparticles.

In Figures 3.41 (1) and (2), DSC thermograms of low molecular weight (and high molecular weight PCL (14 kDa and 65 kDa) were shown, respectively. In order to determine the T_g, values of these two polymers, second heating run was applied. T_m and T_c values were determined and they are listed in Table 3.7. First heating run

values were taken in consideration. In Figure 3.41, derivative of heat flow values are demonstrated as dashed lines. The exact value of T_g could not determined but it was estimated to be under -60°C which was the minimum temperature limit for the system used. Researchers also indicated that the T_g value of PCL was approximately -60°C and the value mainly depended on the molecular weight of the PCL (Acierno, et al., 2006).

From the thermograms, it was observed that low Mw PCL (14 kDa) had higher crystallinity value than high Mw PCL (65 kDa). The low melting temperature, defined as endothermic peak during the second heating scan at 10°C/min and the crystallization temperature, defined as the exothermic peak during a cooling scan at 10°C/min were 67 and 22 °C, respectively. The melting temperature measured in the first scan is always higher than that obtained in the second heating scan after crystallization (Estelle's, et al, 2008). The melting and crystallization points obtained for the commercial PCL was of the same order as mentioned in the literature value (Estelle's, et al, 2008).

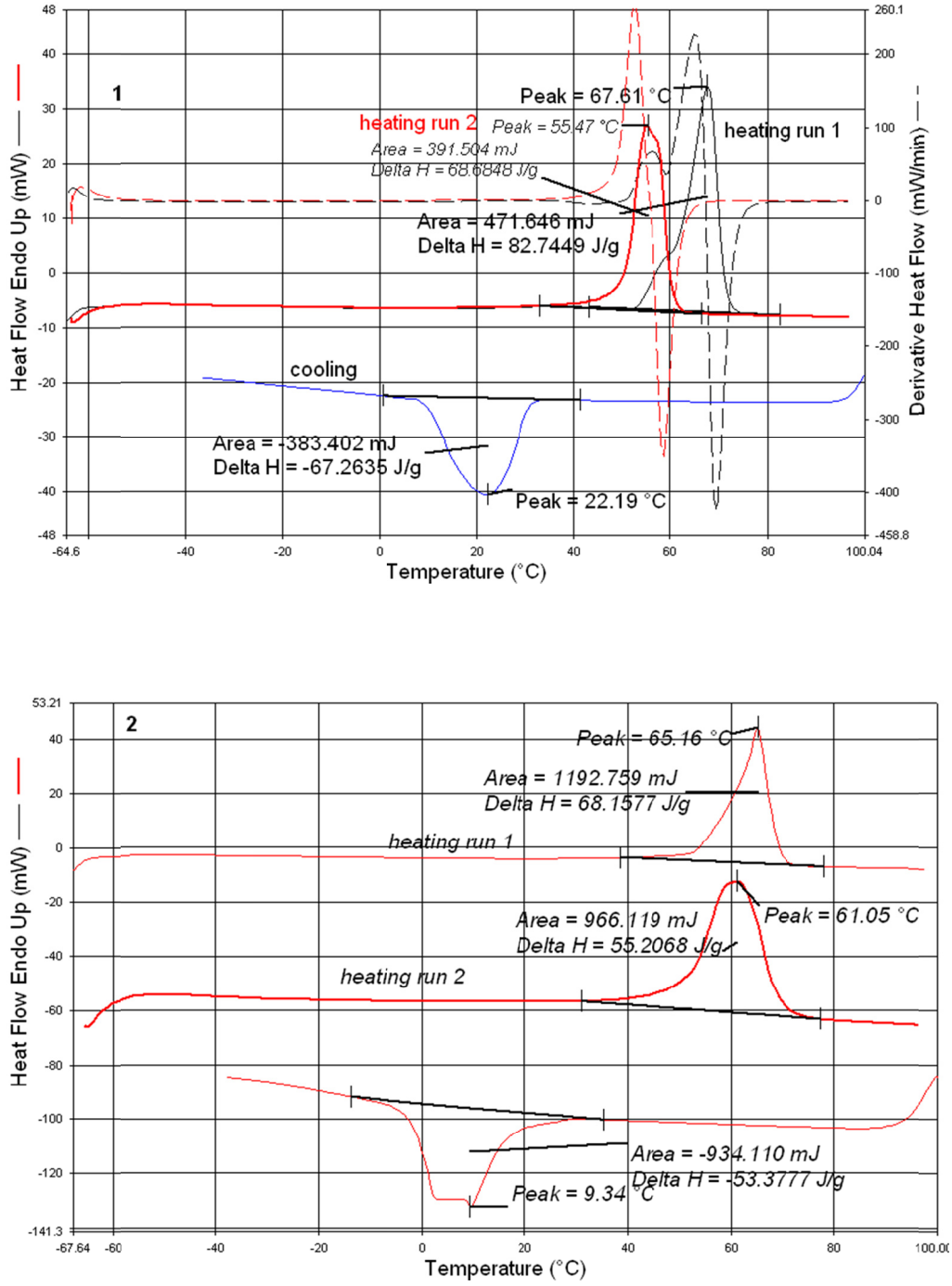


Figure 3.41. DSC thermograms of (1) PCL (14 kDa) (2) PCL (65 kDa)

Table 3.7. DSC analysis results of PCL polymer and PCL microspheres

Sample	T_m (°C)	ΔH_m(J/g)	X_c (%)	T_c (°C)
PCL=14 kDa	67,61	82,7449	59,3153	22,19
PCL=65 kDa	65,16	68,1577	48,8586	9,34
4P MS PCL=65 kDa Before R	63,30	89,2607	63,9862	34,83
4P MS PCL=65 kDa After R	66,31	92,4464	66,2698	35,84
4PMS PCL=14 kDa Before R	60,11	91,2418	65,4063	36,66
4P MS PCL=14 kDa After R	62,96	91,5989	65,6623	38,49
1P MS PCL=14 kDa Before R	60,12	83,6474	59,9623	36,20
1P MS PCL=14 kDa After R	64,98	97,8142	70,1177	38,38
1P MS PCL=65 kDa Before R	63,64	81,9569	58,7505	31,85
1P MS PCL=65 kDa After R	66,00	87,0032	62,3679	36,52
4P PCL=14 kDa empty MS	61,31	97,1950	69,6738	37,71
4P PCL=65 kDa empty MS	62,78	81,2764	58,2627	33,34
1P PCL=14 kDa empty MS	61,75	86,1411	61,7499	36,02
1P PCL=65 kDa empty MS	63,96	83,0273	59,5178	33,00
Doxycycline	201,00	ref:Sigma Aldrich		

(Tm: melting temperature; ΔH_m(J/g): Heat of Fusion; Xc: crystallization degree; Tc: crystallization temperature; 4P:PVA 4%; 1P:PVA-gelatin 1%)

When different molecular weight commercial PCL's (14 kDa and 65 kDa) were compared (Table 3.6), the crystallinity degree of high molecular weight (65 kDa) was found to be lower than that of low molecular weight one (14 kDa). Crystallization temperature of high molecular weight PCL was also lower than that of low molecular weight. However, DSC results of microspheres did not show much difference in crystallinity degrees between microspheres of different molecular weight polymers when they were prepared in same aqueous environment. The degree of polymer crystallinities was found to be more in microspheres than those of original polymers showing that polymer chains get more ordered while being transformed into microsphere form. Also, microspheres after release experiment had higher crystallinity degrees than the latter one which is a common observation in similar

drug delivery studies. This observation can be explained by the loss of small amorphous polymer chains especially on the surface of MS during release experiments.

Crystallinity strongly affects both the drug release kinetics and the degradation (Carcaboso, et al, 2008). When the PCL was used as a polymer in drug release systems, drug release behavior mainly depended on the crystalline microstructure. The molecular weight of polymer affected the crystallinity. When the molecular weight of PCL increased, the crystallinity decreased (Miyajima, et al., 1997; Jeong, et al., 2003). Papaverine loaded microspheres were prepared with molecular weights of 10, 40 and 80 kDa PCL and felodipine loaded microspheres were prepared with molecular weights of 10, 65 and 80 kDa PCLs. Similarly in the present study, it was seen that PCL (14 kDa) and its microspheres were more crystalline than PCL (65 kDa) and its microspheres at Table 3.6.

In order to analyze the drug release mechanism, besides solubility, physical state of the drug within the microspheres was also studied. In Figure 3.42, DSC thermograms of empty and doxycycline loaded PCL (14 kDa) microspheres prepared by using PVA (4 %) were seen. Melting peak of doxycycline was absent on the differential scanning calorimeter thermogram of PCL microspheres containing doxycycline. This phenomenon was also seen at papaverine loaded PCL microspheres. It was estimated that the drug was dispersed in the microspheres as an amorphous form, not crystalline form (Jeong, et al., 2003). Moreover, degradation of PCL in aqueous media was very slow due to the semi-crystallinity and hydrophobicity (Ha et al, 1997). These facts supported the SEM and slow release results that drug release mechanism was not the degradation of polymer and it was mainly based on diffusion of drug through the polymer (Jeong, et al., 2003).

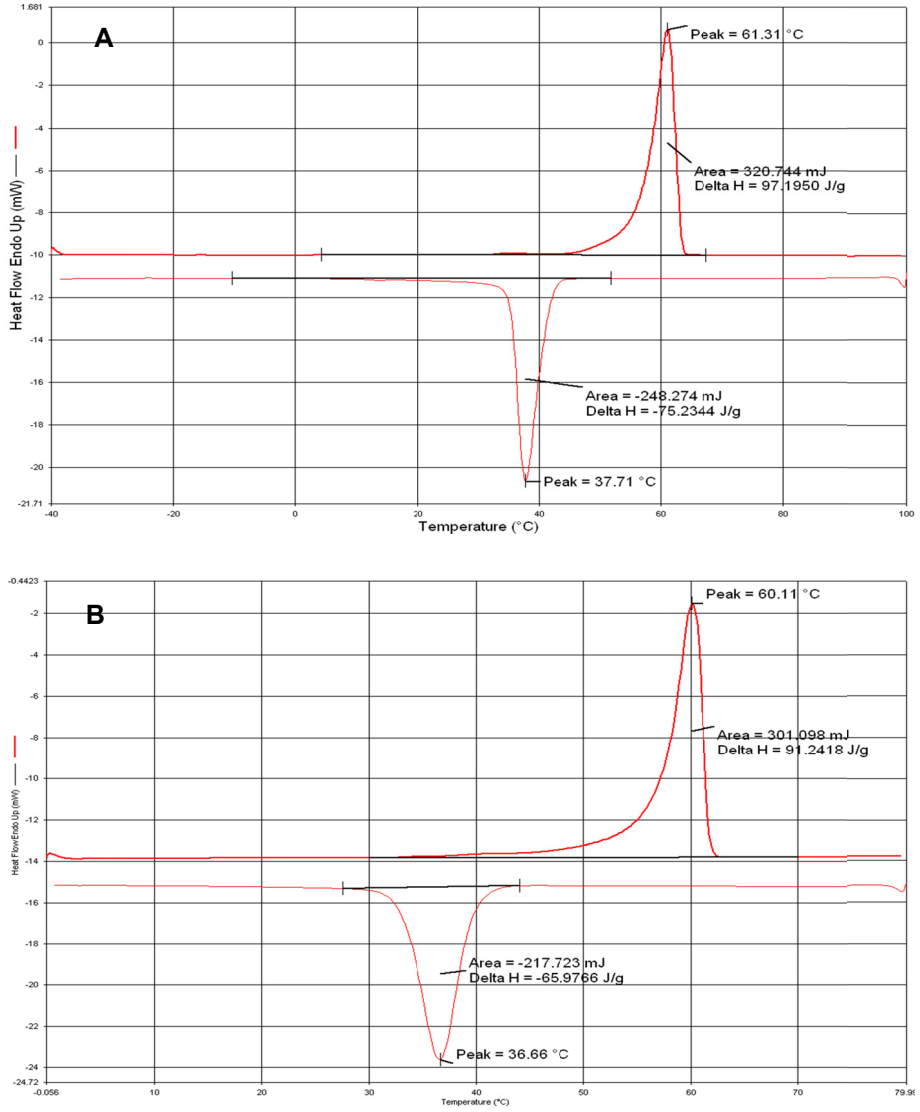


Figure 3.42. DSC thermograms of PCL (14 kDa) microspheres prepared by using PVA (4 %) a) Empty b) Doxycycline loaded microspheres

Figure 3.42 also showed that doxycycline encapsulation did not change the melting and crystallization temperatures of these microspheres. Melting temperature of doxycycline was higher than 185°C. The exact value was not determined by this measurement. The melting temperature was taken from SigmaAldrich and it was 201°C. It can be clearly stated that drug was in its crystalline form and there was no interaction between doxycycline and PCL. Finally, solvent evaporation-emulsion technique did not change the structure of doxycycline. Vanco-loaded chitosan microspheres prepared by using spray drying process and interactions were analyzed with DSC. Process did not change the structure of chitosan and Vanco (Cevher, et al., 2006).

Crystallinity of the polymer is also very important in encapsulation of drugs. The crystalline phase of the polymer is impermeable to water and drug is also encapsulated in the amorphous phase of the polymer. So, the higher the amorphous phase, the higher the encapsulation efficiency (Youan et al, 1999). Kim and coworkers (2005a) indicated that low molecular weight PCL (10 kDa) exhibited higher crystallinity than higher ones (65 and 80 kDa). Therefore, the lowest entrapment efficiency was measured when lowest molecular weight PCL (10 kDa) was used. Entrapment efficiency of PCL (65 kDa) was lower than PCL (80 kDa) and higher than PCL (10 kDa). Youan and coworkers (1999) reported that entrapment efficiency was the balance between the molecular weight and the crystallinity. Doxycycline loaded PCL (65 kDa) microspheres were more amorphous than PCL (14 kDa) microspheres. Hence, they have encapsulated more drug than PCL (14 kDa) microspheres.

3.10. Biocompatibility Studies

3.10.1. Effect of Microspheres on Chondrocyte Viability

The biocompatibility of microspheres was evaluated with in vitro cytotoxicity studies using MTT viability assay. The results are presented in Figure 3.43 as relative viabilities with respect to control. All microspheres had viabilities above 85 % and thus, were found biocompatible.

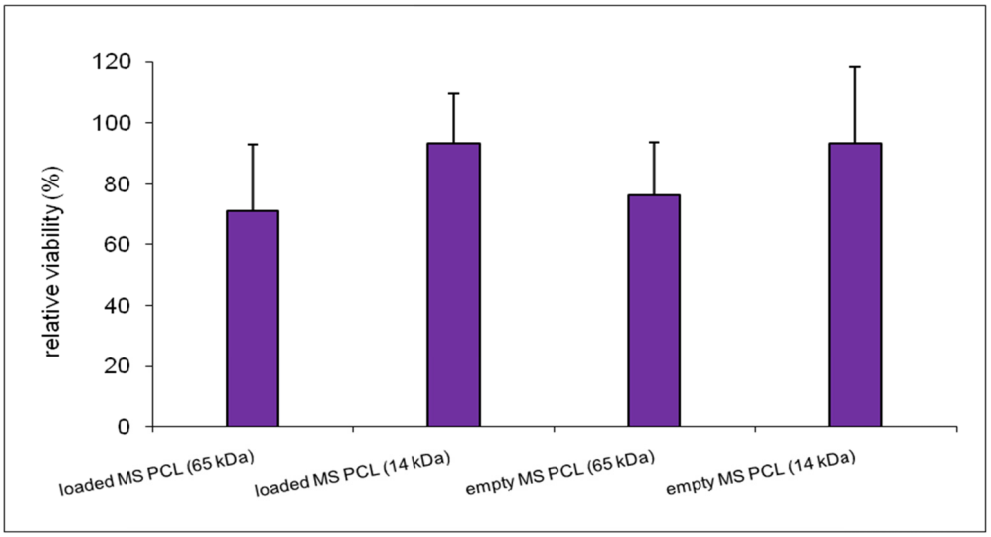


Figure 3.43. Relative viability of empty and drug loaded microspheres prepared by PVA (4 %) after 48 h incubation (n=5).

3.10.2. Effect of Doxycycline on Chondrocyte Viability

Doxycycline (0, 5, 10, 15, 20 µg/ml) dosage formulations were used to determine if doxycycline will have dose dependent toxic effect on 3T3 fibroblast cell line in the concentration range of release studies. Doxycycline is an antibiotic used to inhibit bacterial growth. However, in this study, the anti-collagenase activity (inhibitor of collagenase-3) of doxycycline (D) was used for treatment of osteoarthritis. Tetracyclines (e.g., tetracycline, doxycycline, minocycline) were an inhibitor of collagenases in tissues of osteoblasts, chondrocytes, and tumor cells, as well as extracts of diabetic rat skin and gingiva, human gingiva and synovial tissue, rabbit cornea, and rachitic rat cartilage. Golub (1991) indicated that 20 µM D inhibited 78% of the collagenase activity, *in vitro*. Hence, the dosage formulations were prepared also considering this property of the drug.

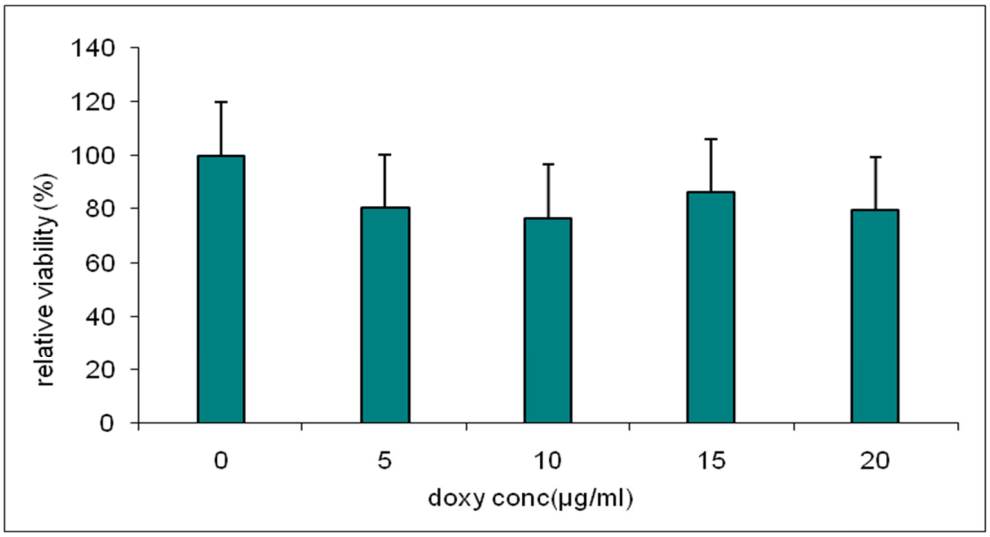


Figure 3.44. Relative viability of 3T3 fibroblast cell line incubated with different concentrations of doxycycline for 48h incubation

As shown in Figure 3.44, although doxycycline at these concentrations did not have anysignificant effect on cell viabilities, it showed slight numerical decrease from the control. Since all cells treated with D had relative higher than 80% it was concluded that D at these doses was not toxic.

3.10.3. Microscopic Examination of Effects of Doxycycline on Chondrocytes

The light microscopy pictures presented below (Figure 3.45) demonstrated that the characteristic morphology of cells did not change upon drug application, which was in agreement with the MTT results.

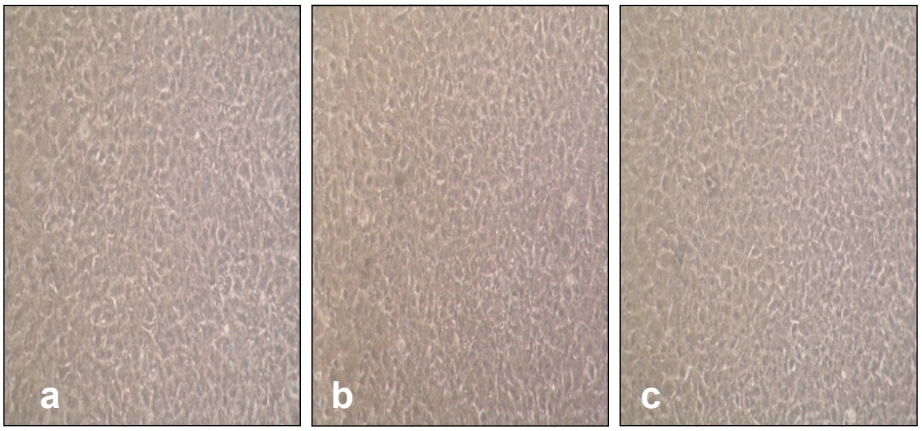


Figure 3.45. Phase contrast micrographs of of3T3 cells treated with a) 0 µg/ml (control) b) 5 µg/ml c) 20 µg/ml doxycycline

3.11. Primary Articular Cartilage Cell Culture

3.11.1. Monolayer Culture

Cells were cultured and expanded in monolayer before embedding into agarose. Treatment potency of the developed controlled release systems in terms of OA like changes was evaluated using chondrocytecells, which were isolated from osteoartritic rabbits. In Figure 3.46, morphology of chondrocytes isolated from healthy and collagen induced osteoarthritis adult rabbits are given at different stages of in vitro cultivation.

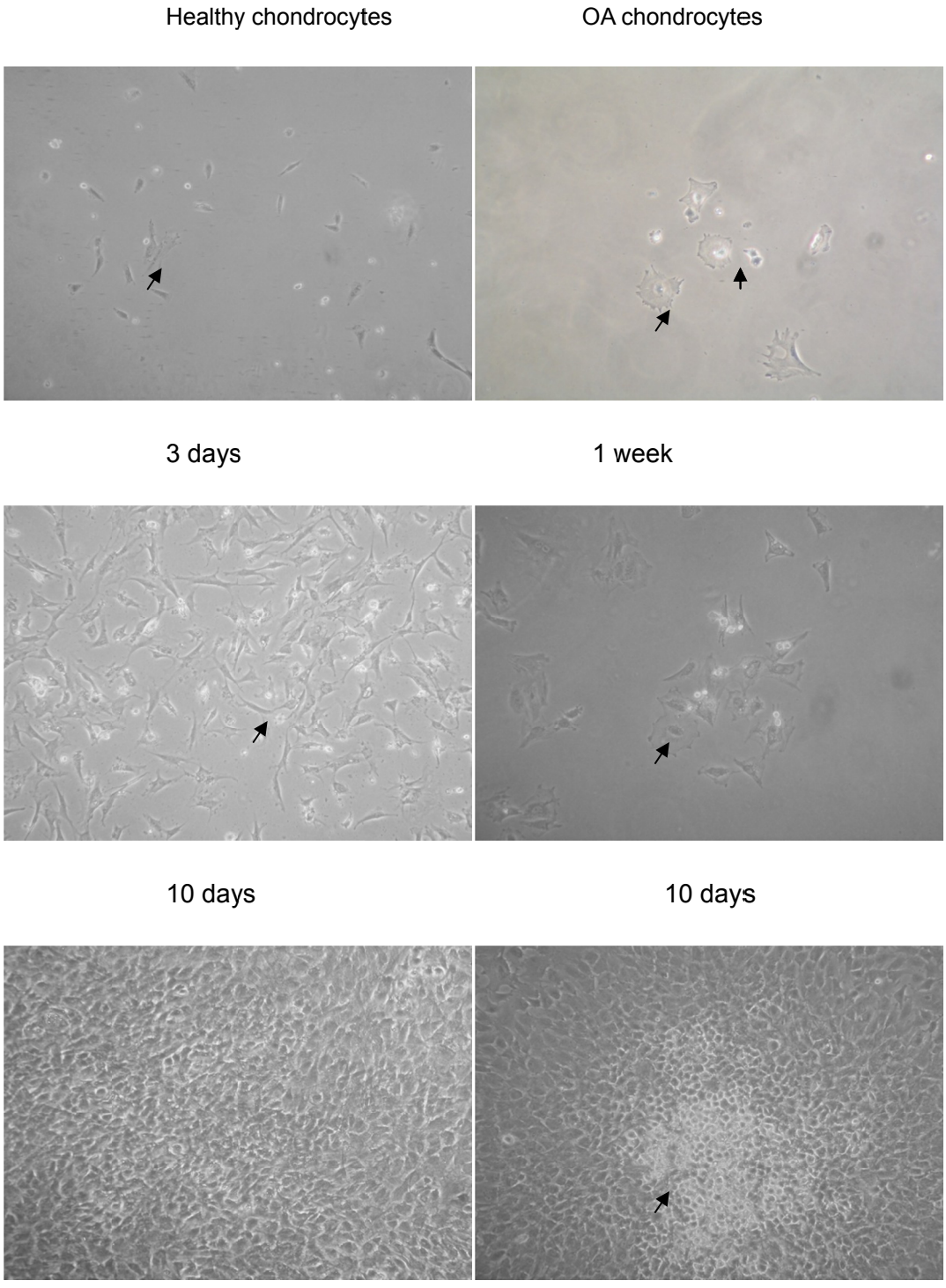


Figure 3.46. Phase contrast photographs of healthy and osteoarthritis induced cartilage cells (Arrows pointed the different zones of the cells (10X).

It was observed that the healthy chondrocytes adhered to the tissue culture flask and gained elongated shape. On the contrary, osteoarthritic chondrocytes kept their round morphology while adhering to the flask surface as seen in image of the cells at 10. This characteristic morphology of the diseased cells in culture has been defined as'fried egg' structure (Omelchenko, et al, 2002). In addition, the adhesion period was also different from each other for healthy and OA chondrocytes. Primary culture of healthy cells adhered only 3 days after introducing to culture flasks, while osteoarthritic cartilage cells could adhere after 1 week. The proliferation stages of these cells were also different from each other (Figure 3.46 c and d). Adhesion period also affected the proliferation stage negatively; proliferation of the cells was low. Cell morphologies at 85 % confluency are also shown in Figure 3.46. The morphological differences between healthy and OA cells were more observable at confluency. Here, both healthy and osteoarthritic cells had their specific polygonal morphology. However, osteoarthritic cells were smaller than healthy cells and showed large zones of "fried egg" structure.

3.11.2. Three Dimensional (3-D) Culture of Chondrocytes

For development of in vitro OA models researchers prefer to use osteoarthritic human articular chodrocytes in studies.(Nerucci, et al., 2000; Julovi, et al., 2004; Barksby, et al., 2006; Julovi, et al., 2008). In this study, instead of human OA chondrocytes, osteoarthritic rabbit articular chondrocytes were used to develop a 3-D model with agarose. While 3-D environment of the cells mimicked the natural environment of the cartilage, the medium took off the role of synovial fluid of the cartilage. Cell-agarose mixture was casted into wells of 24-well tissue culture plate where they gelled to 3-D form. In Figure 3.47, phase contrast photograph of agarose embedded chodrocytes were seen.

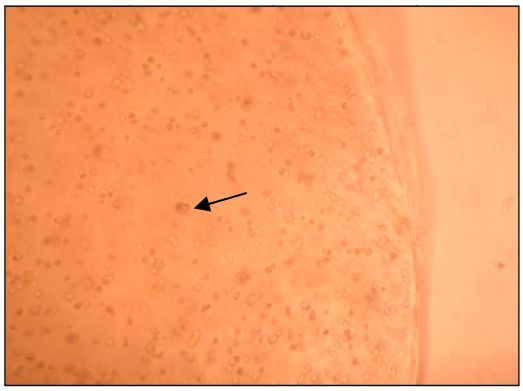


Figure 3.47. Phase contrast photograph of agarose-chondrocytediscs at day 3 (10X magnification) Arrow points cells. Arrow head points the border of agarose disc

3.12. Results of Functionality and Viability Analysis for In Vitro Osteoarthritis Model

3.12.1. DNA Assay

The true cell number in discs was estimated using their DNA contents to observe whether treatments had affected cell viability and also to normalize the amounts of synthesized matrix components (sGAG and collagen) with these values. In order to determine cell viabilites, DNA amounts were found fluorimetrically.

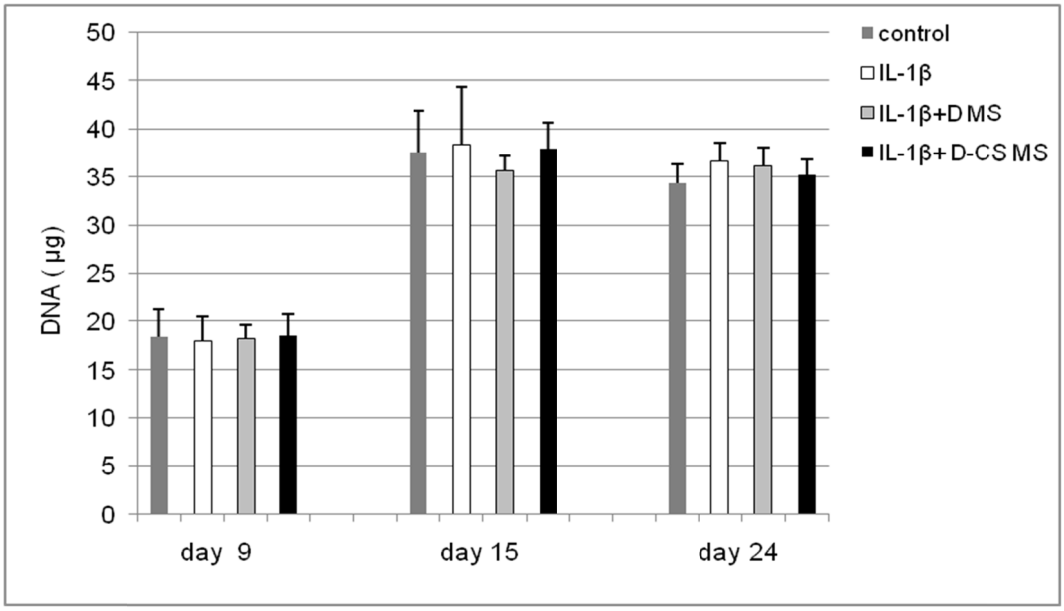


Figure 3.48. DNA amounts of agarose-chodrocyte discs of groups at 9th, 15th, and 24th days. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

According to Figure 3.48, there was no significant difference among groups in terms of DNA amounts within the same time point. DNA amounts of all discs increased significantly from day 9 to 15, which demonstrated the cell proliferated in agarose. For the DNA amounts, the differences between 9th and 24th days were also statistically significant ($p<0.05$). However, for all groups, the DNA measurements did not present a further growth trend from 15th to 24th days and the results here were not significantly different. Similarly, it was shown that IL-1β did not have any negative influence on cell proliferation in agarose culture (Kuroki, et al., 2003)

3.12.2. Glycosaminoglycan (GAG) Assay

The GAG amounts of discs and mediums were evaluated by using Dimethylmethylene blue (DMMB) assay. Total GAG amounts of discs were measured by DMMB assay and the data was normalized with DNA contents of all discs in order to assess the changes arising from differences in cellular activities between groups. In Figure 3.49 the GAG amounts of discs were shown at 9th, 15th and 24th days, respectively.

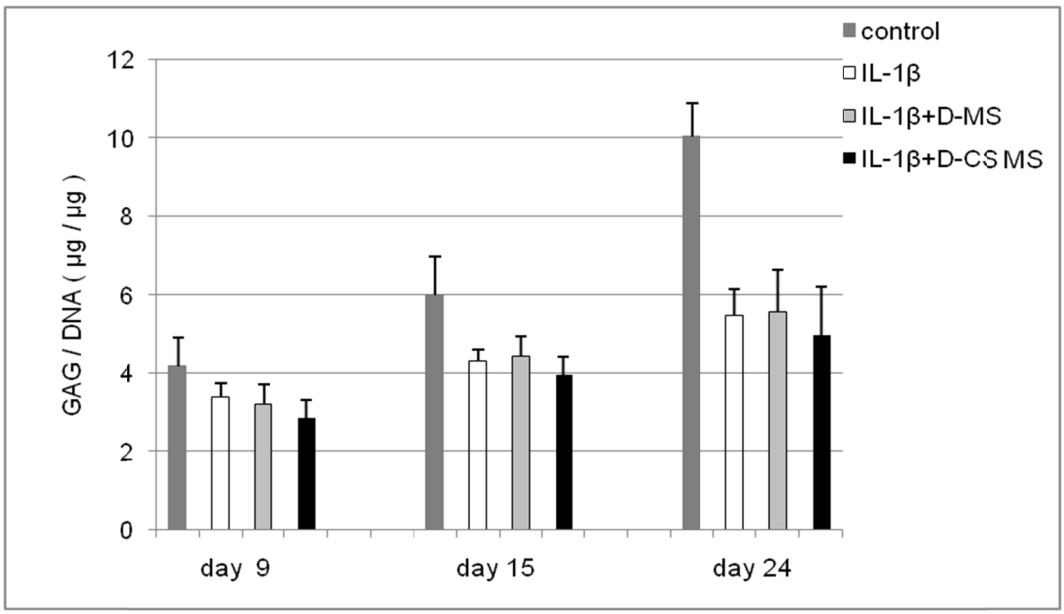


Figure 3.49. GAG content of agarose-chondrocyte discs at 9th, 15th, and 24th days. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

Mean GAG amounts of discs involving control group were significantly different from all IL-1β added groups (IL-1β, D MS, D-CS MS,) at 9th, 15th and 24th days (Figure 3.49). At 9th day, between IL-1β added groups, D-CS MS treated group was only statistically different from control group ($p<0.05$).

Similarly, normalized GAG amounts of discs at 15th day demonstrated that the IL-1β treated groups had less GAG content then IL-1β non-treated group (control). The

differences between IL-1β treated groups, when compared pair-wise, were not statistically significant.

Similar outcomes with other time points were observed for GAG amounts of discs at 24^{th} day; control was statistically different from all of the IL-1β treated groups and there were no significant differences among IL-1β treated groups.

When change in GAG contents of treatment groups according to sample collection time was considered, differences between 9^{th} and 24^{th} days' results were obtained to be statistically significant ($p<0.05$). GAG analysis of 3D agarose-chondrocyte constructs also demonstrated that 20 ng/ml of cytokine (IL-1β) can cause unfavorable effects on matrix accumulation by the cells. The decrease in GAG content upon IL-1β application might be caused by reduced synthesis of GAG (Kuroki, et al, 2005). Differences in GAG content between groups might be a result of decreased production, increased degradation or both (Kuroki, et al., 2003). This result demonstrated that, established drug delivery system did not have any positive effects on GAG synthesis. This result was expected since there was no such information in literature about the effect of antibiotics on GAG synthesis. Cell culture studies reported and suggested therapeutic use of tetracycline group antibiotics in order to inhibit the collagen degradation by MMPs. During OA development, cytokines such as IL-1 produced by activated synoviocytes, mononuclear cells or by cartilage itself significantly up-regulate MMPs (Fernandes, et al., 2002). An agent was needed to stop this reaction (Smith, et al., 1999; Blumberg, et al., 2008).

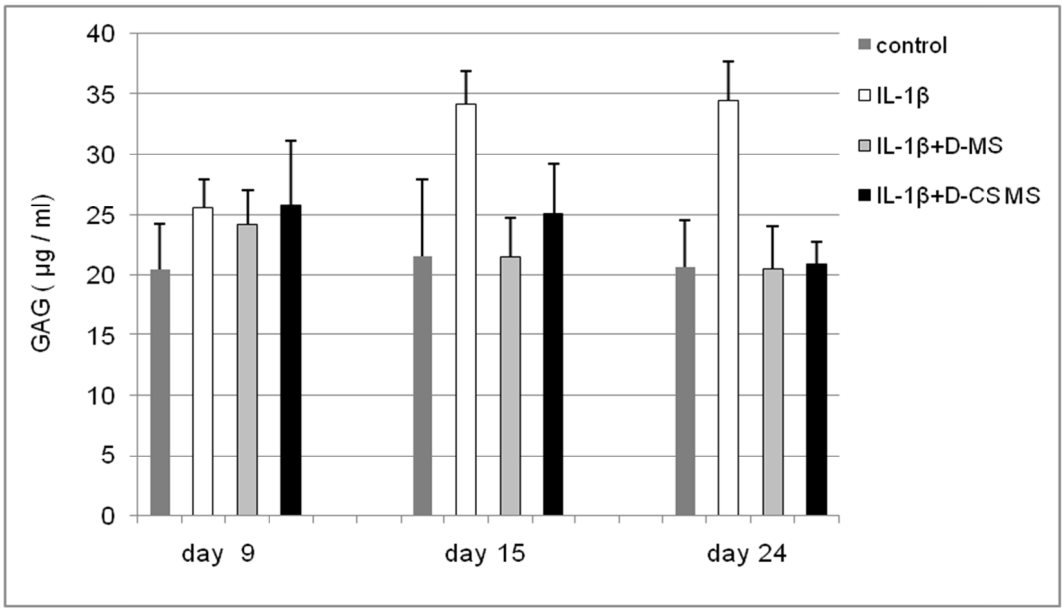


Figure 3.50. GAG amounts released into cell culture media at 9th, 15th, and 24th days. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

The next parameter related to OA development is the released GAG amounts from cells to cell culture media. In Figure 3.50, released GAG amounts into cell culture media results were presented. At the 9th day, released GAG amount of control group was significantly lower than all other IL-1β added groups. At the end of 15th and 24th days, only IL-1β added group was significantly higher than the other groups (control, IL-1β+D MS and IL-1β+D-CS MS groups). Owing to microsphere treatment, released GAG amounts considerably decreased. The differences between control and microsphere treatments were statistically not different ($p<0.05$) demonstrating the positive effect of released drug on this parameter. Doxycycline decreased the negative effects of IL-1β on GAG release into liquid media. Carprofen and dexamethasone have also been demonstrated to reduce the undesirable effects of IL-1β on GAG release into cell culture media (Dvorak, et al., 2002). Decrease in released GAG amount may be due to reduction of active enzymes (aggrecanase) that degrade proteoglycans into smaller units that can be released from the ECM. Researchers reported that, if cartilage is subjected to proinflammatory cytokines, only aggrecanase activity occurs. Thus, MMP activity was not the main outcome of

cytokine activity (Little et al., 1999; Tortorella et al., 2001). In literature, similar results were obtained by using IL-1β (Kuroki et al, 2005; Huh et al., 2009).

3.12.3. Hdroxyproline Assay

Collagen is synthesized from chondrocyte cells and it influences the mechanical properties of cartilage tissue. In literature, osteoarthritic cartilage cells were shown to release collagen into cell culture media (Little, et al. 2005; Palmer, et al. 2009; Wilson, et al. 2006). In order to estimate the total collagen content of the agarose-chondrocyte discs, their hydroxyproline contents were determined. Collagen content is found from hydroxyproline (HYP) content by taking the conversion factor of HYP to collagen as 8 (Reddy and Enwemeka, 1996 and Wilson, et al. 2007). HYP content was normalized by DNA contents as in the case of GAG results (Figure 3.49). In Figure 3.51, HYP content of discs were shown at 9^{th}, 15^{th} and 24^{th} days, respectively. On day 9, the difference in HYP contents of IL-1β and IL-1β+D MS treated groups was significant. On the other hand, HYP content of treatment groups and controls were not significantly different from each other on the three sample collection times ($p<0.05$). HYP contents of all IL-1β added groups showed increase from 9^{th} to 15^{th} and 9^{th} to 24^{th} days were statistically significant ($p<0.05$). Pairwise comparison of HYP contents of 15^{th} and 24^{th} days indicated that treatment groups were not statistically different from each other.

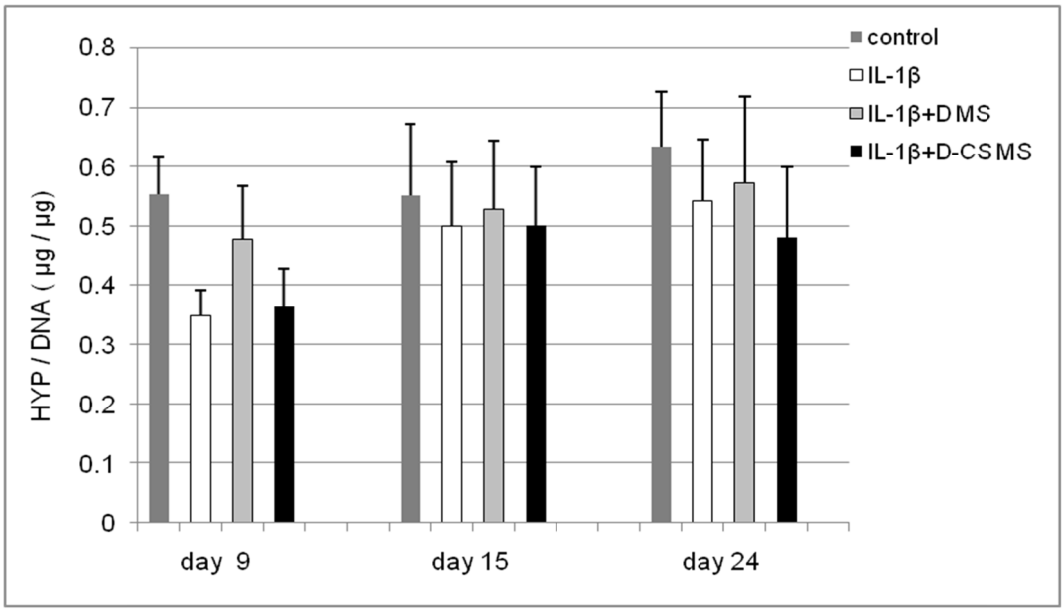


Figure 3.51. Hydroxyproline content of agarose-chondrocyte discs at 9th, 15th, and 24th days. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

The small numerical decrease in HYP content of negative control (IL-1β) compared to untreaed control indicated that IL-1β had adverse effects on collagen contents but the level of this effect was not as pronounced as decrease observed between these groups in the GAG contents. Therefore, we may conclude that the effectiveness of doxycycline (D) and doxycycline-chondroitin sulfate (D-CS) delivery systems were not recognizable at desired levels as these systems mainly aim to improve collagen accumulation by the OA cells rather than modifying GAG production/protection processes of the cells. In addition to this, the observation that decrease in the initial 9th day HYP results compared with control was mostly recovered despite continued IL-1β supply might suggest some other cellular mechanisms to recover from the effects of IL-1β on collagen metabolism.

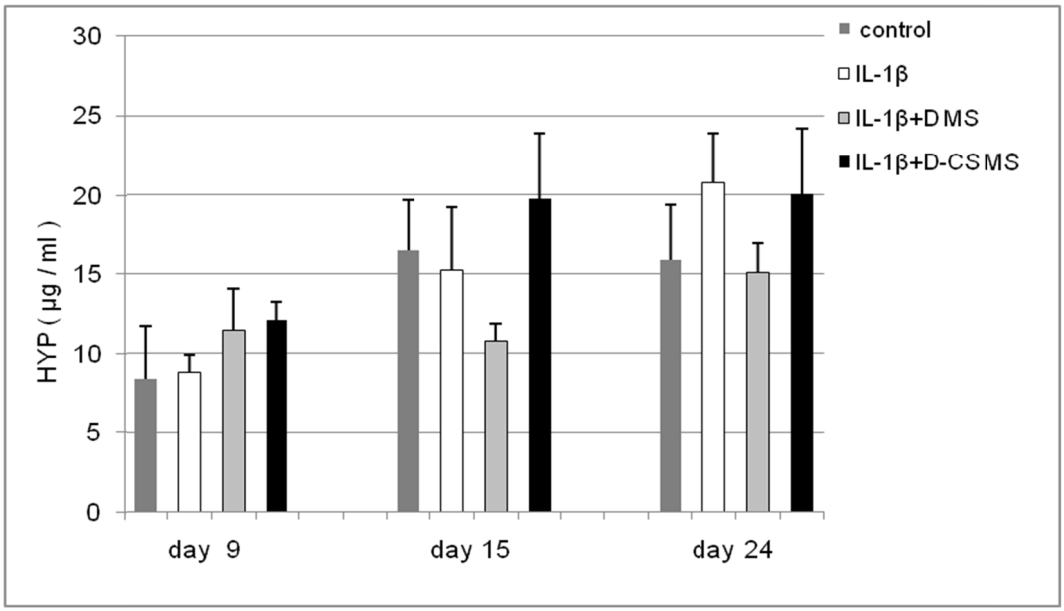


Figure 3.52. HYP amounts released into cell culture media at 9th, 15th, and 24th days. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

The released HYP was shown in Figures 3.52. Released HYP of 9th day; there were no significant changes between treatment groups. Collagen degradation was significantly increased after 15th day. At 15th day, IL-1β+D MS group was statistically lower than the other 3 treatment groups. At 24th day, control and IL-1β+D MS and IL-1β and IL-1β+D-CS MS groups were not statistically significant.

In Figures 3.51 and 3.52, the expected collagen degradation could not be proved. This result was mainly due to the difference in degradation mechanisms of proteoglycans and collagens. Active enzymes that degrade proteoglycans are called 'aggrecanase'. Collagen degredation is however, mostly done by Matrix Metalloproteinases which are induced by inactive proMMPs. These proMMPs inturn are activated upon after several factors. Nevertheless, it was reported that aggrecan degradation was not depended on this MMP activity (Arner, et al., 1997). IL-1β alone is not adequate to degrade collagens. Although, increase in proMMP synthesis is mainly due to IL-1β presence in the system, activation of them requires other factors such as presence of several enzymes (Beekman, et al, 1998). Finally, collagen damage could not be demonstrated clearly in this study. In fact, other

studies suggested that at least 4 weeks culture with 50 ng/ml IL-1 applications made it possible to measure collagen degradation (Kozaci, et al., 1996; Ellis, et al., 1994; Mitcell, et al., 1994). Another prospective contributor to this result might be the serum contained in cell culture media. Serum has an inhibition effect on proteinases and it also inhibits the IL-1 stimulated degradation of collagen in cartilage (Kozaci et al, 1997).

3.12.4. MMP-13 Concentrations

Several MMP members are known to be involved in OA, especially the collagenases; MMP-1, MMP-8 and MMP-13. The collagenase group members degrade type II collagen in the cartilage matrix. Inflammatory cytokines such as IL-1 induced MMPs during cartilage degradation. MMP-3 and 13 are secreted from chondrocytes (Kim, et al, 2005).

In Figure 3.53, MMP-13 concentrations of cell culture media were given. In the 9^{th} day of the experiments MMP-13 concentration of IL-1β treated and non-treated groups were not significantly different from each other ($p<0.05$). However, numerically, IL-1β control had the highest concentration of MMP-13 ($p<0.05$). Yet, Microsphere treatments were observed to have MMP-13 amounts in between two controls, suggesting that released doxycycline inhibited MMP-13 activity to some extent.

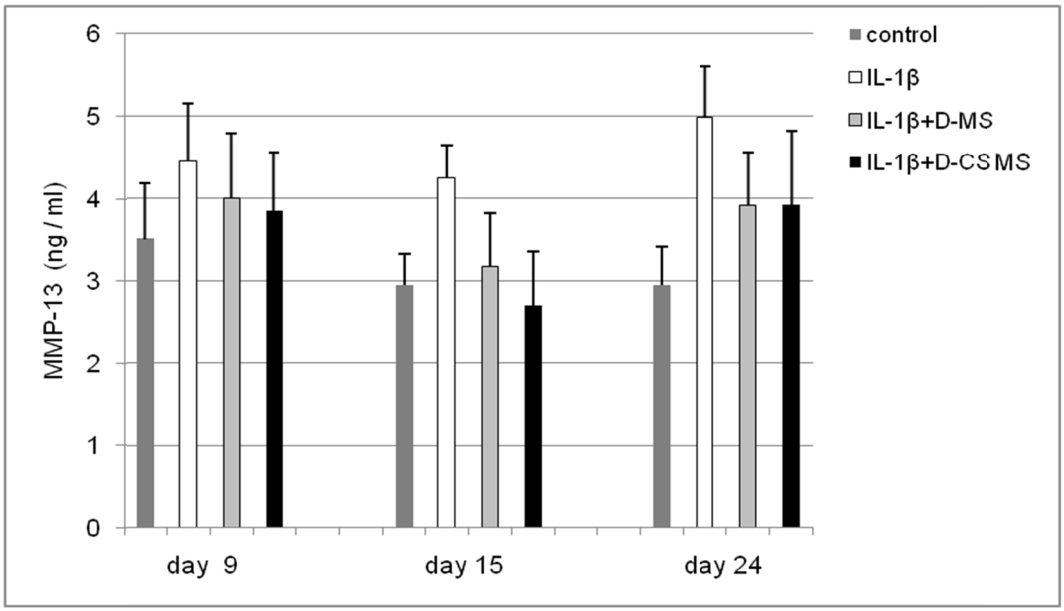


Figure 3.53. MMP-13 levels in cell culture media at day 9, 15 and 24. Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

The MMP-13 results of 9th day were not as pronounced as 15th day results in terms of showing the effect of model. At the 15th day of the experiment, the difference between results of control and IL-1β group was significantly different from each other ($p<0.05$) (Fig 3.53). Also both MS treatments seemed to have more obvious effect in terms of decreasing the MMP-13 amounts to the levels comparable with control. In the 24th day of the experiments MMP-13 concentration of IL-1β treated and non-treated groups were significantly different from each other ($p<0.05$, Figure 3.53). Both MS treatment groups were different from both controls but these changes were not statistically significant.

The MMP-13 results were unexpected at 9th day where inhibition of activity was not very high. This situation, however, was reversed in the expected direction mainly due to enhancement of released amounts of doxycycline into the medium. As explained in the next section, at 15th day, the released doxycycline amount increased more. Due to this outcome and the dynamic behavior of in vivo environment, 5 mg microsphere dosage level was thought to be useful for vivo experiments. In addition, in order to see the dose related effects of the

microspheres, 7.5 mg microspheres dosage was also thought to be investigated in in vivo experiments.

Concentrations of MMP-13 were significantly higher in groups for the OA model than in groups did not contain IL-1 for the first two time points of this study. In literature, similar results were documented for MMP-13 concentrations of negative controls. (Dvorak, et al., 2002).

Finally, it can be concluded, doxycycline release inhibited the MMP-13 activity, but to different degrees at different time points. MS treatments were not significantly different from each other at any of the time points ($p<0.05$). Doxycycline was used to decrease the levels of MMP-13. It has a protective effect against experimental OA (Greenwald, et al, 1994). It was reported that, this effect can be through controlling the MMP levels. Other studies also showed that it inhibits the activity of MMP-13 (Smith, et al., 1999).

3.12.5. Release Profiles of Doxycycline Loaded Microspheres

At the third day of the cell culture experiments, doxycycline loaded microspheres (D MS and D-CS MS) were added to see the effects of released bioactive agents on chondrocytes. Since release media is an important factor in modifying release properties of such polymeric systems, the released drug amounts in media were measured for cell culture experiments. In Figure 3.54, the release profiles of D MS and D-CS MS groups are presented. The D-CS MS microspheres had higher cumulative drug release than D-MS ones probably due to having smaller particle size and for being more hydrophilic than D-MS microspheres.

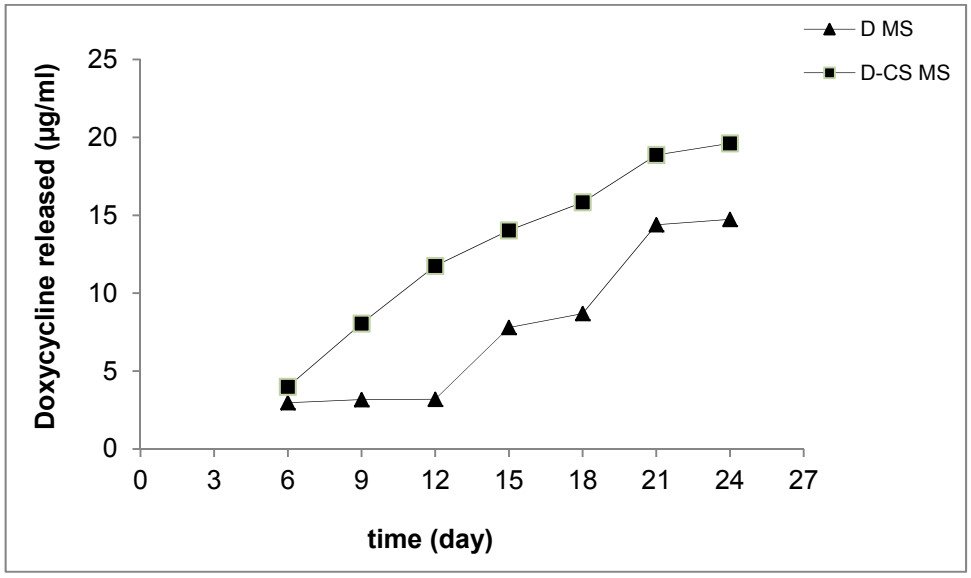


Figure 3.54. Release profile of doxycycline in cell culture media at the end of 24th day

The activity of MMP-13 against collagen type II was inhibited upto 50-60% by 30 µM doxycycline application (Smith, et al, 1999). In another previous study, Golub (1991) indicated that 20 µM doxycycline inhibited 78% of the collagenase activity. According to release profile, released doxycycline amount cannot reach the 20-30 µM level at the 9th and 15th days of the experiment. So, the inhibited MMP-13 percent was lower than 50% at these days. Between 15th-24th day periods, D MS release rate got increased rapidly. However, this rapid increase did not give as high doxycycline amounts as D-CS MS in culture media.

Although D-CS MS released higher than D MS, the decrease of MMP-13 content of these two groups were not significantly different from each other. The main reason of this might be the doxycycline amounts being not very high and it's being replenished in each medium exchange. Thus, for the longer time period release together with other enhancing parameters, the designed drug delivery system would be more efficient in terms of this problem during in vivo conditions.

3.13. Assessment of In Vivo Establishment of Experimental Osteoarthritis and it's Treatment Efficacy with Microspheres

3.13.1. Model Development

For establishment of experimental osteoarthritis (OA), intraarticular collagenase injection was performed twice on day 1 and day 4 (Kikuchi, et al, 1998; Choi, et al, 2002; Huh, et al, 2008)

In Figure 3.55, the radiography results of rabbits 3 weeks after injections of collagenase are presented. Radiological score was 2 in front view, however, in lateral view radiological score was 1, thus Indicating osteophytes with unimpaired joint space with yet doubtful clinical significance.

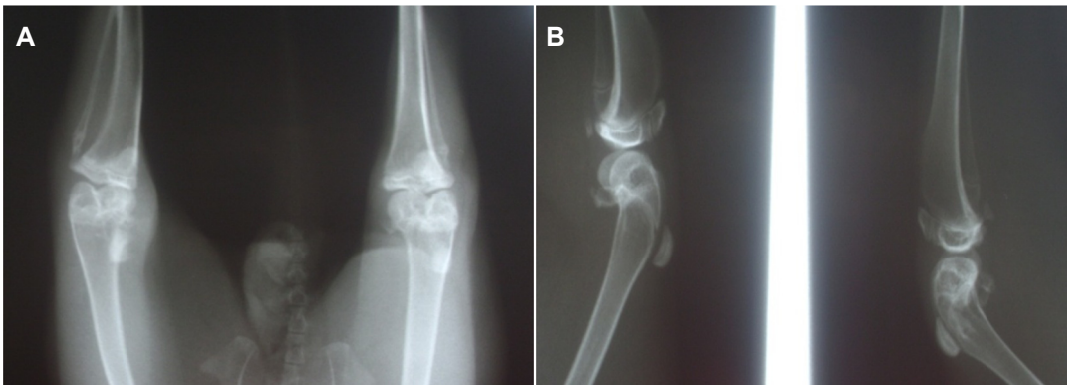


Figure 3.55. The radiograph of joints 3 weeks after collagenase injections a) front b) lateral views

Kikuchi, T et al (1998) indicated that for developing experimental OA, the waiting period after injections was 6 weeks. In Figure 3.56, radiographs of joints 6 weeks after injections are shown. The front and lateral views of joints were evaluated with a score degree of 2 for lateral and front view of right (R) and left (L) joints of 3rd sample. Number 2 right (R) front view and number 4 left (L) front view had a score degree of 0 (zero). However, all other samples had a score degree of 1. Radiography results were not enough to say that experimental OA was established or not.

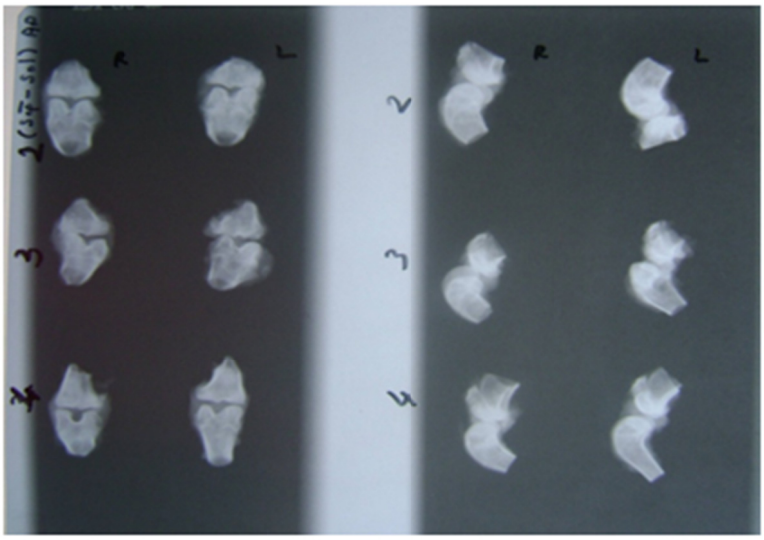


Figure 3.56. The radiographs of joints 6 weeks after collagenase injections

3.13.2. Radiological Evaluations

Figures 3.57-65 represent radiography results of these experiments. Representative radiographs of osteoarthritic joint that had no treatment during 4 weeks period following OA development.were shown (Figure 3.57). These joints had a mean radiological score of 2 in lateral view but 1 in front view.

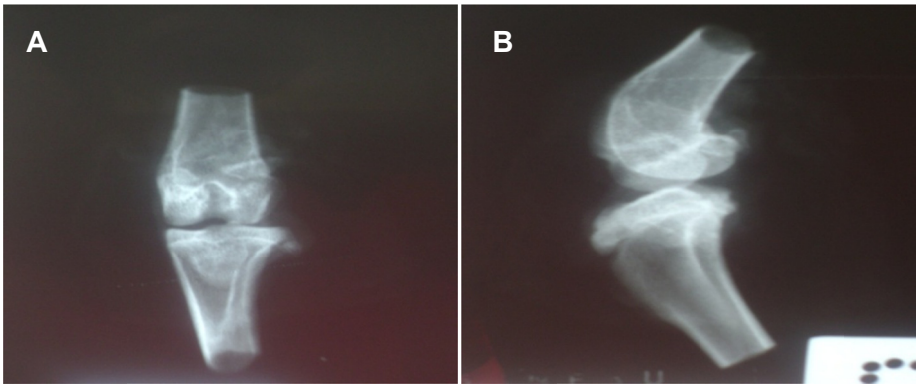


Figure 3.57. OA induced, untreated group at the end of 4 weeks a) front b) lateral view radiographies (10 weeks following collagenase injections)

The next group was hyaluronan injected control group. At the end of 6 weeks of OA development, hyaluronan was injected for treatment. Half of the subjects were left for 4 weeks without further treatment and the other half were kept for another 4 weeks (totally 8 weeks after hyaluronan injection). Rabbits terminated at 4 weeks were scored according to radiographs given in Figure 3.58. The radiological scores were measured as 1 for this treatment. Hyaluronan (HA) is the main component in synovial fluid and cartilage matrix. Lubrication of the joints is provided by HA.

Although the level of the treatment with HA applicationis unclear, the clinical benefit of HA has been indicated with the pain relief of the patients. Accumulation of HA at the surface of the cartilage inhibits the chondrolysis and MMP-3 production into cartilage tissue for the first week. For second or third week MMP-3 blocking could not be detected (Julovi, et al., 2004). HA behaves as a barrier at the surface of the cartilage. It was reported that HA injections did not treat the OA; however, it delayed the OA formation for a short time period following the injections (Julovi, et al, 2004; Julovi, et al, 2008; Hashizume and Mihara, 2009).

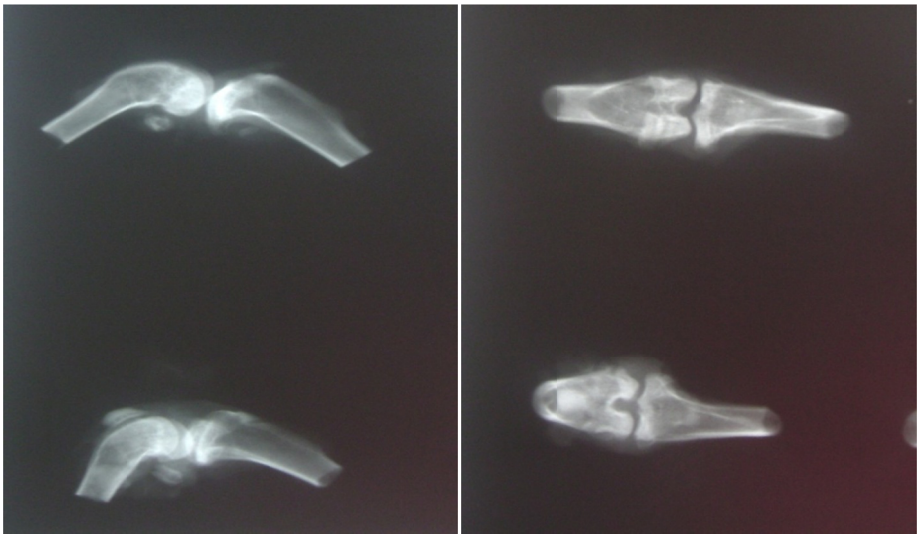


Figure 3.58. OA developed and hyaluronan injected joint radiographs 4 weeks after injections a) lateral b) front view

Example joint radiographs of second group of hyaluronan injected rabbits which were terminated after 8 weeks are given in Figure 3.59. Radiological scores of this treatment varied between 2 and 4. It can be clearly said that the positive effect of HA application observed in 4 week treatment follow up of the joints was lost in longer time period.

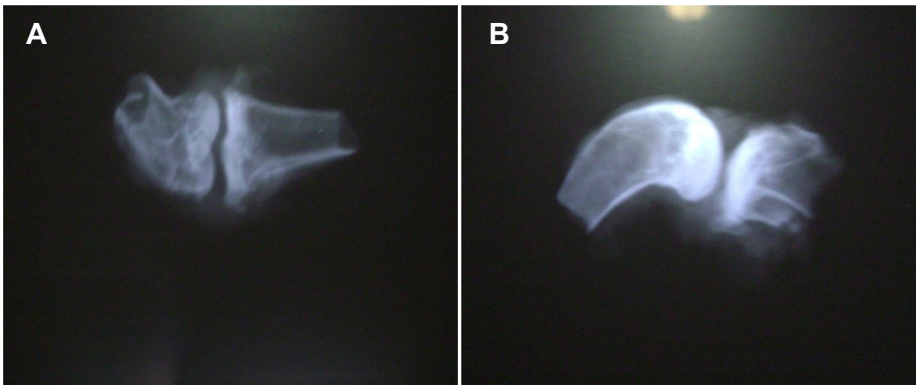


Figure 3.59. OA developed and hyaluronan injected joints 8 weeks after injections a) front b) lateral view radiographs

The other treatment groups were D MS and D-CS MS injected ones. These groups involved drug treatment by means of doxycycline (D) and doxycycline-chondroitin sulfate (D-CS) loaded microspheres. Considering the possible change in release kinetics in the synovial fluid, two dosage forms (5 mg and 7.5 mg) of these microspheres were applied in vivo.

In Figures 3.60 and 61, radiographs of 5 and 7.5 mg D MS injected joints at the end of 8 weeks are given respectively. The radiological score of the front view low dose MS injected cartilage was 3, however; lateral view score was 4. The score values varied between 2 and 4 in 6 replicates.

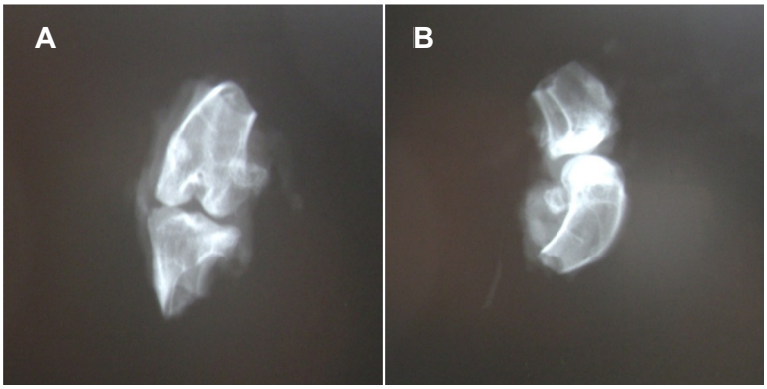


Figure 3.60. OA developed and 5 mg D MS injected joint radiographs 8 weeks after injections a) front b) lateral view

The radiological score of the front and lateral view of higher dose MS applied cartilages (Figure 3.61) was 3. Ostearthritic effects got increased then.

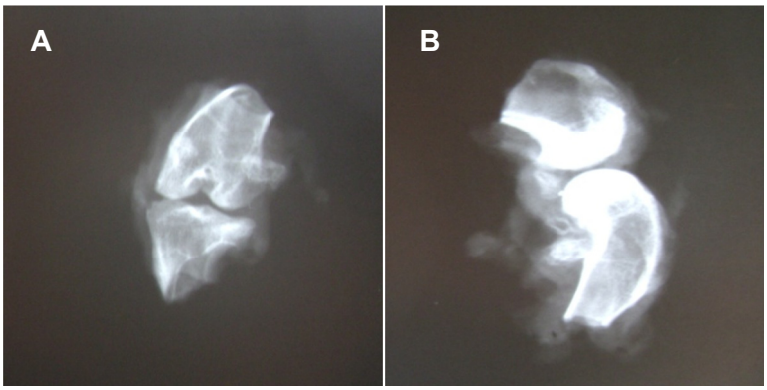


Figure 3.61. OA developed and 7.5 mg D MS injected joint radiographs 8 weeks after injections a) front b) lateral view

In Figures 3.62 and 63, 5 and 7.5 mg D-CS MS applied joints are shown, respectively.

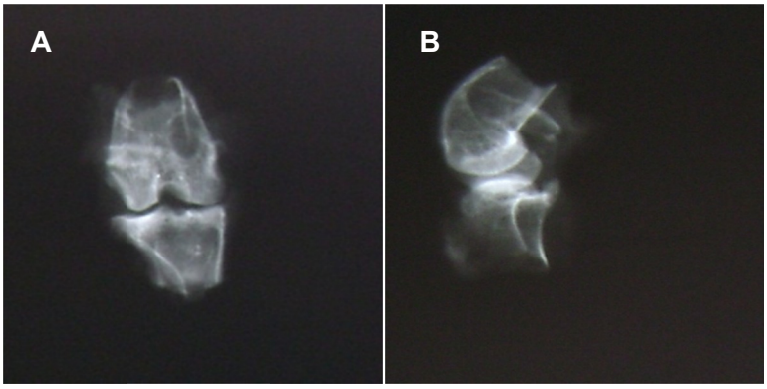


Figure 3.62. OA developed and 5 mg D-CS MS injected joint radiographs 8 weeks after injections a) front b) lateral view

According to radiological evaluations of 5 mg D-CS MS injected joint the cartilage scoring was 2 for front view, and the score was 3 for lateral view. However, when 7.5 mg D-CS MS injected joints' cartilage was scored for front and lateral view, the values were 1 and 2, respectively. It can be suggested that the osteoarthritic development started to draw back now.

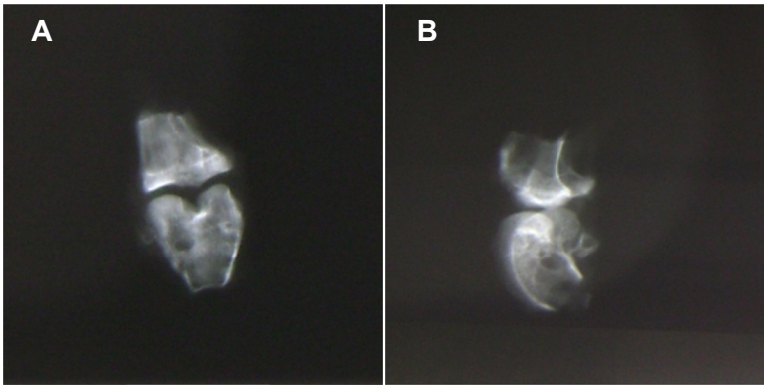


Figure 3.63. OA developed and 7.5 mg D-CS MS injected joint radiographs 8 weeks after injections a) front b) lateral view

In Figure 3.64, front and lateral view samples of the healthy cartilage were presented.

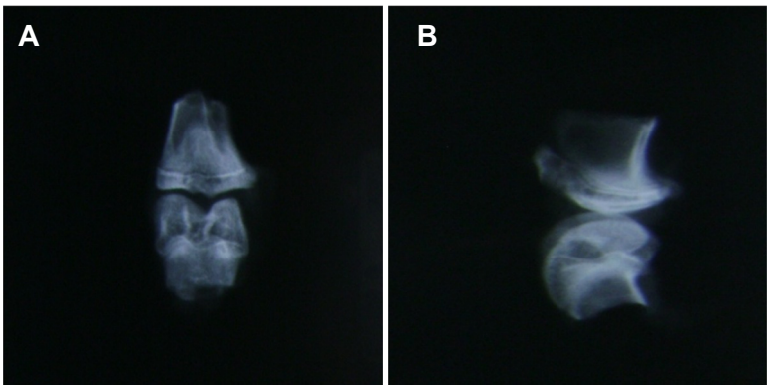


Figure 3.64. Healthy rabbit joint (Positive control) radiographys 8 weeks after injections a) front b) lateral view

In Figure 3.65, OA developed but untreated rabbit joint is shown. The score of this joint was 4 for both lateral and front views. Thus, definite osteophytes with severe joint space narrowing and subchondral sclerosis were present according to radiological observations which were confirming severe OA formation.

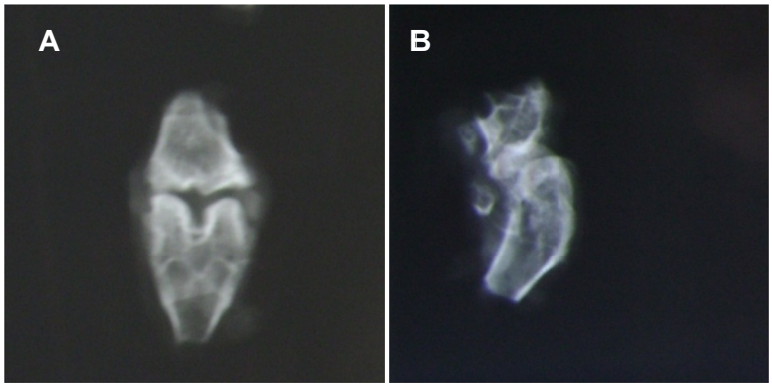


Figure 3.65. OA developed and untreated group joint radiographs (Negative control) 8 weeks after injections a) front b) lateral view

Radiological evaluations were carried out to support the results of histological and mechanical analyses of the joints. However, they were not as conclusive as histological findings of in vivo establishment of the OA model. This was thought to be related with difficulties in scoring these small joints with the limitations in the required degree of degenerations. Yet, in long term follow up, the positive effect of high dose (7.5 mg) D-CS MS on OA treatment was apparently better than other groups according to the radiological analyses.

3.13.3. Indentation Testing

The stiffness and hardness of the cartilage after these treatments were evaluated by using a hardness tester special for viscoelastic materials like cartilage. The comparison of mechanical test results of cartilages of all groups was given in Figures 3.66 and 67 after separate measurements of lateral and medial condyles.

Low values are the sign of the softness of the samples. According to Figures 3.66 and 3.67, the osteoarthritic joints that did not receive any treatment had the smallest mean hardness vales. Thus, osteoarthritis makes the cartilage tissue less resistant to indentation, or softer compared to the healthy state. The heathy cartilage has the highest hardness value for both parts of joints (Bae, et al., 2003, Darmanis, et al, 2006, Niederauer et al, 2004). In another study the hardness values of heathy cartilage of rabbit was measured and compared with static treatment (Periosteal transplantation) groups. The treatment group was lower than untreated group (Darmanis, et al, 2006).

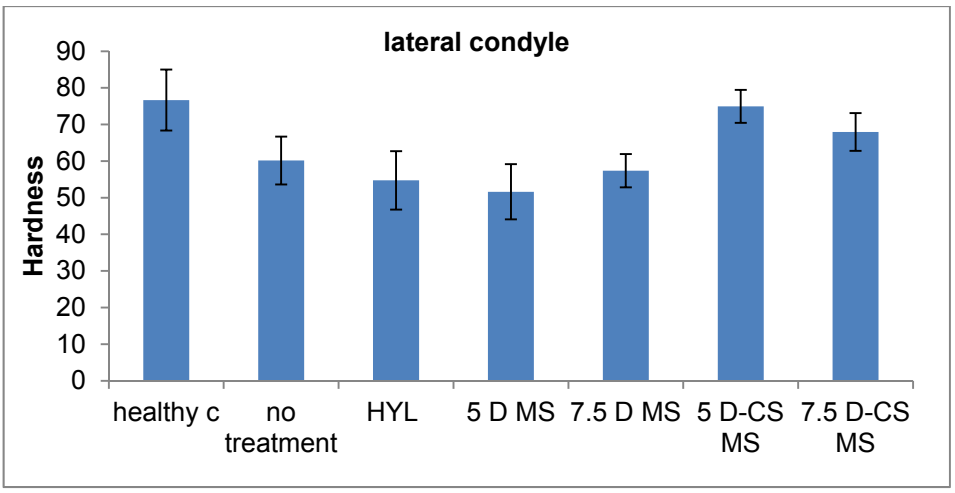


Figure 3.66. Hardness measurement results of lateral condyle of rabbit joints for all groups

In Figure 3.66, hardness values of lateral condyle of joint are given. Healthy cartilage and 5 mg D-CS MS with HYL injected joint had the highest hardness values. According to Mann-Whitney U test, the hardness of healthy cartilage was significantly different from the hardness of cartilage of no treatment, 5 mg D MS and 7.5 mg D-CS MS with HYL injected joints. Except 5 mg D MS and 5 mg D-CS MS with HYL injected values, differences between no treated joint was statistically insignificant (higher than) with other treatments. HYL injected joint was significantly lower than those of both D-CS MS injected hardness values. Microsphere injected joints were statistically different from each other except 5 and 7.5 mg of D MS values ($p<0.05$). According to these results, D-CS MS combination treatment was more effective than D MS combination.

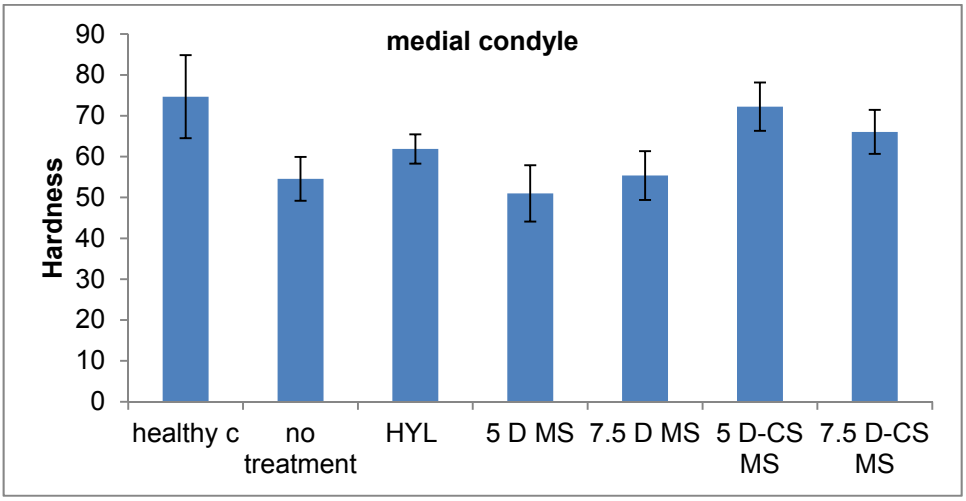


Figure 3.67. Hardness measurement results of medial condyle of rabbit joints for all groups

In Figure 3.67, hardness measurements of medial condyle of joints were compared. Healthy cartilage and 5 mg D-CS MS with HYL injected joints had the highest hardness value. Healthy cartilage was significantly different from no treated, 5 mg D MS and 7.5 mg D-CS MS values. Non-treated cartilage was not significantly different from 5 and 7.5 mg D MS with HYL injections. HYL treatment was statistically different from 5 mg D MS and D-CS MS injected groups. MS injected groups were also analyzed. Differences between 5 mg and 7.5 mg D MS and 5 mg and 7.5 mg D-CS MS values were statistically insignificant. Other two paired groups were statistically significant with each other.

The differences between hardness values were due to the lesions of the cartilage tissue. A representative figure for osteoarthritic cartilage of the rabbit joint is shown in Figure 3.68. Arrow points the cartilage lesion.

Figure 3.68. Osteoarthritic articular joint with arrow pointing the cartilage lesion.

3.13.4. Histological Evaluations

3.13.4.1. Light microscopy

Mankin and the OARSI (Pritzker) scores were significantly correlated in all groups. The descriptive statistical data of both scores are presented in Tables 3.8 and 3.9. The OA groups (OA model group and the OA group with no treatment) received significantly higher Mankin/Pritzker scores exhibiting grade 3 to 5 OA when compared to those of the healthy control (p=0.00) This demonstrated us that the OA model was successfully developed.

The moderate to severe cartilage damage that was noted in all OA untreated and the OA model group samples were observed to be restoring at different levels in the treatment groups. However, none of the joint samples gave the similar scores with the healthy control. The cartilage lesions consisted of the different degrees of the safranin O staining loss, surface fibrillation, vertical clefts, decreased number or the hypertrophy/proliferation/degeneration of the chondrocytes, The cartilage lesions were more severe in hyaluronan only treated groups when compared to other treatment groups.

The 4 week hyaluronan (HYL) treatment (Mankin p=0.005, Pritzker p=0.018), D MS (5 mg -Mankin p=0.017, Pritzker p=not significant-and 7.5 mg-Mankin p=0.00, Pritzker p=0.006-) and, the D-CS MS (5 mg-Mankin p=00.00, Pritzker 0.00- and 7.5 mg-Mankin p=0.00, Pritzker p=0.00) treatments significantly improved the Mankin/Pritzker scores when compared to those of the OA no treatment group (Figure 3.69).

The D-CS MS treatments generally presented better scores comparing to the HYL only groups; but the differences were statistically significant between the HYL 4 week group and the 7.5 mg D-CS MS group (Mankin p=0.022); HYL 4 week and the 5 mg, 7.5 mg D-CS MS groups (for 5 mg treatment Mankin p=0.014, Pritzker p=0.011 and, for 7.5 mg treatment Mankin p=0.005 Pritzker p=0.003 respectively). The D MS and the D-CS MS groups exhibited histologically similar therapeutic performance with usually no statistically significant difference. The only exception was the significantly higher Pritzker score of the 7.5 mg D-CS MS group comparing to that of the 5 mg D-MS group (p=0.013) (Figure 3.70).

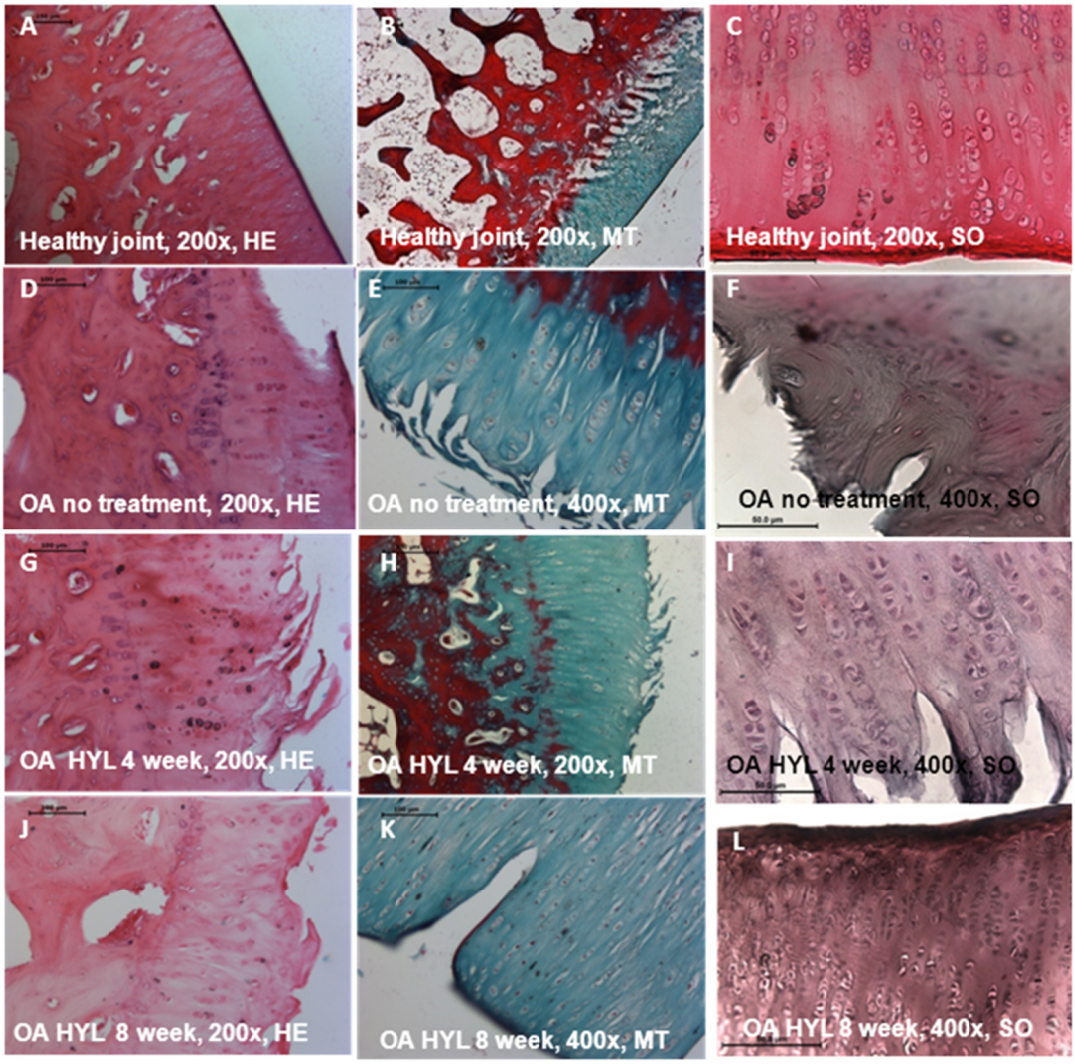


Figure 3.69. The articular cartilage of the healthy joint exhibits normal morphology with smooth cartilage surface, intact tidemark integrity and healthy cartilage cell organization. In D-L, different grades of OA-induced cartilage degeneration with fibrillation, cleft formation (in K), chondrocyte damage and matrix degeneration, loss of safranin O staining (in F, I and L) are observed. Bone is exposed in F. The degeneration severty in hyaluronan groups (G-L)is less than the OA no treatment group. OA: Osteoarthritis, HE: Haematoxylin eosin, MT: Masson's trichrome, SO: Safranin O.

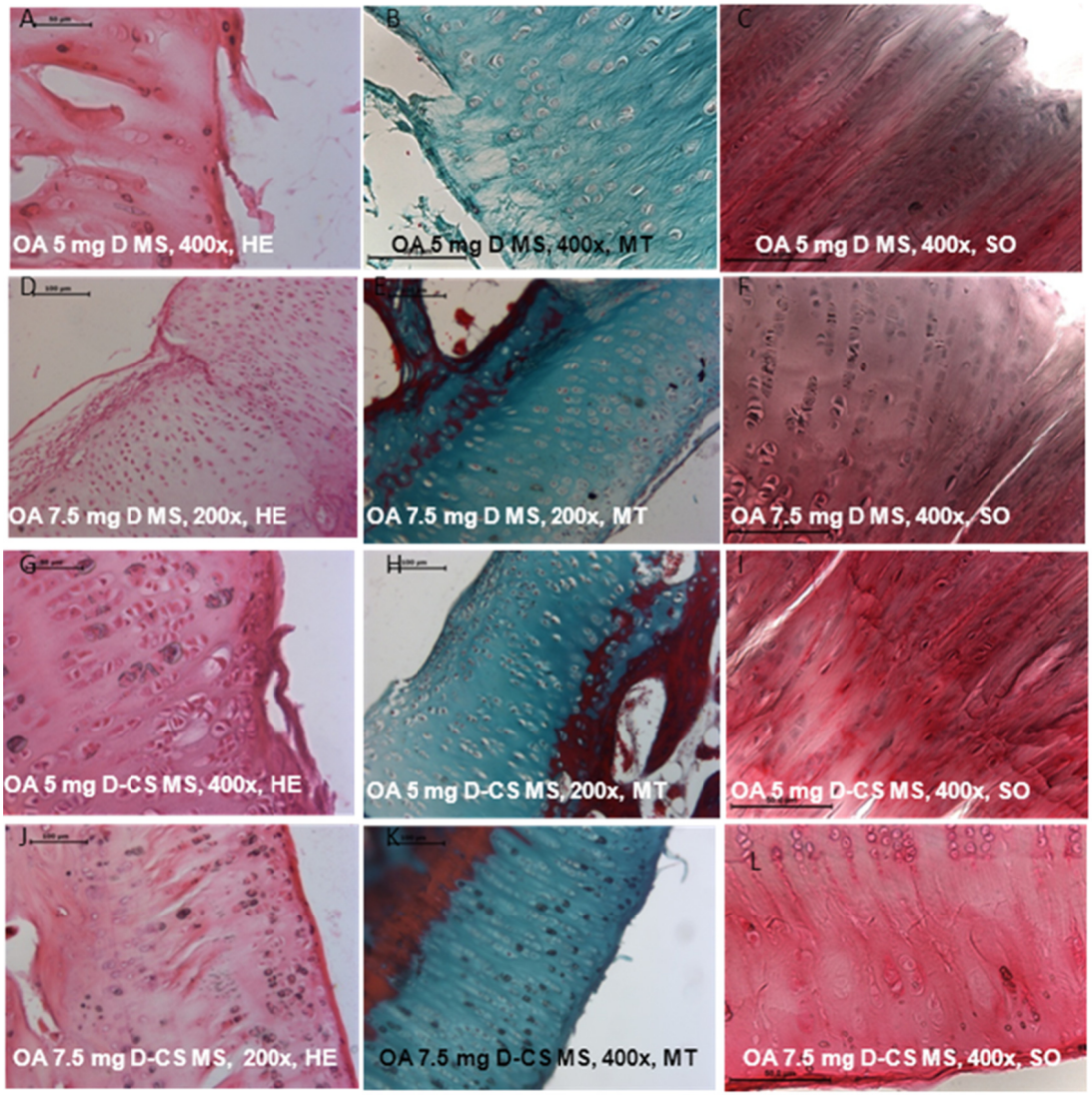


Figure 3.70. The articular cartilage of the different treatment groups exhibits mild to moderate OA-induced degeneration with fibrillation, chondrocyte damage and matrix degeneration. The degeneration severeity is less than that of the OA notreatment in all of them. Note that the safranin O staining is regained to some extent with the treatment modalities. (in C F, I and L). In j to L the mildly rough to smooth articular surface is appearent. OA: Osteoarthritis, HE: Haematoxylin eosin, MT: Masson's trichrome, SO: Safranin O.

Table 3.8. Descriptive statistical data of the Mankin scores

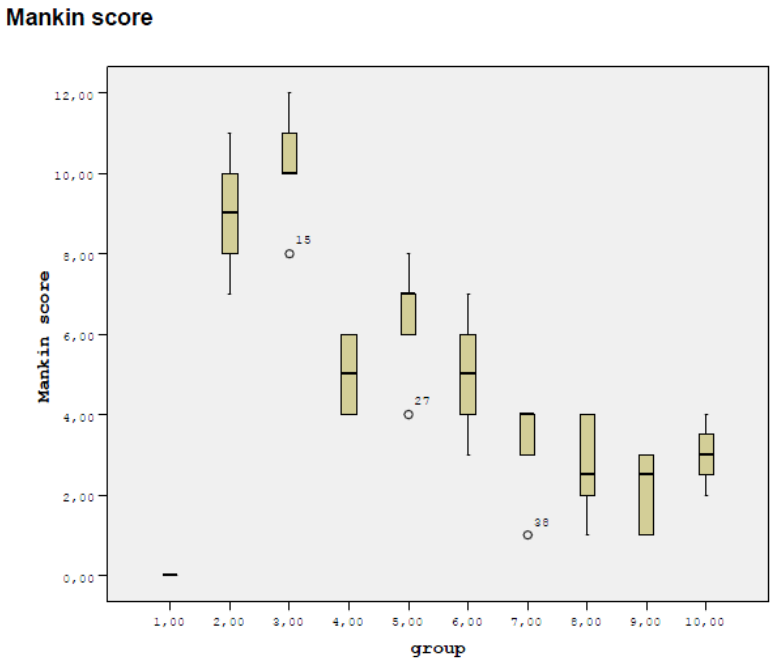
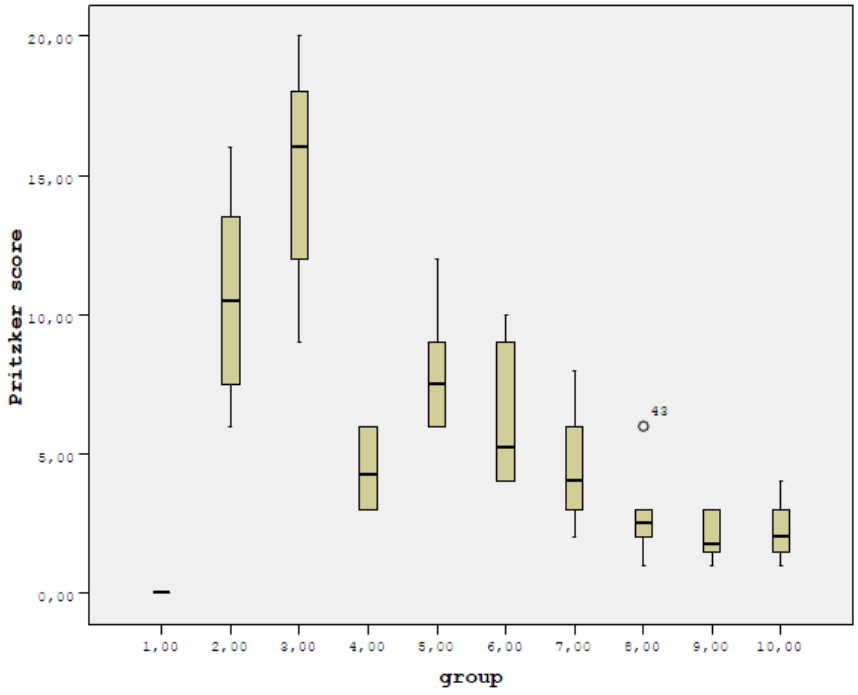


Table 3.9. Descriptive statistical data of the OARSI Pritzker scores



CHAPTER 4

CONCLUSIONS

Polymeric controlled drug delivery systems have many advantages over conventional drug therapies like reduced side effects, possibility of local or targeted application of the treatment and enhanced treatment potency.

In this study, doxycycline or doxycycline-chondroitin sulfate encapsulated polycaprolactone (PCL) microspheres were prepared and studied for their potency as a new treatment approach for osteoarthritis (OA). OA is one of the joint diseases which have the highest negative effects on elderly people. It is widespread all over the world; however, its pathophysiology is not completely understood yet. Conventional therapies are mostly based on alleviation of symptoms like pain. Non-steroidal anti-inflammatory drugs (NSAIDs) which are taken orally are amongst the common conventional therapy. Intraarticular injection of hyaluronan is the best clinically applied medication treatment which still does not provide recovery from the degenerated cartilage. The approach developed here is different from current therapies for; 1) involving sustained delivery of an anti-collagenase agent (doxycycline) for preventing further cartilage damage by collagenases, 2) aiming a local-sustained treatment of OA with single application of polymeric microspheres, 3) providing chondroitin sulfate via release system to enhance cartilage tissue regeneration.

Two different molecular weights of PCL (14 and 65 kDa) were used to obtain desired properties in the developed systems. Modifications on MS preparation conditions were also carried out for this purpose. According to Higuchi and Korsmeyer-Peppas curve fitting models, release of microspheres prepared from PCL (14 kDa) were governed by Fickian diffusion. The experimental release results were mathematically modeled by using diffusion based model. In view of that, the release profile of microspheres prepared from low molecular weight PCL (14 kDa) was more compatible with the diffusion model than those of microspheres prepared

from PCL (65 kDa). Drug release of microspheres which were prepared by using PCL (14 kDa) was therefore diffusion-controlled. The modeling results might indicate that in the case of high drug loadings (25-30 %), the whole drug or a large part of it was in the crystallized form. As a result, the drug release was diffusion-controlled at low loadings (10-17 %) where the solid drug was randomly dispersed in the matrix. The estimated diffusion coefficients of doxycycline were calculated from experimental results and the values varied in range of 0.6-1.25 $x10^{-9}$ cm^2/s.

Although there are many PCL microsphere studies, D MS and D-CS MS PCL (14 kDa) microspheres have been evaluated for in vitro and in vivo OA treatment efficiency for the first time in literature.

According to particle size distribution studies, doxycycline loaded microspheres with smallest mean diameter were prepared by PCL (14 kDa) and using PVA surfactant (4%). These microspheres also had higher encapsulation efficiency than PCL (14 kDa) microspheres prepared by PVA-gelatin (1 %, each). Overall results were used to select optimum preparation parameters as PCL 14 kDa and 4% PVA to obtain D and D-CS microspheres to be used in in vitro cell culture and in vivo OA model experiments.

In order to evaluate the degree of recovery in the cartilage tissue, the matrix structure of the cartilage before and after degeneration needs to be considered. Two effective microsphere groups were decided to investigate the biological efficacy in OA treatment. Firstly, the in vitro OA model was developed by using rabbit chondrocytes. Following isolation from osteoarthritic rabbits, in order to mimic the three dimensional structure of the cartilage, chondrocytes were embedded into agarose. During OA, synovial fluid has proinflammatory cytokines. In order to mimic the diseased synovial fluid, interleukin-1β was added to the media of the agarose-cell constructs. The model development and treatment results were measured by using GAG, collagen amounts and MMP-13 analyses for days 9, 15 and 24. IL-1β induced OA mechanism was found to have different effects on GAG and collagen synthesis. IL-1β added agarose-chondrocyte discs had lower GAG content than control group at 9^{th} day. GAG content of the agarose-chondrocyte discs got increased at 15^{th} and 24^{th} days due to proliferation of chondrocytes. This result demonstrated that, established drug delivery system did not have any positive effects on GAG synthesis. Although, GAG amounts released into media at 9^{th} day were similar for all groups, 15^{th} and 24^{th} days, IL-1β group was higher than D MS

and D-CS MS treatment groups and IL-1 β untreated one indicating the doxycycline's contribution in preventing GAG degradation. This outcome might be suggested to occur by reduction of aggrecanase activity by doxycycline. Hydoxyproline assays for evaluation of collagen amounts of the samples indicated that the GAG and collagen degradation processes were not regulated by the same mechanism. IL-1β alone was found to be not sufficient to result in detectable levels of collagen degradation by MMPs. Thus, IL-1β treated and untreated controls did not show a significant difference in terms of collagen results. This was thought to be related with insufficiency of either studied dose of this cytokine or the time period of follow up for collagen degradation process. On the other hand, in vitro bioefficacy of the released drug in terms of preventing matrix metalloproteinases was observable at enzymatic level with MMP-13 activity decrease. Doxycycline release inhibited the MMP-13 activity, but to different degrees at different time points. While MS treatments were not significantly different from each other at any of the time points, for both groups, the inhibition level of MMP-13 decreased between 20-24th days of the culture. This might be suggested to be owing to washout of the accumulated released drug with replacement of the cell culture media and drug release rate getting slower in time.

Considering the in vitro results, same groups of PCL microspheres were used in vivo treatment groups but at two different amounts of MS (5 and 7.5 mg). Osteoarthritis *in vivo* model was established by using collagenase enzyme. The effectiveness of the treatments was measured by radiography and histology analyses. Biomechanic test was performed by using a special hardness tester called durometer. According to histological analysis, treatment with D or D-CS MS groups improved the recovery from OA compared with untreated OA and/or hyaluronan injection treated groups. The two MS groups: D and D-CS exhibited histologically similar therapeutic performance with usually no statistically significant difference. The only exception was the significantly higher Pritzker score of the 7.5 mg D-CS MS group comparing to that of the 5 mg D MS group ($p=0.013$). Biomechanical properties of cartilages demonstrated improved hardness values upon application of CS together with doxycycline in MS. This effect was also observed in radiological scoring results at 8th week. Therefore, D/D-CS MS were thought to have potential for developing OA treatment strategy via local controlled release system.

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APPENDIX A

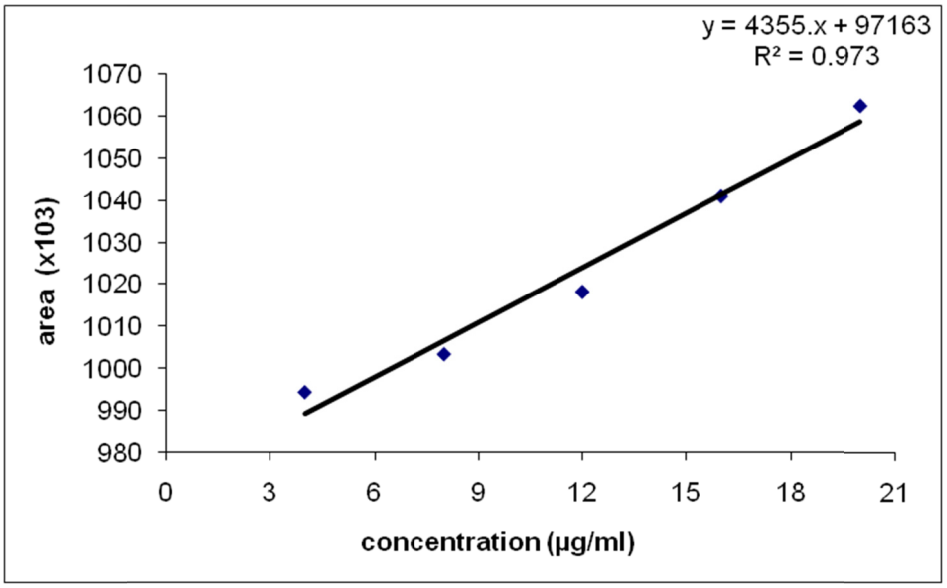


Figure A1. Calibration curve of doxycycline (D) for HPLC analysis

APPENDIX B

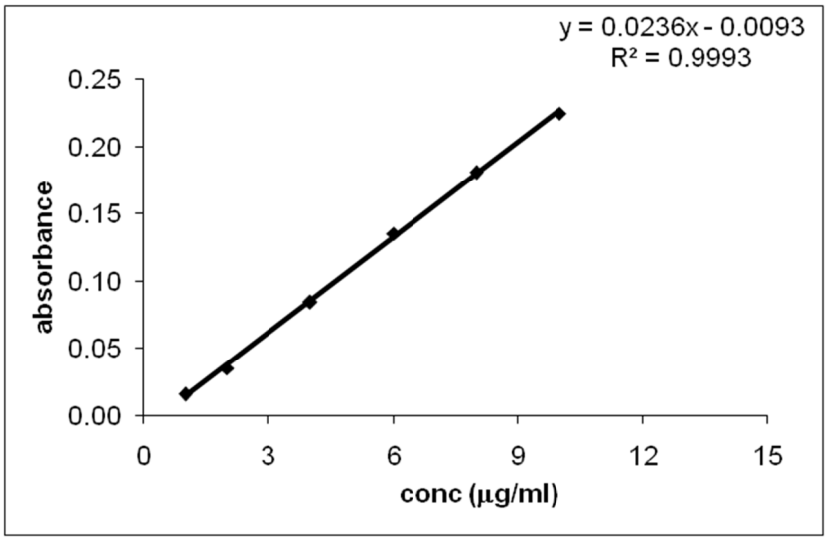


Figure B1. Calibration curve of D in PBS at 274 nm.

APPENDIX C

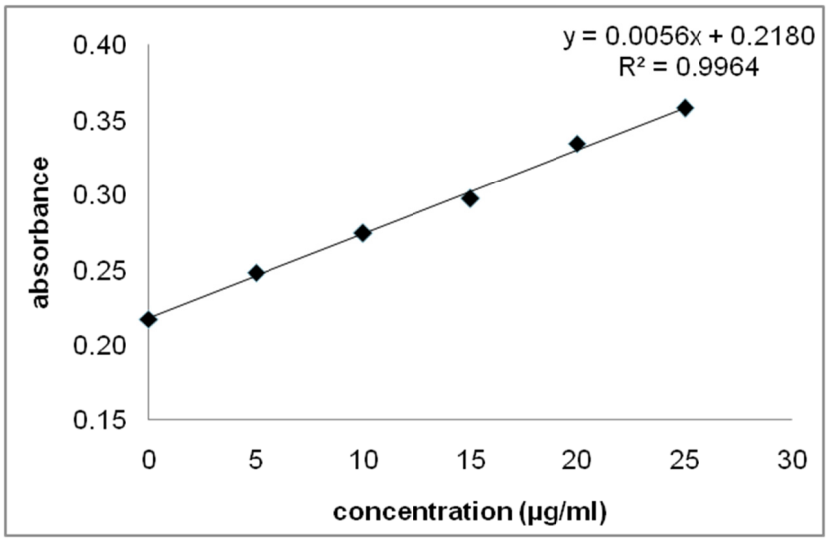


Figure C1. Calibration curve of D in cell culture media at 341 nm.

APPENDIX D

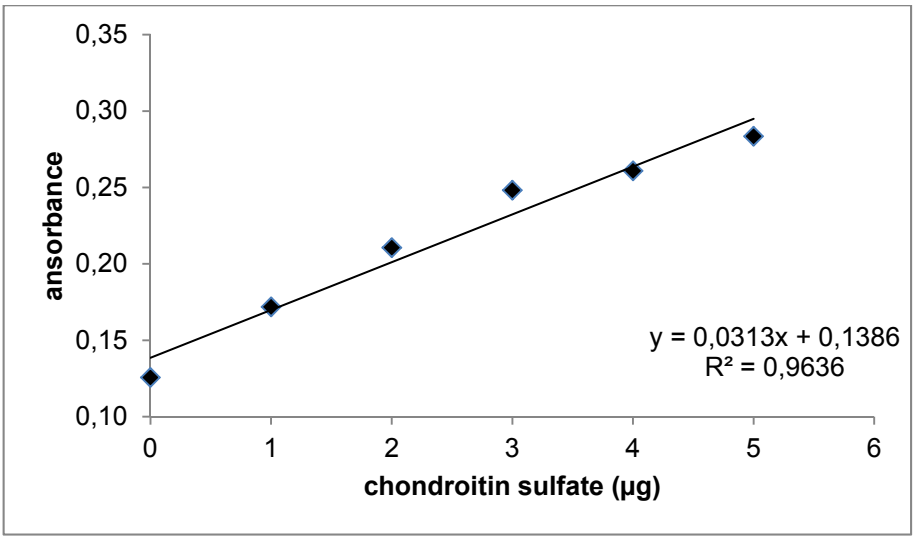


Figure D1. Calibration curve used for determination of GAG amounts

APPENDIX E

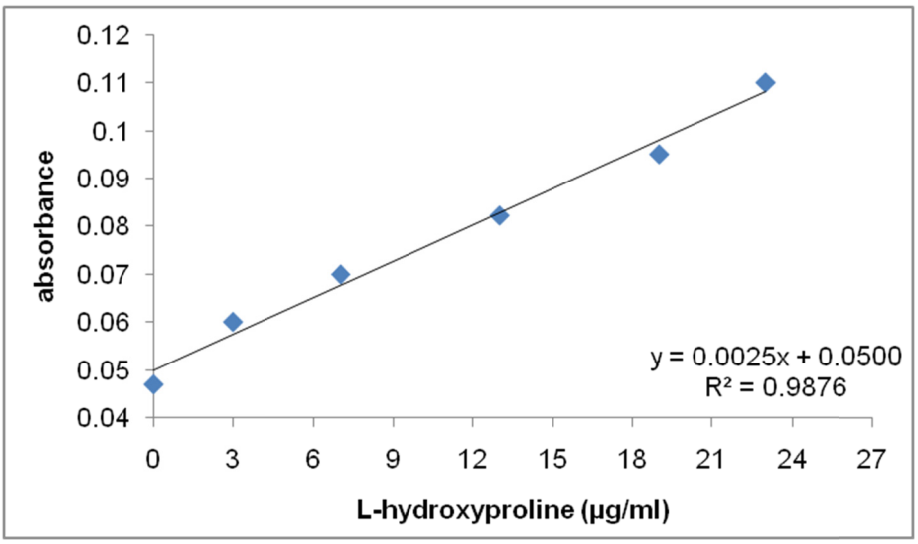


Figure E1.Calibration curve used for determination of hydroxyproline amounts in the experiment groups of agarose-chondrocyte discs

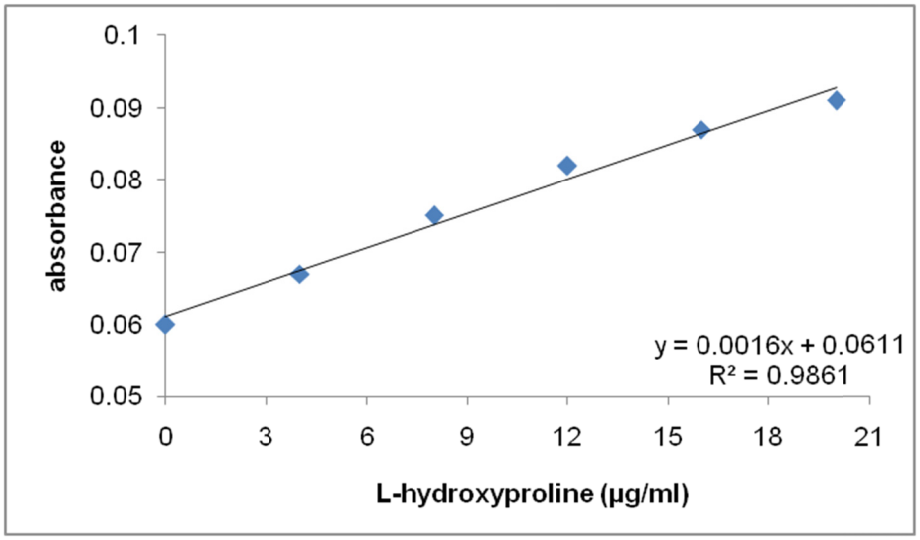


Figure E2. Calibration curve used for determination of hydroxyproline amounts in the experiment groups of liquid media of agarose-chondrocyte discs

APPENDIX F

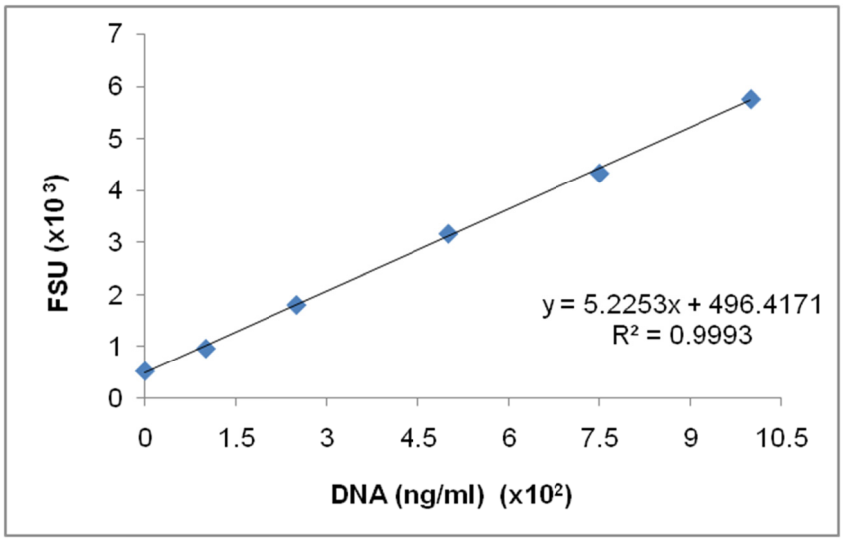
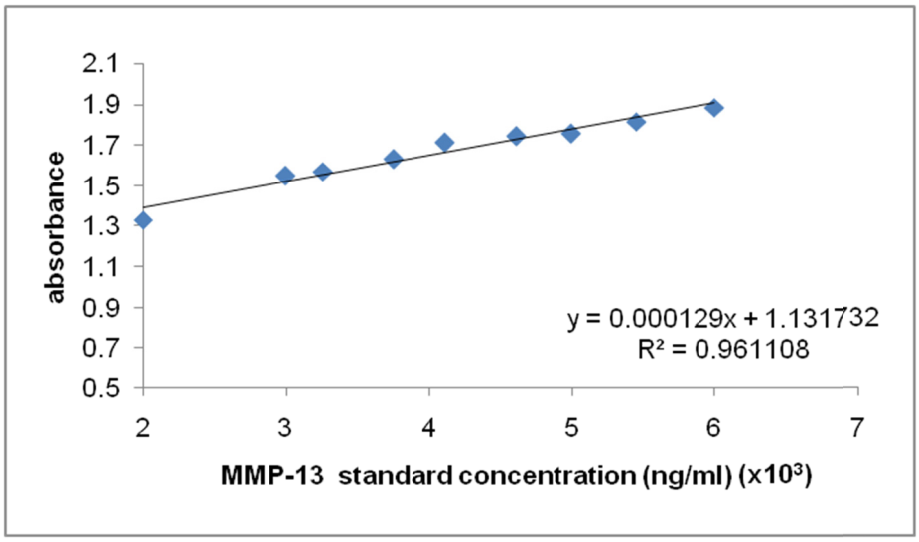


Figure F1. Calibration curve for DNA amounts of agarose-chondrocyte discs.

APPENDIX G



MMP-13

Figure G1. Calibration curve for MMP-13

APPENDIX H

Table H1. GPC values of PCL and PCL microspheres before and after release

No	Sample	Mp	Mn	Mw	Dispersity
1	PCL=14 kDa	17466	9582	20943	2,18562
2	PCL=65 kDa	37816	18847	44942	2,38452
3	PCL=14 kDa PVA 4% Before R	15675	8347	18936	2,2686
4	PVA 4% After R	21409	10964	24558	2,23973
5	PVA-gel 1% Before R	15962	8451	19239	2,27644
6	PVA-gel 1% After R	13805	7059	17044	2,41433
7	PCL=65 kDa PVA 4% Before R	50390	24129	58969	2,44388
8	PVA 4% After R	33160	16283	37392	2,29632
9	PVA-gel 1% Before R	40370	20071	47035	2,34347
10	PVA-gel 1% After R	27624	7351	26690	3,63078

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