DEVELOPMENT AND CHARACTERIZATION OF INJECTABLE ALGINATE/SERICIN/CHONDROITIN SULPHATE MICROSPHERES FOR CARTILAGE TISSUE ENGINEERING

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

NİL GÖL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEPTEMBER 2015

Approval of the thesis:

DEVELOPMENT AND CHARACTERIZATION OF INJECTABLE ALGINATE/SERICIN/CHONDROITIN SULPHATE MICROSPHERES FOR CARTILAGE TISSUE ENGINEERING

submitted by **NİL GÖL** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____

Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Filiz Bengü Dilek _____

Head of Department, **Biotechnology**

Assoc. Prof. Dr. Dilek Keskin _____

Supervisor, **Engineering Sciences Dept., METU**

Prof. Dr. Feza Korkusuz _____

Co-supervisor**, Faculty of Medicine, Hacettepe University**

Examining Committee Members:

Assoc. Prof. Dr. Ayşen Tezcaner _____

Engineering Sciences Department, METU

Assoc. Prof. Dr. Dilek Keskin _____

Engineering Sciences Dept., METU

Prof. Dr. Feza Korkusuz _____

Faculty of Medicine, Hacettepe University

Assoc. Prof. Dr. Eda Ayse Aksoy _____

Faculty of Pharmacy, Hacettepe University

Assoc. Prof. Dr. Sreeparna Banerjee _____

Biology Department, METU

Date: 29.09. 2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Nil, Göl

Signature:

ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF INJECTABLE ALGINATE/SERICIN/CHONDROITIN SULPHATE MICROSPHERES FOR CARTILAGE TISSUE ENGINEERING

Göl, Nil

M.S., Department of Biotechnology

Supervisor: Assoc. Prof. Dr. Dilek Keskin

Co-Supervisor: Prof. Dr. Feza Korkusuz

September 2015, 87 pages

Osteoarthritis (OA) is one of the joint diseases with the highest prevalence worldwide. Eighty percent of the OA patients present limitation in joint movements and 25% cannot perform their daily activities. Due to loss of regenerative potency in OA joints, treatments with drugs and bioactive agents can provide only limited recovery in the damaged cartilage tissue. Current studies and clinical approaches therefore, mostly focus on cell based treatments with hydrogel carriers.

The microcarrier systems supply an efficient method of delivery of cells and drugs within non-invasive, injectable systems. The aim of this thesis was to develop and optimize Cartilage Cell containing injectable Alginate/Sericin/Chondroitin sulphate microspheres (ALG/SER/CS) to modify the natural course of OA. In this study Alginate (ALG), sericin and Chondroitin sulphate materials were mixed at specific concentrations and ALG/SER/CS microspheres with average size of 192±46 μm were prepared by Electrospraying method. Due to high water uptake and less aqueous stability these microspheres needed to be further modified with silk fibroin (SF) using two methods; incorporation and coating.

The degradation analyses showed that ALG/SER/CS microspheres with SF showed a slow degradation profile in two weeks. During this period, slow, sustained release of Sericin and CS were achieved. By this approach it is thought that CS will be released continuously and as a component of native cartilage tissue ECM, it will enhance regeneration. Released sericin will provide a protein surface for attachment and proliferation of cartilage cells.

The cell culture studies were done with the cartilage cell line and the microspheres were shown to increase the cell attachment and proliferation.

According to these results, it can be suggested that the developed microcarriers can be for used as injectable systems and have potential for application for Osteoarthritis (OA). In vivo studies on the developed cell microcarriers should be done in further studies to confirm the usability of the microsystems.

Keywords: Osteoarthritis, cartilage, alginate, microspheres, sericin, fibroin, chondroitin sulphate.

ÖZ

KIKIRDAK DOKU MÜHENDİSLİĞİ İÇİN ALJİNAT/SERİSİN/KONDROİTİN SÜLFAT MİKROTAŞIYICI SİSTEMLERİN GELİŞTİRİLMESİ VE KARAKTERİZASYONU

Göl, Nil
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Doç. Dr. Dilek Keskin
Ortak Tez Yöneticisi: Prof. Dr. Feza Korkusuz

Eylül 2015, 87 sayfa

Osteoartrit (OA), dünya çapında en yüksek prevalansa sahip eklem hastalıklarından biridir. OA hastalarının yüzde sekseninde eklem hareketlerinde kısıtlılık görülmektedir ve %25'i günlük aktivitelerini devam ettirememektedirler. Rejeneratif potansiyelin kaybından dolayı OA 'lı eklemlerde ilaçlar ve biyoaktif maddeler ile tedavi, hasarlı kıkırdak dokusunda sadece sınırlı bir iyileşme sağlayabilir. Son zamanlardaki çalışmalara ve klinik yaklaşımlara bakılırsa hidrojel taşıyıcılarla hücre tabanlı tedavi yöntemleri çoğunlukla kullanılmaktadır.

Mikro taşıyıcı sistemler non-invaziv enjekte edilebilir sistemlerle hücrelerin ve ilaçların taşınmasında faydalı bir metot sağlamaktadır. Bu projenin amacı, OA 'nın doğal seyrini değiştirebilmek için kıkırdak hücre hatlarıyla çalışılmış enjekte edilebilir Aljinat/Serisin/Kondroitin Sülfat (ALG/SER/KS) geliştirmek ve optimize etmektir. Bu çalışmada Aljinat, serisin ve kondroitin sulfat malzemeleri belirli konsantrasyonlarda karıştırılmıştır ve elektrosprey metodu ile ortalama 192±46 μm boyutlarında ALG/SER/KS mikroküreleri üretilmiştir. Yüksek su emilimi ve düşük

su stabilitesi sebebiyle bu mikroküreler, ipek fibroin (SF) ile birleştirme ve enkapsule etme teknikleri kullanılarak modifiye edilmesi gerekmektedir.

Degredasyon analizleri iki haftalık dönemde ALG/SER/KS mikrokürelerin yavaş degrede olduğunu göstermiştir. Bu dönemde, serisinin ve kondroitin sülfatın (KS) yavaş, sürekli salımları sağlanmıştır. Bu yaklaşımla devamlı CS salınması doğal kıkırdak dokusunda bulunan ECM bileşenlerinde varlığı sebebiyle, rejenerasyonu arttıracaktır. Salınan Serisin Kıkırdak hücrelerinin protein yüzeyinde yapışmasını ve çoğalmasını arttıracaktır.

Hücre kültürü çalışmaları kıkırdak hücre hattıyla yapılmıştır ve mikroküreler hücrelerin yapışma ve çoğalma kapasitelerini arttırmıştır.

Sonuçlara göre, geliştirilen mikrotaşıyıcılar enjekte edilebilir sistem olarak kullanılması önerilir ve Osteoartrit (OA) uygulamalarında kullanılma potansiyeline sahiptir. Gelecek çalışmalarda, mikrosistemlerin kullanılabilmesinin onayı için geliştirilmiş olan hücre mikrotaşıyıcıların in-vivo çalışmaları yapılmalıdır.

Anahtar Kelimeler: Osteoartrit, kıkırdak, aljinat, mikroküre, serisin, fibroin, kondroitin sülfat

To My Precious Family…,

ACKNOWLEDGMENTS

I would like to feel of thankfulness and appreciation to my supervisor Assoc. Prof. Dr. Dilek Keskin for her encouragements, patience and conduct the insights throughout study, co-supervisor Prof. Dr. Feza Korkusuz for his positive attitude and permanent support during my master life and Assoc.Prof. Ayşen Tezcaner for her cooperative.

Also thanks to Ayşe Begüm Tekinay lab, in UNAM, at Bilkent University for ATDC5 cell lines.I wish to thank to BIOMATEN for allowing me to use their laboratory for my FTIR studies.

I would like to add my precious thanks to Hazal (Aydoğdu) Kayadelen for her never-ending effort for support and care. She always behaves positive about my theses during master life. Also Derya Gokcay for her energetic and positive attitude.

Deniz Atilla for not leave alone in MODSIMMER and for innovative thinking and graceful sharing with me. I have to thank to my lab mates. I'm very grateful to them for all their help. In particular, thanks to my seniors and friends: Dr. Aysegul Kavas for her kindness and share of experiences, Dr.Ömer Aktürk for his teaching on electrospinning, and continuous information exchange, Dr.Özge Erdemli for all her advices with instructiveness, Dr. Aydın Tahmasebifar and Ali Deniz Dalgıç for their gentle support, Engin Pazarçeviren for positive appearance, Zeynep (Barcin) Gürtürk for her friendship and relaxing motivation, Sibel Ataol for her tenderness and share of experiences, Merve Güldiken for being extra-colorful room-mate, Yagmur Calıskan for her everlasting smile and Reza Moonesirad for his entertaining motivation all the time.

I would also thank to my dearest friend, Gül Altınbay for being endless support and encouragement.

I would like to thanks to my special & old friends Erkan Cırak, Yıldız Marangoz, and Irmak Görgülü. for showing an appreciation of kindness. Since 1997…….

A special thanks to my family….

I would like to thank to Berk Berker for support during my thesis study, also for his patience to me and his love to me for all the time.

I wish to say my grateful feelings for my mother Ilknur Göl for supporting me, patience to me and she was always with me for all the time

I would like to express my grateful feelings for my father Nail Göl with his endless encouragement, for his positive attitude and support not only during my thesis study but also during my lifetime.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

AA	Acetic Acid
ALG	Alginate
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
BSA	Bovine Serum Albumin
CS	Chondroitin Sulphate
DMEM	Dulbecco's Modified Eagle Medium
D-WATER	Distilled water
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FT-IR	Fourier Transform Infrared
hMSC	Human mesenchymal stem cells
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
SEM	Scanning Electron Microscopy
SER	Sericin
SF	Silk Fibroin
TCPS	Cells seeded on polystyrene tissue culture wells

CHAPTER 1

INTRODUCTION

1.1 Tissue Engineering

Tissue engineering is an interdisciplinary field, which combines the principles of engineering, and life sciences for the development of biological substitutes that restore, maintain or improve tissue function (Vacanti et al., 1993). Over the past decade, stem cell biology, bioengineering, and animal models have combined in the area of **regenerative medicine (**Bernstein et al., 2011).

Every day, thousands of surgical procedures are carried out to replace or repair tissues that have been damaged through disease or trauma. Tissue engineering (TE) aims to regenerate damaged tissues by combining cells with a scaffold designed as substitutes for skin, cartilage or bone other tissues with varying degrees of success (O'Brien, 2011; Priya et al., 2008) . The field of tissue engineering has the advantage of using living cells that are either from the patient's own tissues or from another person together with availability and immunogenicity considerations.

Tissue Engineering provides the development of scaffolds or cell carriers with properties; suitable surface chemistry for cell attachment, proliferation, and differentiation (Hutmacher et al. 2000) larger surface area for cell attachment, combined with porous structure for cell to cell interaction and colonization, besides

transport of nutrients and metabolic waste; biocompatible and bioresorbable with a controllable degradation kinetics; 3-dimensional architecture which mimics extracellular matrix (Horner et al., 2010; Subbiah et al., 2005; Yang et al., 2013). The scaffolds should support cell adhesion, proliferation, migration and differentiation (Venugopal et al., 2005; Venugopal & Sharon Low, 2005).

Microspheres are carriers that immobilize cells inside and provide surface for cell attachment that would support the cell activities. They should protect the structural unity and the mechanical integrity of cells during application to the target site. If microspheres are compared with the other cell carrier systems for tissue engineering applications like scaffold or sponges (Dorotka et al., 2005), microspheres have better mechanic stability and longer stability duration because of its large surface area to volume ratio (Yao et al., 2012).

1.2 Cartilage Tissue Engineering

1.2.1 Cartilage Tissue and Its Properties

One type of connective tissue is supportive connective tissue and cartilage is one of them. Three types of cartilage are distinguished: hyaline, elastic and fibrous (Figure 1.1).

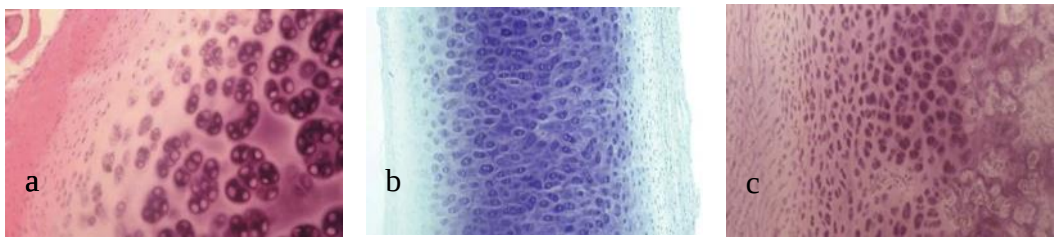

Figure 1. 1 Types of cartilage tissues in human body a) Hyaline cartilage, b) Elastic cartilage c) Fibrocartilage (Intervertebral Disc) (www.histol.chuvashia.com)

Cartilage consists of a gel-like matrix that is composed of collagen (mainly type II) and glycosaminoglycan's (chondroitin sulfate, hyaluronan) within which chondrocytes are embedded. Unlike other connective tissues, cartilage has no blood vessels or nerves except in the perichondrium which is a membrane covering the cartilagenous tissue. So, adult cartilage tissue has poor capability of self-repair, especially in case of serious cartilage damage due to trauma or age-related degeneration (Zhang et al., 2009). Also the discontinuous distribution of highly differentiated and non-dividing chondrocytes, slow matrix cycle, low supply of progenitor cells are the other reasons of poor capability of self-repair of articular cartilage (Wanga et al., 2005).

There are chondrocytes in 10% or less of the total volume of cartilage. Consequently cartilage has stiffness and durability, which rely on the extracellular matrix. Therefore, matrix is important in the distribution of load and the functional properties of cartilage. In human, about 1% of the volume of hyaline cartilage is composed of cells but they are important since they replace degraded matrix molecules with newly synthesized ones to maintain the correct size and mechanical properties of the tissue (Temenoff & Mikos, 2000).

1.2.2 Articular Cartilage

Articular cartilage is a shiny, slippery, pearly-blue-white tissue that covers the articulating ends of the bones (Figure 1.2) (Kutner et al., 1991).

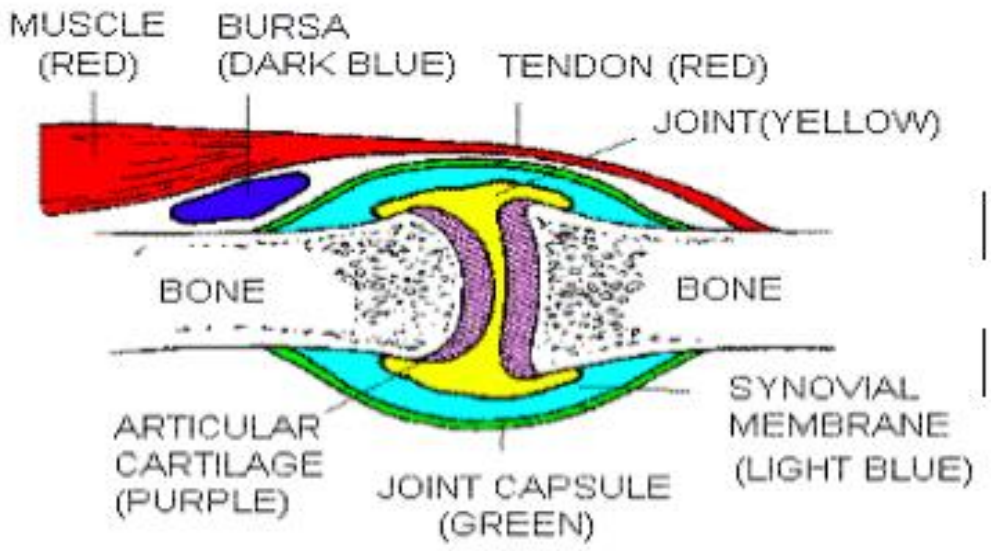

Figure 1. 2 Anatomical structure of an articular joint (www.medicalphoto.com)

It is an avascular tissue and it mainly consists of chondrocytes encapsulated in a dense matrix of proteoglycans and collagens so it has limited capacity for self-repair (Madeira et al., 2015). It is typically depicted in 3 zones (Figure 1.3): The tangential or superficial zone, the transitional zone (the intermediate zone) and the deep zone. They are based on the shape of chondrocytes and distribution of the type II collagen. They have their own matrix region. The tangential zone has flattened chondrocytes, tangentially arranged and condensed collagen fibers, and relatively rare, proteoglycan. In the intermediate layer, the chondrocytes are round and tend to be oriented in vertical columns, which are parallel to the collagen fibers. The deepest layer is the basal layer where chondrocytes are round in morphology (Figure 1.3) (Kuetner et al., 1995). The deep zone contains chondrocytes that are similar to those of the transitional zone and also contains the largest collagen fibrils of articular cartilage and the highest content of proteoglycans. There is also lamellar bone called the subchondral bone plate (Machado et al., 1995).

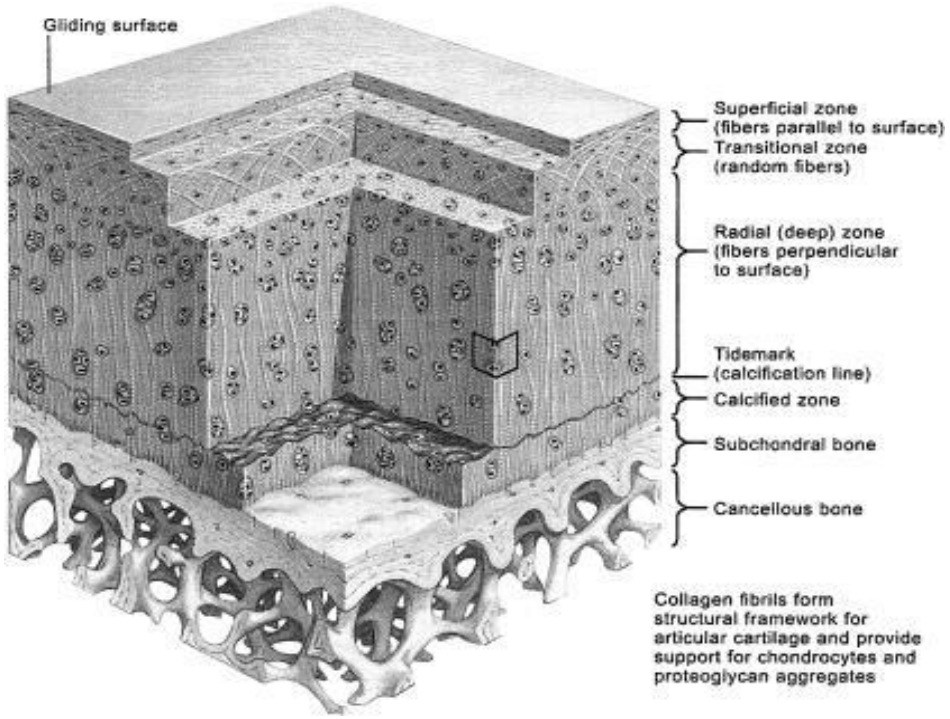

Figure 1. 3 Structure of articular cartilage (Kuetner et al., 1995)

The material properties of articular cartilage depend on its extracellular matrix network, which is composed of a hydrated collagen (~ 60 % of the tissue dry weight), a highly charged proteoglycan gel (PG, ~ 25% of the tissue dry weight), and other proteins and glycoproteins (~ 15 % of the tissue dry weight). The existence and maintenance of the matrix depends on the chondrocytes (Train et al., 2001; Sminoff & Mikos 2000).

Cartilage has high water content; 70 to 80 % of the tissue-wet weight is water which enables to withstand the sudden compressive, tensile, and shear forces correlated with joint loading (Kuettner et al., 1995). This tissue is generally subjected to high and varying loads (Hall et al., 1991). The ability of cartilage to resist high loading is due to collagen fibers and its resilience property is due to the presence of chondroitin sulfate (Caplan et al., 1984).

1.3 Articular Cartilage Defects

Among articular cartilage degenerative diseases, arthritis is one of the most widely observed in the world. It is an inflammatory disease in the joint(s) that could be due to lack of fluid, autoimmunity (your body attacking itself), infection, or a combination of many factors. Arthritic symptoms are mainly joint pain, sensitivity, warmth, swelling, redness and related other complications like difficulty in movement (Gordon et al., 1993).

There are two forms of arthritis:

1) The atrophic form with synovial inflammation, loss of cartilage, and bony rupture occurs in diseases such as Rheumatoid Arthritis (RA). Rheumatoid Arthritis involves rheumatoid pannus (inflammatory penetration) and uncontrolled inflammation, which can be seen at a microscopic level by synovial cell hyperplasia and endothelial cell proliferation (Scott et al., 2000)

2) Cartilage damage in focal areas, thickening of capsule and overgrowth of subchondral bone are the characteristics of osteoarthritis.

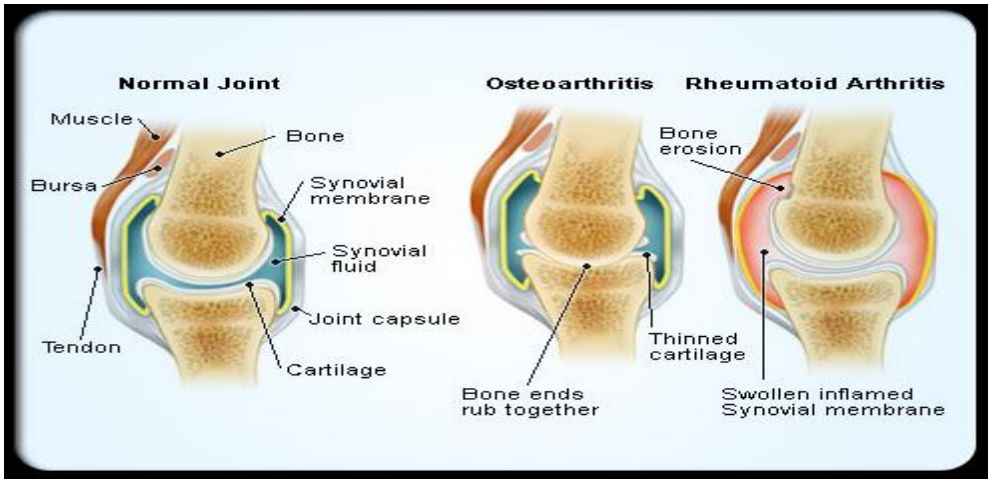

Figure 1. 4 Different types of arthritis (www.medicinenet.com)

1.3.1 Osteoarthritis

Osteoarthritis (OA) is one of the joint diseases with the highest prevalence worldwide (Felson, 1990). According to the Centers for Disease Control and Prevention (CDC), OA affects 70% of women and 55% of men. As the population ages, arthritic conditions are expected to affect an estimated 67 million adults in the United States by 2030 (www.cdc.gov/arthritis/data_statistics).

It is an articular joint disease that is characterized by chondrocyte and extracellular matrix molecular alteration, degradation, and degeneration which results due to the failure of chondrocytes for repair of the damaged articular cartilage in synovial joints (Wolfstadt et al., 2015). Main symptoms of OA are (a) pain, (b) deformity, and (c) loss of function.

Chondrocytes are metabolically active but after adolescence they cannot divide. Only small defects related with matrix components or minimal loss can be regenerated by cartilage. However, the excessive defects pass over the healing capacity and so become permanent (Lorenz & Richter 2006). Progressive loss of articular cartilage and formation of osteophytes are the characterization clues of OA. These signs of OA lead to chronic pain and the functional restrictions can appear in these affected joints (Lorenz & Richter, 2006).

Multiple factors (e.g., heredity, trauma, and obesity) interact to cause OA. Any event that changes the environment of the chondrocytes has potential to cause OA too **(**Table 1).

Table 1. 1 Risk factors for osteoarthritis (Sinusas et al., 2012).

- *Age older than 50*
- *Crystals in joint fluid or cartilage*
- *High bone mineral density*
- *History of immobilization*
- *Injury to the joint*
- *Joint hypermobility or instability*
- *Obesity (weight-bearing joints)*
- *Peripheral neuropathy*
- *Prolonged occupational or sports stress*

Classic twin studies conducted by Osteoarthritis Research Society Twin Research & Genetic Epidemiology Unit in London have shown that the influence of genetic factors is between 39% and 65% in radiographic OA of the hand and knee in women, about 60% in OA of the hip, and about 70% in OA of the spine. Taken together, these estimates suggest a heritability of OA of 50% or more, indicating that half the variation in susceptibility to disease in the population is explained by genetic factors (Spector et al.,2004). Pain usually results from degeneration of the joint's cartilage due to primary osteoarthritis or from trauma causing loss of cartilage. Since cartilage shows very little tendency for self-repair, these injuries are maintained for years and can eventually lead to further degeneration (secondary osteoarthritis) (Temenoff & Mikos, 2000).

Joint pain due to cartilage degeneration is a major cause of disability in middle-aged and older people. Although many techniques, like surgery and hyaluronan injection to the knee are currently employed to treat this suffering, none shows complete success in result (Makris et al, 2014).

1.3.2 Treatments of OA in Medicine

There is no known definite treatment for OA. Drug therapy for the pain and inflammation is most effective one. At the same time non-pharmacological techniques like exercise, ice massage, joint bracing and weight control of patient should be combined with the oral therapy (Felson et al., 2000). Intra-articular injections of hyaluronic acid, opioids, glucosamine sulfate, chondroitin sulfate which is also relieve pain, selective and nonselective NSAIDs (non-steroidal anti-inflammatory drugs) are the pharmacological therapies for OA. However, these therapies frequently cause serious adverse effects (Scott et al., 2000). Patient education interventions are important for the quality of life and sometimes surgery can be applied to patients of OA. Chondroitin sulfate-hyaluronic acid are now seen as disease modifying agents as a result of animal, in vitro and short run clinical studies (Henrotin et al., 2010).

Huskisson and Donnelly showed that five weekly intra-articular injections of Na-Hyaluronate (Hyalgan) were successful than placebo and well tolerated in OA patients. At the same time NSAID usage are low (Huskisson & Donnelly, 1999). Finally, according to animal studies, (Brandt et al., 2000) it was reported that although intra-articular injection of hyaluronan therapy seems safe and decreases pain of the knee in the short-run, it could cause overloading of the damaged joint, and reduction of proteoglycans in articular cartilage and thus, rise in structural damage in the long-run (Fernandes et al., 2002). Clinical trials showed that use of glucosamine for relief from disease symptoms has a moderate treatment effect, whereas chondroitin sulfate has more treatment effect (Leeb et al., 2000; McAlindon et al., 2000; Richy et al., 2003).

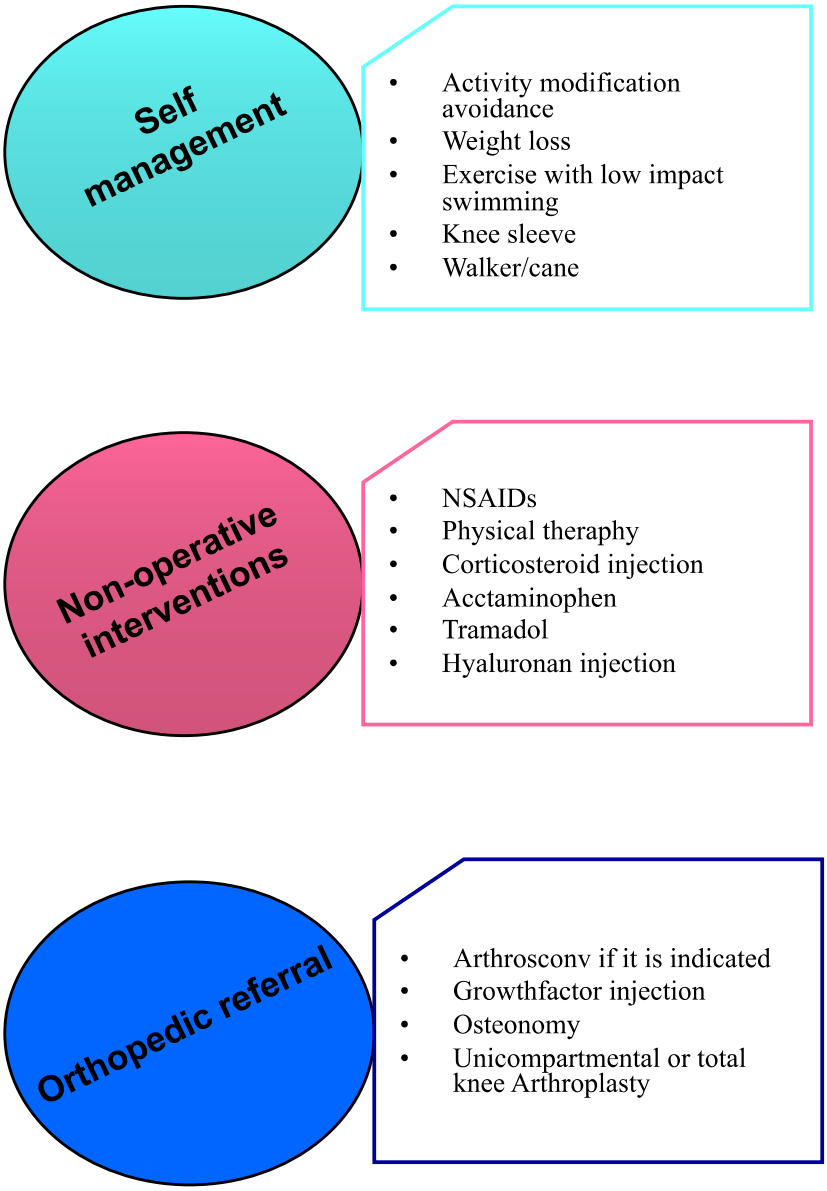

Figure 1. 5 Knee osteoarthritis treatment recommendations. (Zhang et al., 2008)

Bolded items showed strong evidence in AAOS (American Academy of Orthopedic Surgeons) analysis and guidelines. (An update on primary care management of knee osteoarthritis Cody Sasek, MPAS, PA-C) (Lippincott Williams & Wilkins, 2015)

1.3.3 Surgical Treatment

Cartilage loss is irreversible, so the goals are symptom control and appropriate function in daily life. The aim of the surgical treatment of OA is to decrease or eliminate the pain and improve the function of the patient. Total number of knee arthroplasties has increased by 162% from 1991 to 2010 (Cram P et al, 2012).

Up to now, the surgical procedures of OA include obliteration of the joint-by-joint fusion (arthrodesis) or replacement of the joint with an artificial one. Although joint replacement provides excellent short- and medium-term relief and it provides functional range of joint motion, it has poor long-term results in functionally active patients (Kuettner, 1991).

Cartilage engineered via autologous chondrocyte implantation (ACI) was first reported by Brittberg and colleagues in 1994. ACI is performed using chondrocytes isolated from non-weight-bearing parts of intact joint regions. After isolation cells are expanded in culture, and transplanted into defective joint areas; however, chondrocytes should be extracted directly from the patient, thus increasing the probability of donor-site morbidity of healthy articular cartilage. Other disadvantages are loss of the chondrocytes' phenotype in cells expanded in monolayer culture, not enough cell proliferation for application to the large lesions due to the limited multiplication capacity of chondrocytes; poor functionality and quality of the extracellular matrix (ECM) synthesized. Additionally, the technical efficacy in patients older than 40 years is limited (because of poor cellular activation) (Wang et al., 2015).

There is an increasing interest in stem cells usage for treating degenerative diseases such as leukemia, liver failure, Parkinson, diabetes, OA like diseases for which there are no established cure strategies (Zaher et al., 2015).

As shown in Table 1.2, Yoshimura et al. compared advantages of rat mesenchymal stem cells from various tissues in his article (2007).

Table 1. 2 Sources for mesenchymal stem cells (Sakaguchi et al., 2005)

Tissue Type	*Advantages*
Bone marrow	➢ Good chondrogenic and osteogenic potential ➢ Easy to harvest and exist in large numbers ➢ High proliferative activity
Synovium	➢ Best chondrogenic potential ➢ High proliferative activity
Adipose tissue	➢ Easily harvested ➢ Available in large numbers
Umbilical cord	➢ No morbidity with collection ➢ Large capacity for ex-vivo expansion ➢ Full potential to differentiate into chondrogenic, adipogenic and osteogenic lineages

1.4 Cartilage Tissue Engineering Approach

Recent advances in biological and materials sciences have forced tissue engineering to the forefront of new cartilage repair techniques. Techniques are being improved to generate new cartilage, using a "scaffolding" material like microparticles with cultured cells to grow artificial cartilage (Cruz et al., 2012).

There are three principal therapeutic tissue-engineering strategies for treating diseased or injured tissues in patients (Griffith & Naughton, 2002):

(i) Implantation of freshly isolated or cultured cells;

(ii) Implantation of tissues combined in vitro from cells and scaffolds;

(iii) In situ tissue regeneration

Ideally, a scaffold should have: 3-D, porous structure with an interconnected pore network for cell growth and mechanical properties that match those of the tissues at the site of implantation.

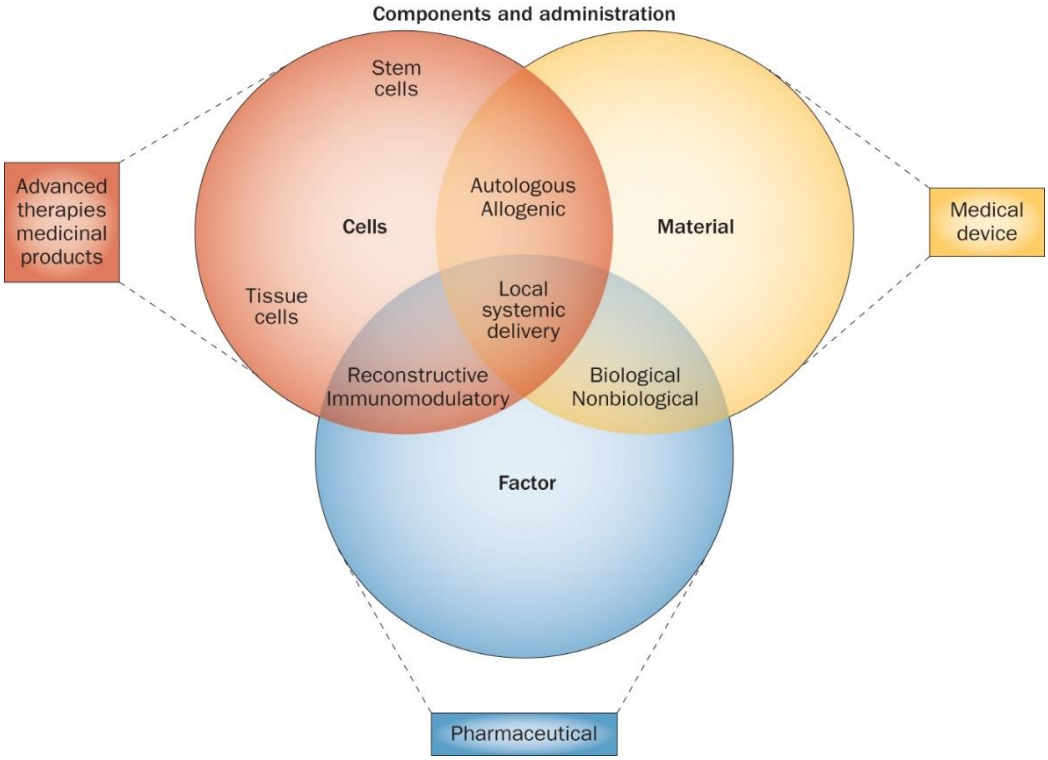

Figure 1. 6 Components, mode of administration and regulatory categories of regenerative therapies (Ringe et al., 2012)

Tissue engineering based cartilage repair with different cell and biomaterial combinations (delivery of cells and growth factors for regeneration) are also under intense search to develop new healing methods for the damaged cartilage (Robert et al., 2003, Mizuta et al., 2004; Na et al., 2006; Mouw et al., 2005). One of the recent animal studies showed that bone marrow-derived MSCs could be a promising cell source for the treatment of OA. In this study, the scaffold-free mesenchymal stem cells (MSCs) obtained from bone marrow of rabbits were used in an experimental animal model of OA by direct intra-articular injection into degenerated knees. The knees were examined at weeks 16 and 20 following surgery.

Results confirmed that rabbits receiving MSCs showed a lower degree of cartilage degeneration, osteophyte formation, and subchondral sclerosis than the control group 20 weeks post-operatively (Singh et al., 2014).

One of the strategies to regenerate tissue in tissue engineering as a possible therapy for damaged tissue and organs is the use of biomaterials for scaffolding (Langer & Vacanti 1993). Recent studies have focused on seeding cells onto three-dimensional (3D) structures, such as porous scaffolds, hydrogels, sponges, fibers, films or microspheres (Hutmacher et al., 2000; Bian et al., 2011).

Nowadays microspheres have also been used as a scaffold for tissue engineering applications that have the required properties such as physical, chemical, and mechanical for inducing cell penetration and formation of tissues (Karp et al., 2003). Microspheres can be prepared using ECM components such as collagen (Lee et al., 2004), fibrin (Eyrich et al., 2007), hyaluronic acid (Bian et al., 2011) as well as materials derived from other biological sources such as chitosan (Cruz et al., 2008), alginate (Nayak et al., 2014) or silk fibroin (Sinha & Trehan, 2003).

In the last 20 years, micro- and nanoparticulate systems have been investigated as an alternative to simplify the application of substances in a less invasive way (Sinha, & Trehan, 2003). The microparticle usage in tissue engineering can be by injection of a cell suspension or drugs, which guarantees drug gradients. The use of microcarriers may be done by encapsulating cells inside or by allowing attachment of cells onto their surface (Orive et al., 2003).

Biomaterials are not generally phagocytized by neutrophils or macrophages because they are mostly greater than the size of the cell. Particles, microcapsules, microspheres, or liposomes less than 10 μm in size may undergo phagocytosis (Anderson et al., 2007).

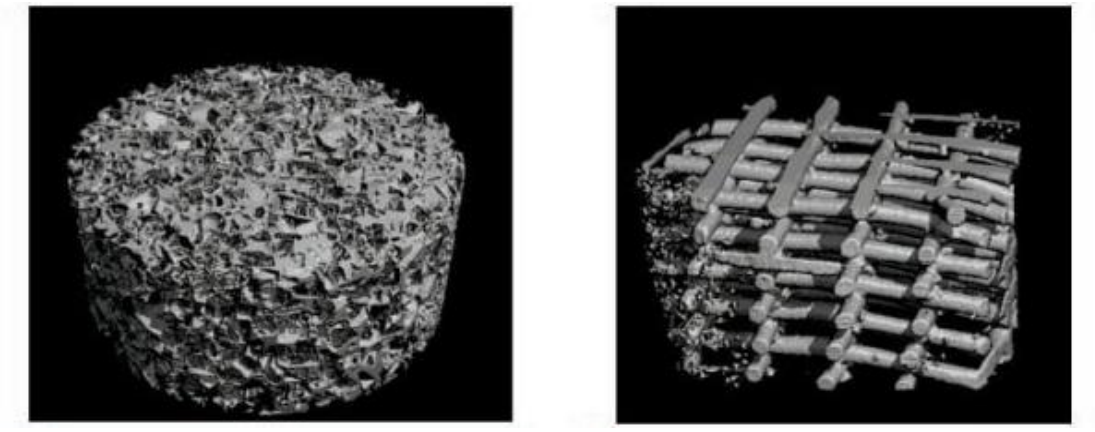

Figure 1. 7 Examples for scaffolds used in tissue engineering (Hollister et al., 2005)

When the molecular composition and organization of articular cartilage change, the material properties and structural integrity of the tissue deteriorate (Ringe, et al., 2012). Autologous cell-based tissue engineering using three-dimensional (3-D) porous scaffolds has provided an option for the repair of full thickness defects in adult cartilage tissue. Mesenchymal stem cells (MSCs) and chondrocytes are the two major cell sources for cartilage tissue engineering (Train, 2001; James and Uhl, 2014).

1.4.1 Materials Used in Repair of Articular Cartilage

Hydrogels are compliant and permeable structures, which are mostly composed of water. They resemble the native ECM providing an ideal 3-D microenvironment for cells. As a consequence, hydrogels have emerged as a valuable platform for examining the effects of ECM properties on cell behavior.

1.4.1.1 Alginate

Alginate is a seaweed derived anionic polysaccharide. It is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues and covalently linked together in different composition and blocks (Figure 1.8) (Draget et al., 1997; Wang et al., 2007).

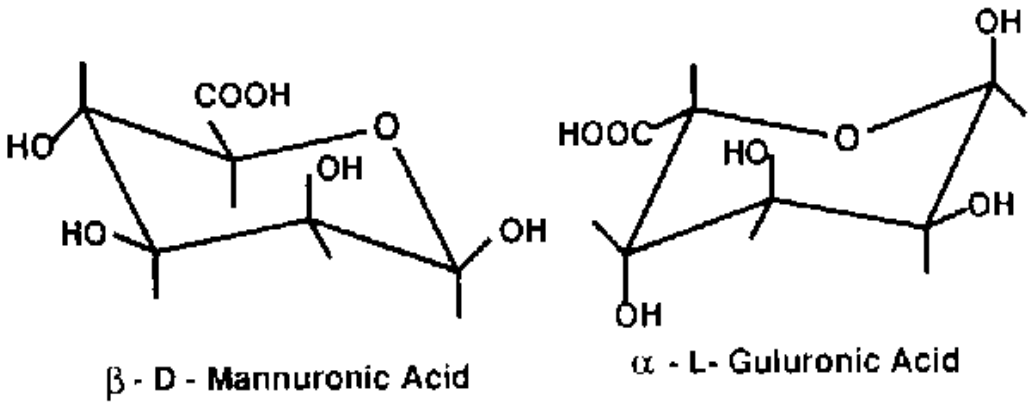

Figure 1. 8.Structure of main units that form alginate (Draget et al., 1997)

The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks) or randomly organized blocks (Smidsrød et al.,, 1996). The relative amount of each block type varies with the origin of the alginate (Leea & Mooneya, 2011**).**

Na-Alg has been used as controlled drug delivery systems and extracellular matrix material in medicine (Kulkarni et al., 2000). Alginates (polysaccharides) are hemacompatible and they do not accumulate in organs of the human body.

Alginate is nontoxic so that cells can be encapsulated while maintaining their viability. Alginate can form spherical gel beads by ionic network formation via ionic cross-linking of polyelectrolytes with multivalent counter-ions like calcium (Smidsrød O & Skjåk-Braek G, 1990).

Moreover, in many studies, alginate microspheres cross-linked in the presence of multivalent cations have been used widely for controlled delivery of growth factors because of their advantages such as biocompatibility, high encapsulation efficiency, and mild fabrication conditions (Chan et al., 2012). The fact that organic solvent is not needed while preparing alginate microparticles make them also preferable for cell encapsulation and suitable carrier for protein/peptide molecules. Alginate microspheres show high shear stresses. Moreover, they let the macromolecules with high diffusion rates (Strand et al., 2002). This diffusion can be controlled with a simple one step coating procedure (Gaserod et al., 1999; Zhai et al., 2011).

1.4.1.2 Sericin

Silk consists of two types of proteins; fibroin and sericin (Padamwar & Pawar, 2004). Sericin is a silk protein obtained from silkworm cocoons (Bombyx mori) comprising 20-30% of total cocoon weight (Sasaki et al., 2000). It is a highly hydrophilic protein consisting of 18 amino acids of which 32 % is serine.

It envelops fibroin (silk) fibers (Figure 1.9) and it is an antibacterial agent that resists oxidation and absorbs moisture and UV light (Hazeri et al., 2012). Its recovery provides significant economic and social benefits. Sericin is a biocompatible and biodegradable material due to its protein nature (Veparia & Kaplan, 2007). The size of sericin appears to be important for its activity (Wray et al., 2011).

Silk sericin has been successfully used as a biomaterial for tissue regeneration (Wray et al. et al. 2010). Sericin has a high moisture absorbency, antioxidant and, hence, antitumor activity, as well as wound-healing capacity (Hazer, et al., 2012). In one of the in vitro study of sericin it was also shown that sericin suppressed lipid peroxidation and had tyrosinase inhibitory activity (Kato et al., 1998). Additionally, it was suggested that it could provide benefit to colon cancer treatments (Kaewkorna et al., 2014).

Figure 1. 9 Composition of Silk Filament (Gulrajani et al., 2008)

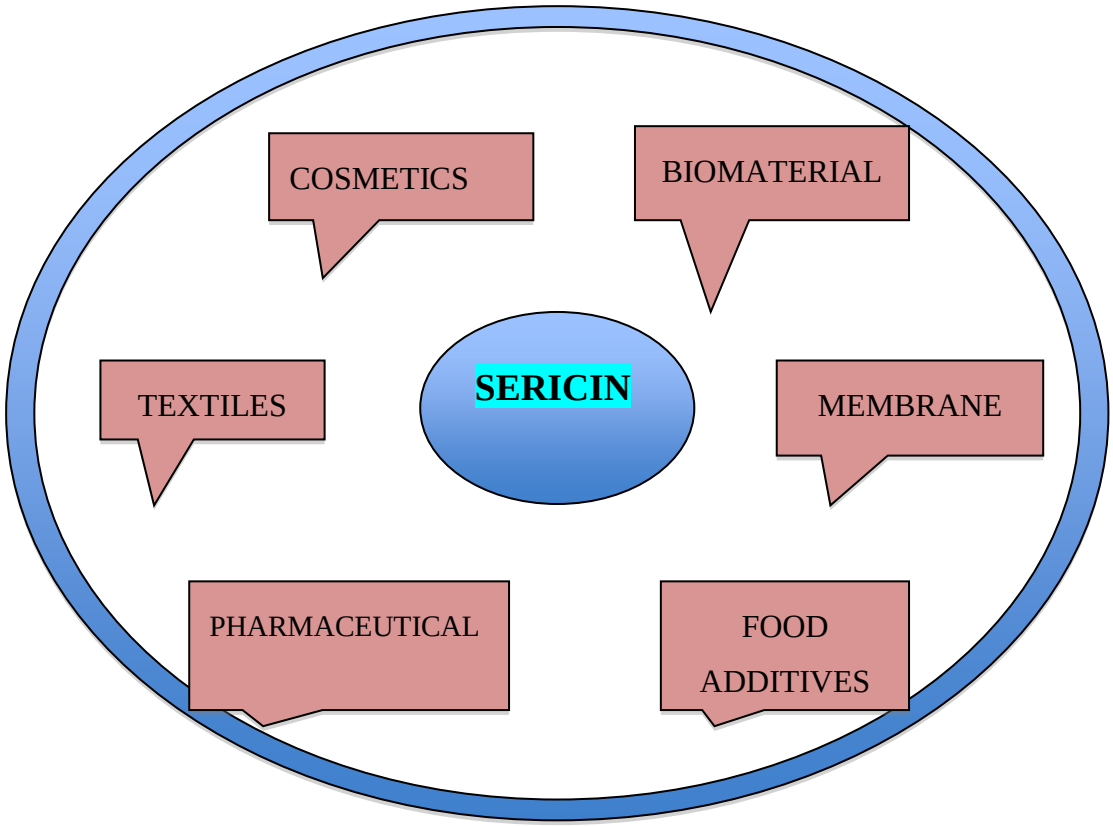

Figure 1. 10 Application of sericin in various industries (Gulrajani et al., 2008)

Moreover, the properties of sericin can be enhanced by applying modifications such as cross-linking, copolymerization and blending with other polymers (Kundu et al., 2008).

1.4.1.3 Chondroitin Sulphate

Glycosaminoglycans (GAGs) are found in the lubricating fluid of the joints and as components of cartilage, bone and heart valves. (Pieper et al., 1999; Malafaya et al., 2008). Furthermore, GAGs are practically non-immunogenic and degrade to non-toxic oligosaccharides (Doppalapudi et al., 2013) .

CS includes repeating disaccharide units of D-gluronic acid and N-acetyl galactosamine sulfated at either 4- or 6- positions (Lee et al., 2005).

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 inflammatory cytokines such as interleukin-1, and inhibition of cartilage matrix degradation (Jiang et al., 2011).

CS has been shown as the most potent prochondrogenic factors for cartilage tissue engineering (Dinescu et al., 2013). In addition to this, it interacts with cells and proteins improving cell behavior of the developed materials. Also it provides an appropriate environment for prospective cartilage tissue engineering (CTE) applications. CS can interact with various growth-active molecules (Malafaya et al., 2008).

CS can attach with core protein to make highly absorbent aggregan, named as cartilage-specific proteoglycan core protein (CSPCP). CS can absorb shock, or it can let to produce syndecan, which shows the sensing flow direction. Moreover, it is a cell receptor, which makes interaction with adhesion proteins, cells and the extracellular matrix (ECM) (Lee et al., 2005).

In vitro studies of Bassleer et al. showed that chondroitin sulfate was also able to increase matrix component production by human chondrocytes (Bassleer et al., 1998). It can reduce the pro-inflammatory cytokine, interleukin (IL)-6 (Cho et al. 2004). However, CS generally limits its application with a solid-state delivery system and crosslinking treatment, but it can combine with other polymers. CS is negatively charged and it interacts with positively charged molecules such as polymers or growth factors. It can show anti-coagulant, antitumor, anti-arthritis and anti-HIV activities. It was used for treating atherosclerosis, angina, myocardial infarction, hyperlipemia and hypercholesterolemia because of its activity of bringing down blood lipids (Herrero-Beaumont et al. 2008).

1.5 Electrospraying Method for Production of Microcarriers

Electrospraying developed on electrospinning systems and principles, is one of the simple and efficient methods to produce nano/microspheres from wide variety of polymers (Ramakrishna et al., 2005; Bhardwaj & Kundu, 2010; Khajavi and Abbasipour, 2012). Micro- and nanospheres have specific, unique properties such as high surface area to volume ratio and high porosity with various pore sizes.

The electrospraying process was interest in tissue engineering field and drug delivery because of the possibility of microsphere preparation with a variety of natural and synthetic polymers. These microspheres mimic extracellular matrix. The use of electrospinning is the most promising fabrication method for biopolymers or biocomposite scaffolds in replacement of injured tissues (Rogina et al., 2014).

Microspheres prepared with electrospraying method can be injected to the required site without requiring an invasive operation compared to conventional strategies.

Ideally, these systems can provide control over release rates, which is needed by certain therapies. Also the control of particle morphology, size and polymeric matrix properties are possible (Sokolsky et al., 2007). The other advantage of electrospraying is not making use of an external dispersion/emulsion phase, which often includes undesirable ingredients for biomedical applications (Arya et al., 2008).

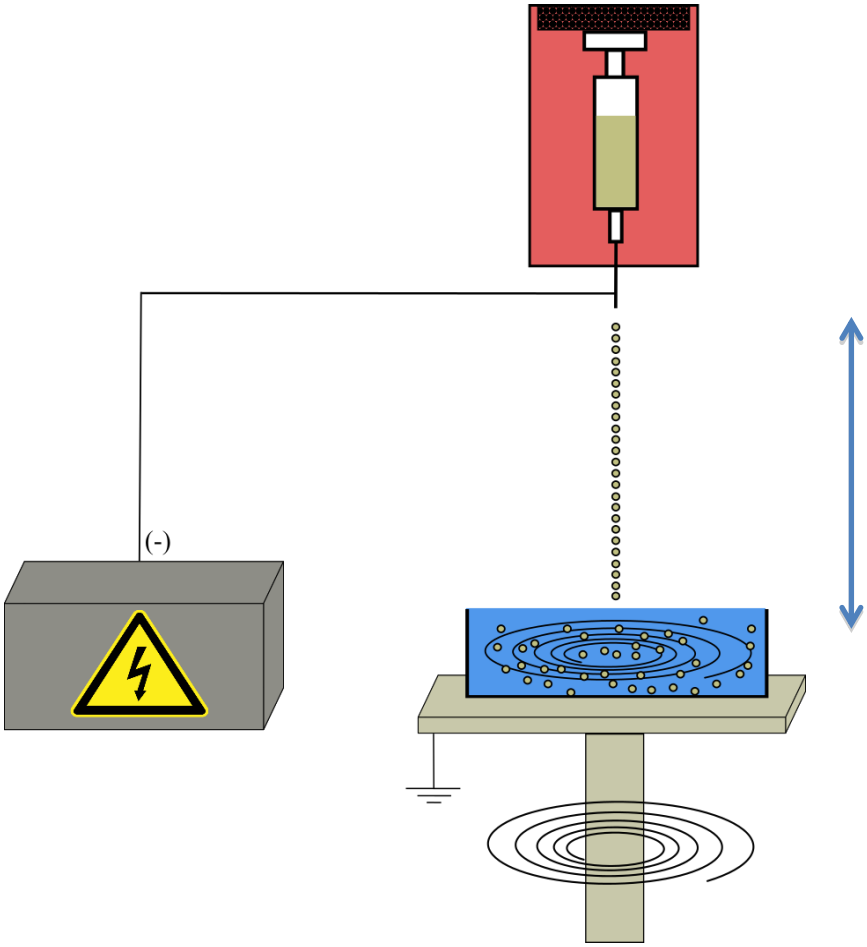

Figure 1. 11 Electrospraying set-up

The electrospraying process involves a polymer solution loaded into a syringe and dropped at a constant rate using a syringe pump through highly charged capillary (Figure 1.11). The applied voltage used is up to 30 kV and the collector/collection media might be placed at a 7 to 30 cm distance from the capillary. Once the droplets have detached from the Taylor cone, the solvent evaporates, generating dense and solid particles, propelled towards the collector. In some studies encapsulation of hydrophilic and hydrophobic drugs (Xie, et al., 2008), proteins (Yixiang et al., 2006), anticancer agents (Zheng et al., 2013), and cells (Xie et al., 2007) were accomplished by electrospraying process.

Solution viscosity and electrical conductivity are the important parameters during the electrospraying process (Theron et al., 2003).

Other parameters include voltage, distance to collector, needle gauge, flow rate, polymer, drug and solvent properties, and organic/aqueous solvent ratio.

1.6 Aim of the Study

In this study it is aimed to develop and characterize of injectable ALG/SER/CS microspheres for cartilage tissue engineering.

OA is an articular cartilage disease characterized by the progressive degradation and loss of cartilage. There is no effective treatment for the disease. The currently used strategies aim to decrease the pain and symptoms of OA.

The research question of this study is whether injectable chondrocyte containing microspheres could be developed to carry the cells successfully and support their proliferation. By their efficient sizes, spherical shape and the cell carrying potential to repair cartilage defects were also optimized for their suitability for injection, releasing profiles, swelling properties, degradation capacities and mechanical properties etc. required for cartilage tissue engineering. The protein and CS release properties of the microspheres were also investigated. The microspheres' suitability as cell carrier for new treatment approaches was also evaluated with cell culture studies using L929 and ATDC5 cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Grades and properties of Materials

Alginic acid sodium salt from brown algae was purchased from Sigma Adrich (USA). Sericin was obtained as dry form from Kirman İplik Limited Company (Turkey). Chondroitin sulphate was purchased from Sigma (USA). Calcium chloride was from Riedel-de Haen (Germany). Silk fibroin was purchased from Akman Ipek Bursa, Turkey. Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/L) with L-glutamine, DMEM high glucose (4.5 g/L) with L-glutamine, DMEM/F-12 (1:1), heat inactivated fetal bovine serum (FBS), trypsin EDTA and penicillin/streptomycin were purchased from Biochrom (Germany). Dimethyl sulfoxide (DMSO) and Triton X-100 were supplied by AppliChem GmbH (Germany). Dimethyl methylene blue (DMMB) were obtained from Aldrich (Germany). Presto blue was purchased from U.S.A, Bovine serum albumin (BSA), bicinchoninic acid (BCA) reagent were purchased from Sigma (Germany). Acetic Acid was purchased from Sigma-Aldrich (Germany).

2.2. Methods

2.2.1 Preparation of Alginate/Sericin/Chondroitin Sulphate Microspheres by Electrospraying Method

Electrohydrodynamic (EHD) spraying of solutions is the electric force applied to the surface of liquid cause a physical process. The Alg/Sericin/Chondroitin Sulphate microspheres with and without Silk Fibroin were produced by electrospraying technique. The microsphere forming device is shown in Figure 2.1. This devise conducts electrohydrodynamic atomization (EHDA). In EHDA the tip of the polymer solution jet from a syringe tip was divided into microspheres with the help of the joint action of electrostatic force, gravity and surface tension forces (Vaol and Zhang et al., 2012).

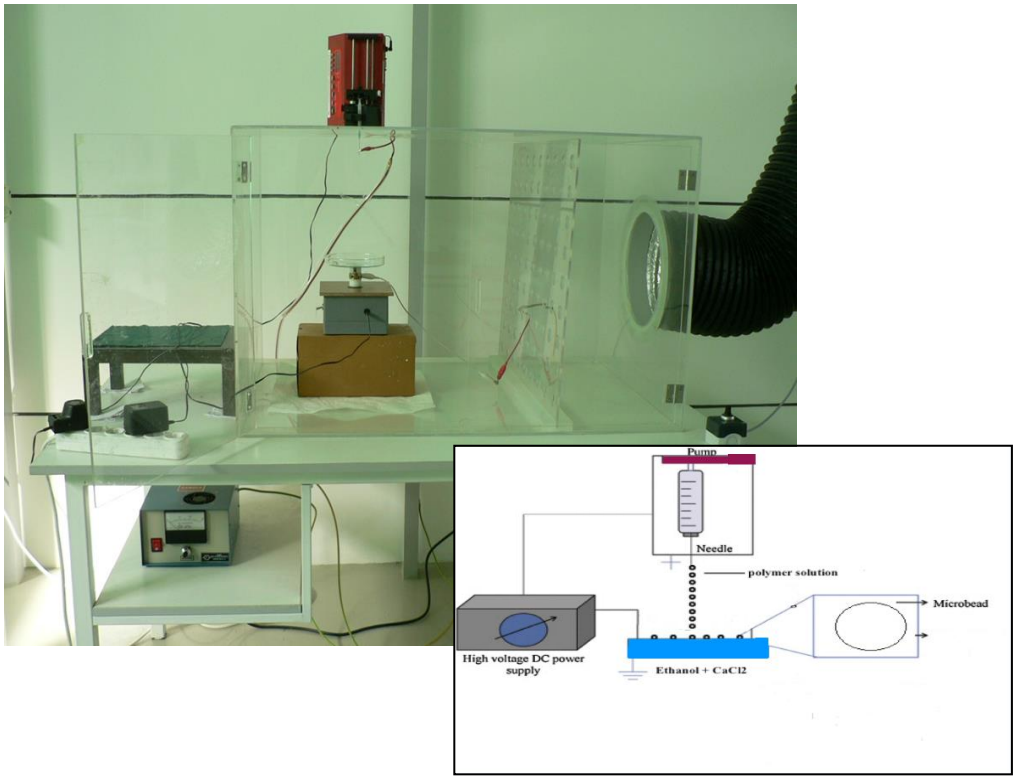

Figure 2. 1 Electrospraying set-up used for microsphere preparation and its schematic drawing taken from Nayak et al., 2014.

Basically, the setup includes syringe pump equipped with a syringe, a needle connected to high voltage power supply, (1 kV-30 kV) and collection solution in an ethanol bath including $CaCl_2$ (Figure 2.1). The needle was connected with the positive end of the high voltage generator, whereas the collection solution was grounded.

Figure 2. 2. Illustration of (a) a single-cone spraying mode (also called stable cone jet mode) and (b) a multiple-cone spraying mode (Hartman et al., 1999).

Table 2. 1 Preparation procedures for different microsphere groups.

	Solvent	*Solvent ratio*	*SF (w/v %)*	*Polymer concentration (w/v %)*	*Polymer ratio*
ALG	d-water	–	-	2	
ALG/SER	Acetic acid/ d-water	3:1	-	2:1	80/20
ALG/SER	Acetic acid/ d-water	2:1	-	1:1	80/20
ALG/SER	d-water	1:1	-	1:1	50/50
ALG/SER/CS	d-water	-	-		
ALG/SER/CS	Acetic acid/ d-water	2:1	-		
ALG/SER/CS	Acetic acid/ d-water	1:1	-		
ALG/SER/CS	d-water	1:1	-		
ALG/SER/CS/SF	Acetic acid/ d-water	1:1	0.25%		
ALG/SER/CS/SF	Acetic acid/ d-water	1:1	0.50%		

-$CaCl_2$ was used as crosslinker in all groups.

Alginate-sericin-chondroitin sulfate (ALG/SER/CS) solution was pumped out through the needle orifice at different rates (2, 4, 6 ml/h) and at different voltages (9-20 kV). Fine droplets of the solution were collected in the ethanol bath containing 1.2 % (12 g in 1000 ml ETOH) $CaCl_2$ solution in a container and left for crosslinking overnight. The resulting cross-linked microspheres were washed with pure water ethanol and collected by filtration through 100µl filter. The microcapsules formed were observed under light microscope (Nikon Eclipse TS100) and its attachment (Nikon Digital sight DS-U3) for determination of their shape and diameter. The microcapsules having optimum size and diameter were selected for further experiments. Electrospraying parameters like pump rate, distance, and voltage as well solution properties were determined for production as microspheres.

Different sericin and alginate concentrations were used to obtain spherical, stable microspheres during optimization studies. As a result the concentrations of materials were optimized as; ALG (40 mg), 0.8 w/w% and 1% w/w, SER (1 w/v %, 0.5 ml),

also SER (2w/v %, 1 ml) (100:50:10) and chondroitin sulphate (CS) (4 mg) were dissolved in Acetic acid solution (0.5M, 1 ml) at RT. A homogenous solution was obtained by stirring overnight. For preparation of CS containing microspheres, several concentrations (1 mg, 2 mg, 4 mg, 10 mg and 20 mg) of CS were tried for obtaining spherical stable microspheres 4 mg CS was chosen as the suitable CS amount for preparing microspheres. The protocol for preparation is detailed in Table 2.2. Microspheres were also coated with SF for improving the structural stability of microspheres in degradation studies. Apart from surface coating with SF, SF was also added to the composition of ALG/SER microspheres (Table 2.1, Table 2.2).

Table 2. 2 SF coated ALG/SER/CS microspheres

ALG (%)	*SER (%), (ml)*		*CS (mg)*	*Type of Solvent (ml)*	*SF (w/v)*
1	2	1 %2	4	3 ml d-water	-
1	2	1 2%	4	1:3 (v/v, AA /d-water)	-
1	2	1 2%	4	3 ml d-water	%2.5 coated
1	2	1 2%	4	3 ml d-water	%0.25 incorporated
1	2	1 2%	4	1:3 (v/v, AA /d-water)	%2.5 coated
1	2	1 2%	4	1:3 (v/v, AA /d-water)	0.25% incorporated

2.2.2 Characterization of Microspheres

The formed microparticles with different polymer compositions were evaluated; for the stability in aqueous media by degradation studies, for morphological and size properties by light microscopy (Nikon Eclipse TS100) and Scanning Electron Microscopy (SEM) (Stereoscan S4-10, Cambridge, UK and JSM-6400 Electron Microscope, Jeol Ltd., UK), for chemical composition and properties by Fourier Transform Infrared Spectrometry (FTIR) (Perkin Elmer, Inc., UK)

2.2.2.1 Degradation Studies of Microspheres

The microspheres were placed in distilled water and also in cell culture media (high glucose DMEM with 10% FBS and 1% penicillin medium and DMEM/F12 without phenol red) and examined with a light microscope (Nikon Eclipse TS100) and photographed with the attachment (Nikon Digital sight DS-U3) for determining the change in morphology and size of the microspheres.

2.2.2.2 Chemical Characterization of Microspheres

FTIR-ATR analysis of the microspheres was conducted using a transformed infrared spectrophotometer FTIR-ATR - Perkin Elmer Spectrum to determine the chemical properties of the particles. The ALG/SER/CS microspheres with fibroin encapsulation and without fibroin encapsulation were examined by FTIR-ATR-Perkin Elmer Spectrum Two 2 (Perkin Elmer, Inc., UK).

FTIR- ATR analysis was conducted to determine the presence of Sericin and CS around the microspheres via a spectrometer of FTIR - Perkin Elmer Spectrum Two 2 (Perkin Elmer, Inc., UK) using spectrum 100/100N software program in transmission mode within the wavelength range 4000-600 cm^{-1}, with a resolution of 4 cm-1 were used to analyze them.

2.2.2.3 Morphology of Microparticles

The surface and general morphology of microparticles were studied by Scanning Electron Microscopy (SEM S4-10, Cambridge, UK and JSM-6400 Electron Microscope, Jeol Ltd., UK), equipped with NORAN System 6 X-ray Microanalysis System & Semafore Digitizer (Thermo Fisher Scientific Inc., USA) in the Department of Metallurgical and Materials Engineering at METU (Ankara, Turkey) and Quanta 400F Field Emission SEM device (FEI, USA) in Central Laboratory at METU (Ankara, Turkey). Samples were prepared for analysis by coating with gold using a sputter coating device (Hummle VII, Anatech, Istanbul, Turkey). The ALG/SER/CS microspheres with and without fibroin were taken to the SEM in Central Lab.

2.2.2.4 Particle Size Analysis

The sizes of at least 100 wet microspheres were measured by light microscope (Nikon Eclipse TS100) using the magnification of 4X and 10 X to obtain the size distribution of the microspheres and determine the average diameter of microspheres.

2.2.2.5 In Vitro Release of Sericin and Chondroitin Sulfate from Microspheres

2.2.2.5.1 Determination of CS Amounts Released from Microspheres Using DMMB Assay

For the release study 10 mg EtOH sterilized microspheres were placed in 5 ml DMEM/F-12 media without phenol red under sterile conditions. Aliquots from the release media were collected at different incubation periods (1, 3 and 7 days). The CS amounts released from microspheres were determined with dimethyl methylene blue (DMMB), sulfated glycosaminoglycan (sGAG) assay (Farndale et al., 1986). 16 μg DMMB/ml (pH 3) was prepared in Glycine/NaCl solution including 3.04 g Glycine, 2.37 g NaCl, 95 mL 0.1 M HCl and 905 mL distilled water. The Chondroitin Sulphate (CS) from shark cartilage was used as the standard for constructing the calibration curve (0-5 mg).

CS was prepared in phosphate buffered EDTA (PBE) solution, which includes 14.44 mM cysteine, 100 mM di-sodium hydrogen phosphate, 10 mM EDTA, pH 6.5.

For the assay 50 μL of the release media was mixed with 1.25 mL DMMB dye solution in a 24-well plate and the absorbance at 525 nm was determined immediately with a microplate spectrophotometer (BioTek μQuant USA). CS amounts released from the microspheres were calculated according to the calibration curve.

2.2.2.5.2 Determination of SER Amounts Released from Microspheres Using BCA Assay

EtOH sterilized microparticles (10 mg) were placed in 5 ml DMEM/F-12 without phenol red and incubated at 37°C in water bath (Nuve Bath NB 5, Turkey). The release media were collected at different time points (1, 3 and 7 days) and the amount of silk sericin released into the media was measured using a BCA protein assay.

Bovine serum albumin was used as a standard protein for drawing a calibration curve (Appendix B). Firstly, 50 μL of each standard solution was mixed with 1 ml cupric sulfate for preparing BCA solution. 25 μL of BSA standards and samples into 96 well plate and incubate them for 15 min at 60 C°**.** After cooling step, absorbances at 562 nm were read with a microplate spectrophotometer (BioTek μQuant, USA) (National Institute of Health 31 Sciences, 2006). The protein content of the microspheres (SER content released) was determined by the equation of the calibration curve. All experiments were performed in triplicates.

2.2.3 Cell Culture Studies

All microparticles were sterilized in wet state (in EtOH) by UV (365 nm) exposure for 45 min. Non-adherent plates were used for easy cell attachment on the microspheres. Microsphere suspension (200 μL) was added to each well of 48-well plate (n=4 for each group).

Cartilage cells (ATDC5, UNAM Lab, Bilkent University) were seeded at a density of 1.5x104 cells/cm^2 onto microspheres in each well and incubated for 1 h for cells to attach. Finally, 300 μL of high glucose DMEM with 10% FBS and 1% penicillin medium was added to wells and incubated at 37°C in 5% CO_2 atmosphere in carbon dioxide incubator (5215 Shel Lab., Cornelius, OR, USA). Medium was refreshed carefully without touching microspheres.

2.2.3.1 Cell Viability Study (Presto Blue)

Presto Blue assay was performed to study the cell proliferation on microspheres after 1, 3 and 7 days of incubations. At each time point, the culture media in the 48-well plate were replaced by 200 μL medium containing 15% (v/v) serum and 9:1 Presto Blue (Sigma, U.S.A.). The cells were incubated under standard cell culture conditions for 1 to 8h. After that the optical densities were obtained at 500, 570 nm and 570-600 nm against a medium of negative control set with Presto Blue. Unseeded microspheres were used as blank. The results are presented as cell number according to standard curve constructed with known number of cells.

2.2.3.2 Cell Proliferation

For determining cytocompatibility of microspheres, L929 (L cell, L-929, derivative of Strain L) and ATDC5 cells were used. L929 cells are subcutaneous connective tissue cells with fibroblast morphology (www.atcc.org). The chondrogenic cell line ATDC5 has been derived from teratocarcinoma AT805.

For cell viability studies ATDC5 were used. It is used for chondrogenic studies. Its biological source is unknown from Mouse and its origin was teratocarcinoma. Its morphology is epithelial like (www.sigmaaldrich.com).

In ATDC5 studies, firstly the surface areas of microspheres were calculated based on the formula of surface area of spheres ($4\pi r^2$). It was equal to the $4.1*10^{-3}cm^2$. For cell culture studies firstly microspheres were sterilized with U.V. for 30 minutes (Umeki et al., 2010).

226 ALG/SER/CS microspheres were put in 100 μL in 48-well plate. Cells were seeded at an initial density of 30.000 to 200 μL microspheres in each well. For cell culture studies, the following experimental groups were used:

I.	ALG/SER/CS in AA and dH_2O with 0.25% SF encapsulation
II.	ALG/SER/CS in AA and dH_2O without SF
III.	TCPS control

2.2.4 Measurement of Microparticle Stability

The mechanical stability of the microparticles was tested by centrifuging (n=3) at various centrifugal forces 1000 G at 10 min for observing their maintenances of structural stability under centrifugal force. 100 microspheres were observed after centrifugation under light microscopy.

2.3 Statistical Analysis

All data were expressed as mean ± standard deviation. SPSS software was used for the One-Way Analysis of Variance (ANOVA), with Tukey's multiple comparison test for post-hoc analysis. Differences were considered as significant for $p \leq 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of ALG/SER/CS microspheres

In order to obtain microparticles of ALG/SER/CS with optimum properties different amounts and combinations of the materials (alginate, sericin, and chondroitin sulphate) were used with various solvents (d-water, acetic acid) in electrospraying process.

Optimizations were also done on crosslinking and coating conditions and materials as well as on electrospraying parameters (voltage, distance, flow rate, etc.). Microparticles were initially characterized for their sizes, morphologies and aqueous stability.

In preliminary studies the weight blend ratio was decided according to photos that were taken by light microscopy.

The experiments about alginate and sericin concentrations were done and the ratios of concentrations ALG: SER were (1:0), (0.8:0.2), (0.6:0.4), (0.4:0.6),(0.2, 0.8), (0,1). At last according to the light microscopy photos the ratio of ALG: SER were chosen as (0.2:0.8). The collection solvent type, flow rate and voltage were constant during these optimization studies.

The most suitable microspheres were obtained with ALG/SER/CS ratio of 0.64:0.32:0.06, in 0.5 M acetic acid solution by electrospraying under 17 kV electrical voltages with 2 ml/h flow rate and at a distance of 12 cm from ethanol bath involving 1.2 % $CaCl_2$ (Figure 3.1).

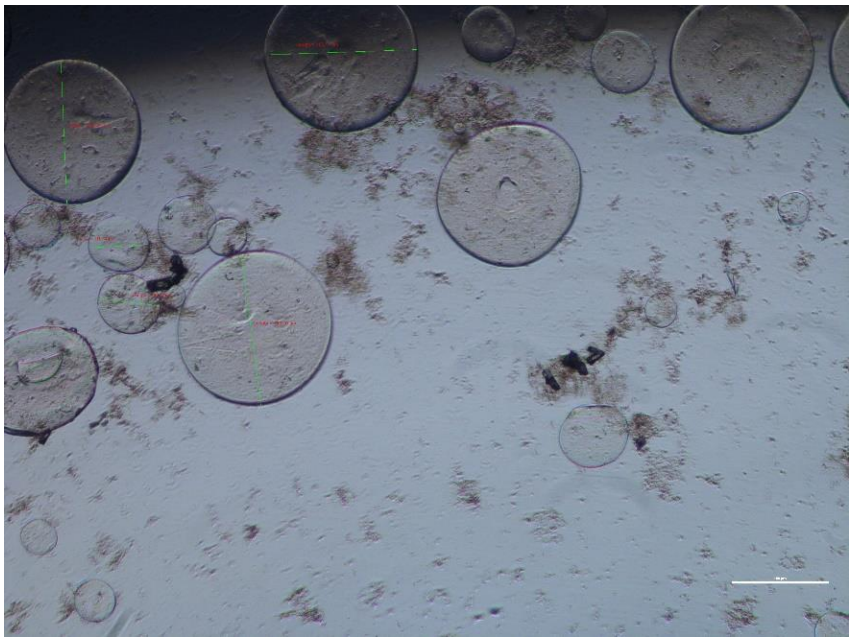

Figure 3. 1 Light Microscopy images of initially optimized ALG/SER/CS microspheres (10X) (scale bar= 100 μm)

These microspheres were found suitable for cartilage tissue engineering applications regarding to their shape, size and formulations. The spherical shape was important because of the stability of cartilage, the size should appropriate for injectable with syringe and formulations of microspheres were beneficial for cartilage.

Electrospraying method was carried out using several concentrations and ratios of ALG/SER/CS for the preliminary studies of microspheres. As it can be observed in Table 3.1, the sizes of microspheres changed very little according to the presence of CS and SER. Also the presence of acetic acid changed the size more effectively than the other materials.

In addition to these modifications, fibroin coating and fibroin incorporation into microspheres were also applied in later optimization studies. The encapsulation of ALG/SER/CS in H_2O with fibroin is crucial because the microspheres should remain at the injection site and the degradation of them should be slow. Moreover SF has the other advantages like enabling specific ligands attachment to the surface by fibroin coating to the surface of microspheres (Wang et al., 2007). Fibroin encapsulated microspheres had more dense appearance as shown in microscopy images with darker regions (Fig. 3.2.)

Table 3.1 Average particle sizes of microspheres obtained with different combinations and solvent systems

Alginate (%, w/v)	*Sericin (%,w/v)*	*Chondroitin sulphate (mg)*	*Solvent System (ml)*	*Size (µm)*
0.8	1	-	1:3 (v/v, AA /d-water)	270
0.8	1	4	1:3 (v/v, AA /d-water)	258
1	2	4	d-water (3ml)	500
1	2	4	AA (1ml)	222
1	2	-	d-water (3ml)	760
1	2	-	1:3 (v/v, AA /d-water)	400

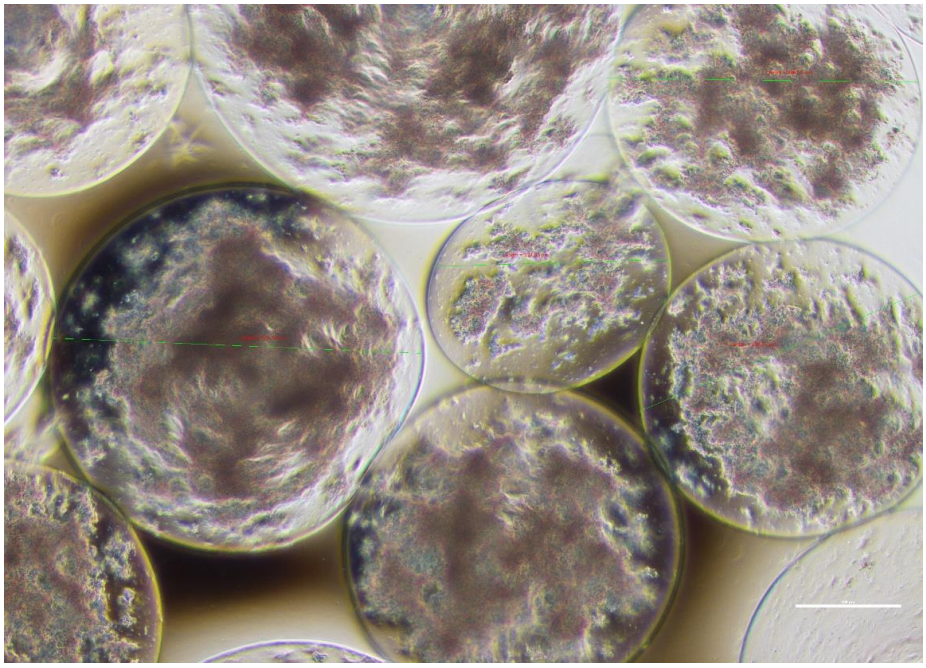

Figure 3. 2 Microscopy image of wet electrosprayed ALG/SER/CS microspheres with fibroin encapsulation (10X) (scale bar: 100 μ).

Cartilage tissue engineering approaches mostly focus on injectable 3D-microspheres in combination with cells to simulate cartilage ECM since microspheres are favorable as carrier of cells with their spherical morphology, porous structure, and suitable dimensions (Shalumon et al., 2015). So optimizations of microspheres were done considering these properties. In literature there is one study on SER/ALG microparticles. However, Wantanasiri and his coworkers also studied 3D of silk sericin/alginate microparticles prepared by electrospraying technique, however the aim in their study was just controlled release of SER.

In preliminary studies the concentration of polymers was set to obtain microsphere form. The distance of collector system was decided as 12 cm after several trials. However, during initial experiments when water was used as the solvent of polymers, at the end of experiment the sizes of microspheres were not appropriate for injection. There were several other problems about these microspheres such as shape and size homogeneity; they were mostly within 700-1000 μm size range.

When these microspheres were removed from ethanol and put into PBS (pH 7.4) 37°C, they ruptured and dissolved in 1-3 days (Figure 3.3). So there occurred need for another modification for increasing stability of the microspheres.

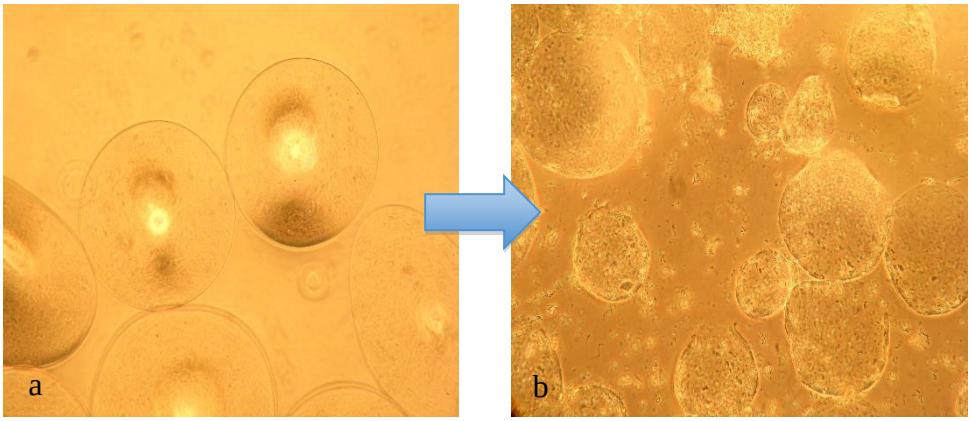

Figure 3. 3 Electrosprayed optimized microspheres (a) Before (b) After 1 day incubation in PBS at 37°C

Sodium alginate is the sodium salt of alginic acid (also alginate). It has gained much research interest in targeted drug delivery for the release of both small and large molecules (Bowersock et al., 1995). Its applications in Biotechnology industry are continuously increasing (Wayne et al., 2012; Baimark et al., 2014).

Alginate is a versatile material for biomedical applications. This is because it is biocompatible for human and it has good stiffness property. So it is suitable for biomedical applications. In the beginning of the study several amounts and different percentages of alginate have been tried. At last 1% (40 mg) of powder form of alginate was decided to be suitable for the electrospraying process.

In our study a cell carrier system based on alginate was aimed. The encapsulation of protein and cells within the alginate-based microparticles had high encapsulation efficiencies in literature (Fukui et al., 2010; Suksamran et al., 2010). Sericin was added to alginate during microsphere production for its novel properties like high water retention, antibacterial properties and it enhance cartilage regenerative potency (Goudarzi et al., 2014).

So, initially microspheres were produced by electrospraying these two polymers and microspheres having good spherical structures and homogenous size distributions could be obtained at conditions: 12 kV flow rate, 12 cm distance between needle tip and ETOH bath collector, standard stirring rate and 2 ml truncated syringe. (Figure3. 4).

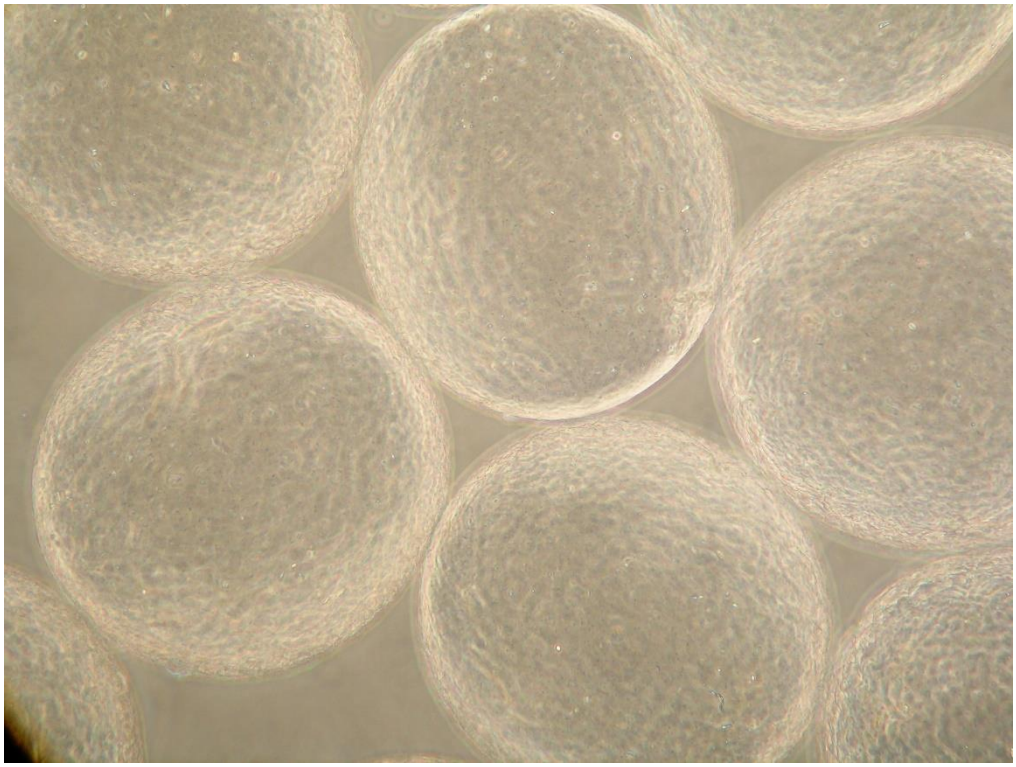

Figure 3. 4 ALG/SER microspheres of a size distribution of 600-1000 μm (10 X) (600 -1000 μm)

The microspheres were cross-linked to increase their stability, because both polymers were highly water-soluble. It is known that for alginate microspheres the ionic crosslinking is important. To achieve alginate gelation and crosslinking, the exchange of sodium ions from guluronic acid units with divalent cations such as calcium (Ca^{2+}) is needed (Figure 3.5).

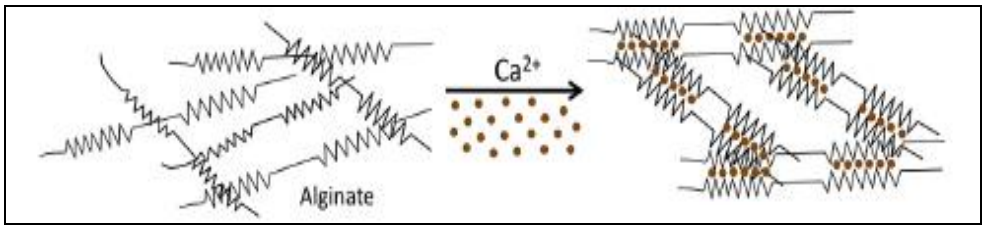

Figure 3. 5 Schematic representation of alginate hydrogel formation by crosslinking via calcium (Bidarra et al., 2013).

Ionic crosslinking with calcium-induced chain–chain association of guluronate blocks forms the junction zones responsible for gel formation (Bidarra et al., 2013).

Besides Sericin, in order to enhance cartilage regenerative potency of the microspheres it was decided to add CS, a native component of ECM of cartilage. These ALG/SER/CS microspheres are thought to provide release of the regenerative agent, CS, besides being cell carrier. After preliminary studies with 25 mg, 15 mg, 10 mg, 8 mg and 4 mg CS, at last 4mg CS was chosen as the best amount considering the shape/structure of microspheres. The effect of CS addition on microspheres on morphology was evident (Figure 3.6); CS affected the surface topography by creating more smooth appearance on the surface of microspheres

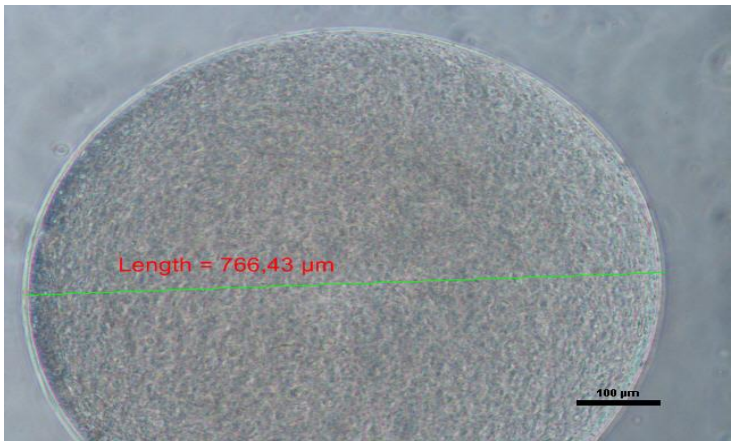

Figure 3. 6 Light microscopy image of ALG/SER/CS microsphere (10X) (100 µm)

In addition to this, addition of CS was thought to decrease the immunogenicity of the microspheres considering the results obtained in literature after addition of CS to treat OA where immunogenicity, size and stiffness of microspheres changed CS. gave minimal foreign body reaction and minimum sense of immune cells (neutrophils and macrophages), however microspheres with no CS showed a relatively increased inflammation (Lee et al., 2007).

Electrosprayed 3D-microspheres could be produced reproducibly. However the sizes of spheres were around 700-1000 µm. Microsphere sizes need to be reduced with additional optimizations. For this purpose, polymer concentrations were altered several times while keeping the same electrospinning parameters; 10-12 kV electrical voltages, 2 ml/h flow rate, and 12 cm distance from the needle tip and EtOH bath (Figure 3.7)

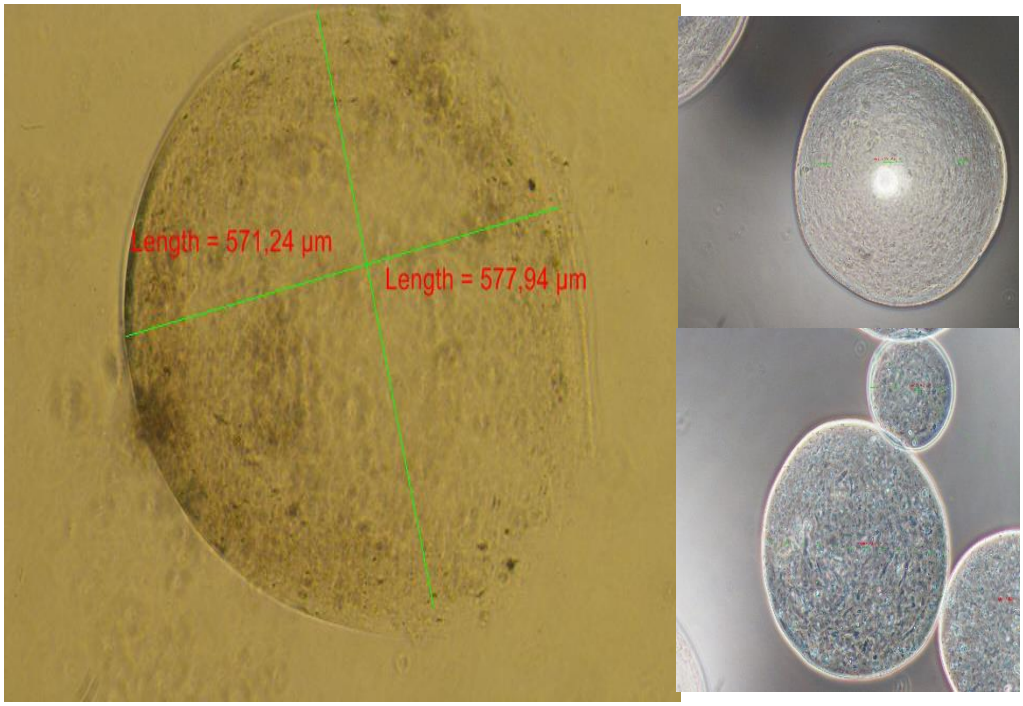

Figure 3. 7 Light microscopy images of ALG/SER/CS microspheres (10 X).

Scale bar: 100 µm.

Jet stability is important in electrospraying. When the cone–jet formation occurred the concentric droplets were generated because the jet breaks up. The concentric droplets dropped from the pumping machine were full of charge and lose their charge when dropped due to the presence of grounded electrode (Soc et al., 2010). By increasing electric field strength, stable jetting was tried and the dripping mode increased at the same time. The dripping mode changed according to the materials that were used. One of the reasons of adding d-water to the solution was to increase the electrical conductivity and thus spinnability as d-water has high dielectric constant. As the conductivity of the solution was increased by the addition of HCOOH or CHCOOH to water or to aqueous protein solutions, the reduction of flow rate was needed to obtain stable electrospray. Smith et al reported that the optimum flow rate and decrease in the optimum flow rate for electrospray occurs with the increase in solution conductivity. After all preliminary studies, acetic acid (AA) was added to solution and the size reduction in microspheres were observed (Figure 3.8) The handicap of adding AA into solution was the change of shapes of some microspheres to less spherical forms. However, generally the microsphere sizes were in the range of intended sizes.

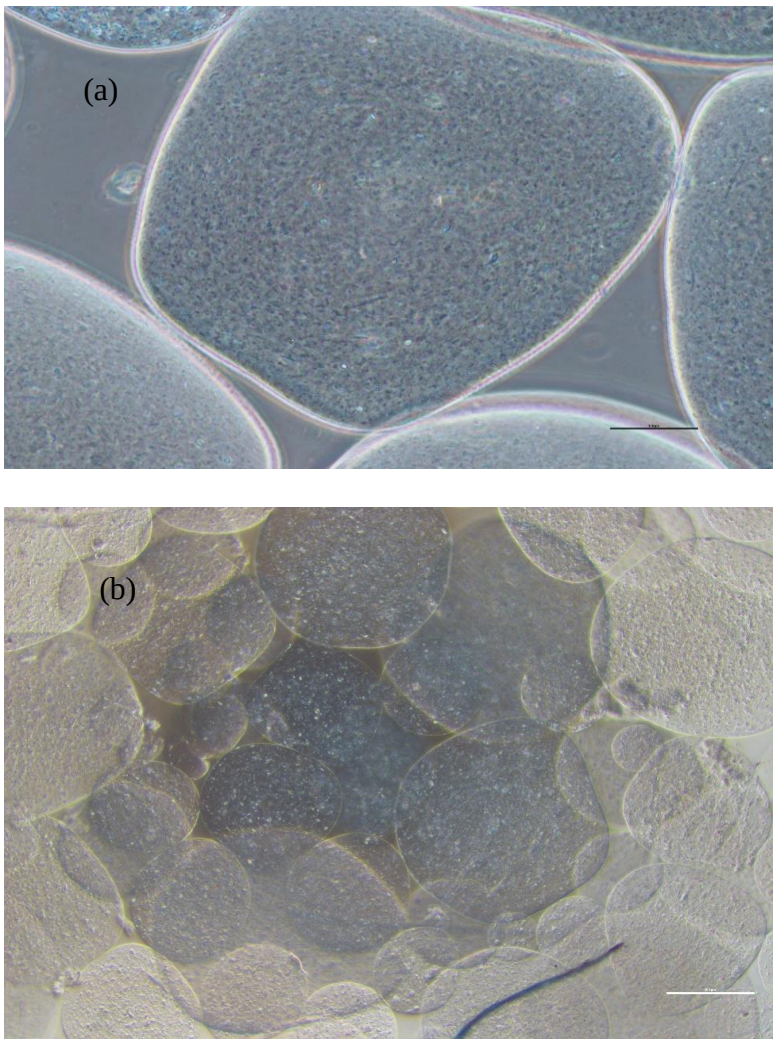

Figure 3. 8 ALG/SER/CS microspheres prepared with addition of (a) 2 ml AA (b) 1 ml AA to the polymer solution

Scale bar: 100 μm.

When 1 ml 0.5 M acetic acid was used, the sizes and shapes of microspheres were affected by the acetic acid solution as also observed in 10%(v/v), 30 % (v/v), 50% (v/v), 70%(v/v) and 90% (v/v) acetic acid concentrations that were studied by Arya et. al (2007). Since AA decreased the size of microspheres, after several trials the usage of 1 ml AA at 1% 0.5 M concentration was decided.

Moreover, various crosslinking studies were conducted with chemical crosslinking agents as EDC/NHS (Figure 3.11), borax (Figure 3.12) and glutaraldehyde, at different concentrations and time durations.

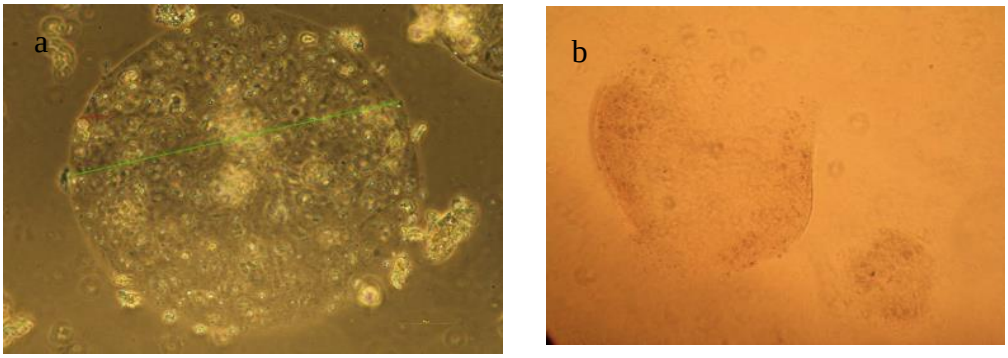

Figure 3. 9 Microscopy image of ALG/SER/CS microspheres crosslinked with EDC/NHS (a) Immediately after crosslinking and (b) 2 days after awaiting in PBS

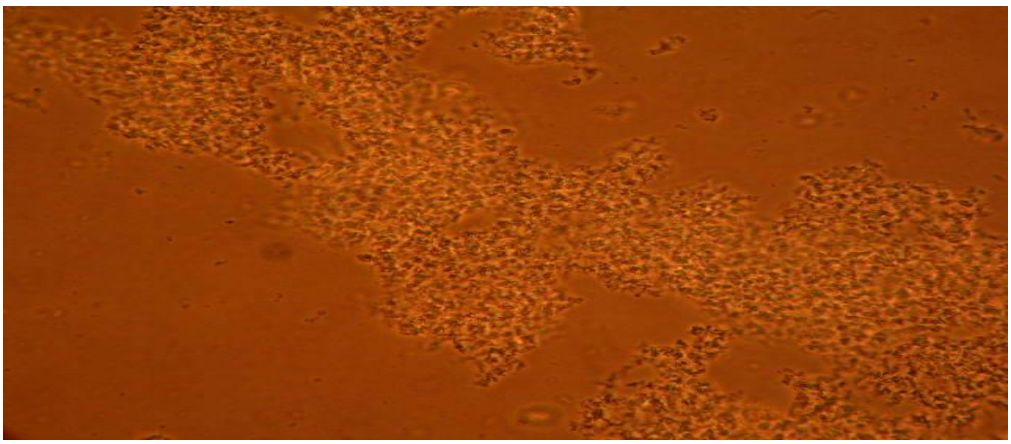

Figure 3. 10 Microscopy images of forms obtained after alginate dialdehyde (ADA)/SER oxidation reaction with 0.1 M Borax

First stock solutions were prepared with Alginate, sericin, acetic acid and dH_20. As there is no amine groups in ALG structure for making crosslinking with Borax stock solution included Alginate dialdehyde (ADA) instead of Alginate. After mixing stage, the Borax solution was added to ALG/SER solution and the electrospraying process was done. Several concentrations of Borax were tried for effective crosslinking between aldehyde groups.

Although the oxidation reaction of Alginate dialdehyde was made, there was no microsphere formation by electrospraying (Figure 3.10). Also crosslinking reactions with several concentrations of Glutaraldehyde were tried, but it was not successful too. Microspheres were not durable. At last the SF incorporation into microspheres were decided to increase their stability.

In the experiments silk fibroin concentrations were changed. There was microsphere formation when 0.25 % SF was used within the experiment solution for electrospraying (Figure 3.11).

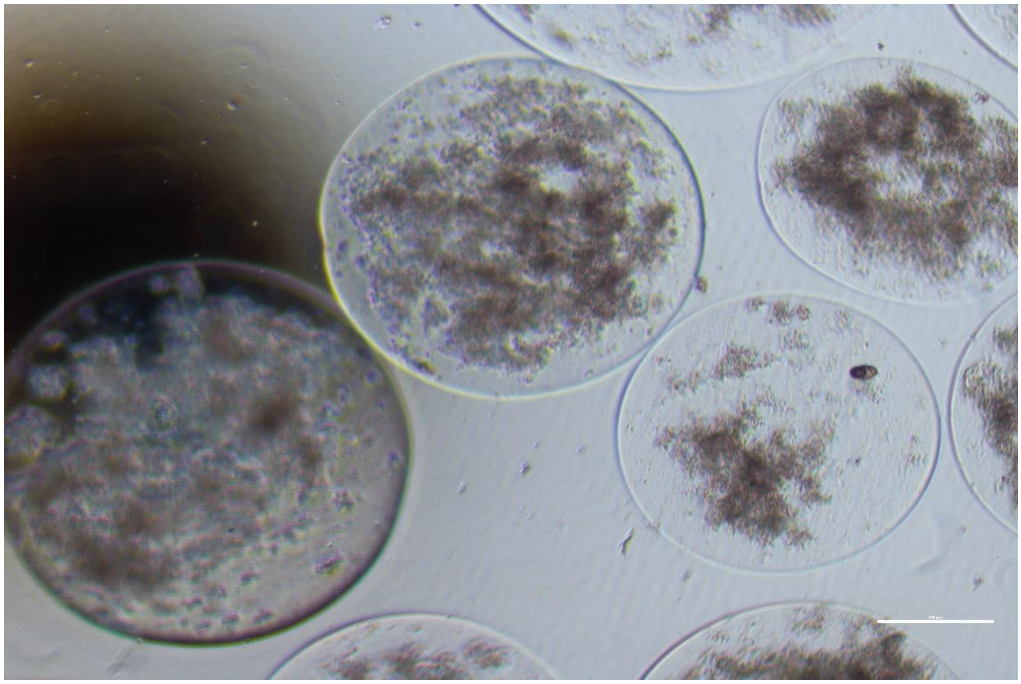

Figure 3. 11 Light microscopy images of microspheres prepared with ALG/SER/CS incorporated with 0.25 % SF (10X)

Scale bar: 100 μm.

However, no microsphere formation occurred when 0.50 %, 1% and 2.5 % SF were added to polymer solution during electrospraying because the syringe was congested during electrospraying of the solution. Alternatively, ALG/SER/CS microspheres were awaited inside 2.5% SF solution for 30 min for coating with SF.

The shapes of microspheres and stiffness of them were enough; however the sizes of microspheres were not efficient (Figure 3.12).

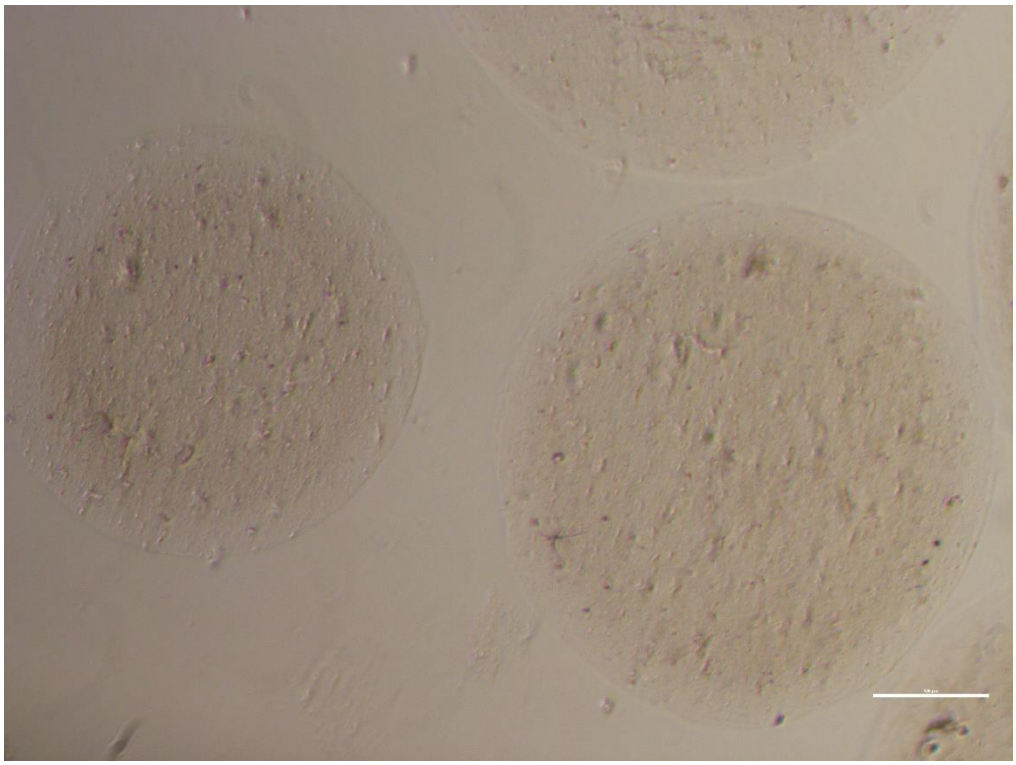

Figure 3. 12 ALG/SER/CS microspheres coated with 2.5 % SF solution (4X).

Scale bar: 100 μm.

Generally, when a material is covalently cross-linked, it is not available for injection. Also release from covalently crosslinked microspheres is usually less than non-crosslinked ones (Bidarra et al., 2013; Lee et al., 2012). There are some exceptions of alginate crosslinking. Also there are disadvantages of these crosslinking methods and the major one is toxicity of the reagents used (Bidarra et al., 2013). While the encapsulation methods need several processing conditions they can affect the materials stability (Pritchard et al., 2013; Hu et al., 2011). The SF encapsulation renders the microspheres as possible carriers for long-term carrier of sensitive biologicals (Wen et al., 2011). The SF also affects the cell adhesion and spreading (Servoli et al., 2005).

For improving aqueous stability of microspheres 0.25% fibroin was incorporated to microspheres experiment solution. It is known that fibroin gets β-sheet conformation in ethanol, which is highly stable in this form (Zheng et al., 2009). Yet, the sizes of microspheres (Figure 3.13) were not small enough for injection with a needle. They were even larger than previous groups (Figure 3.7).

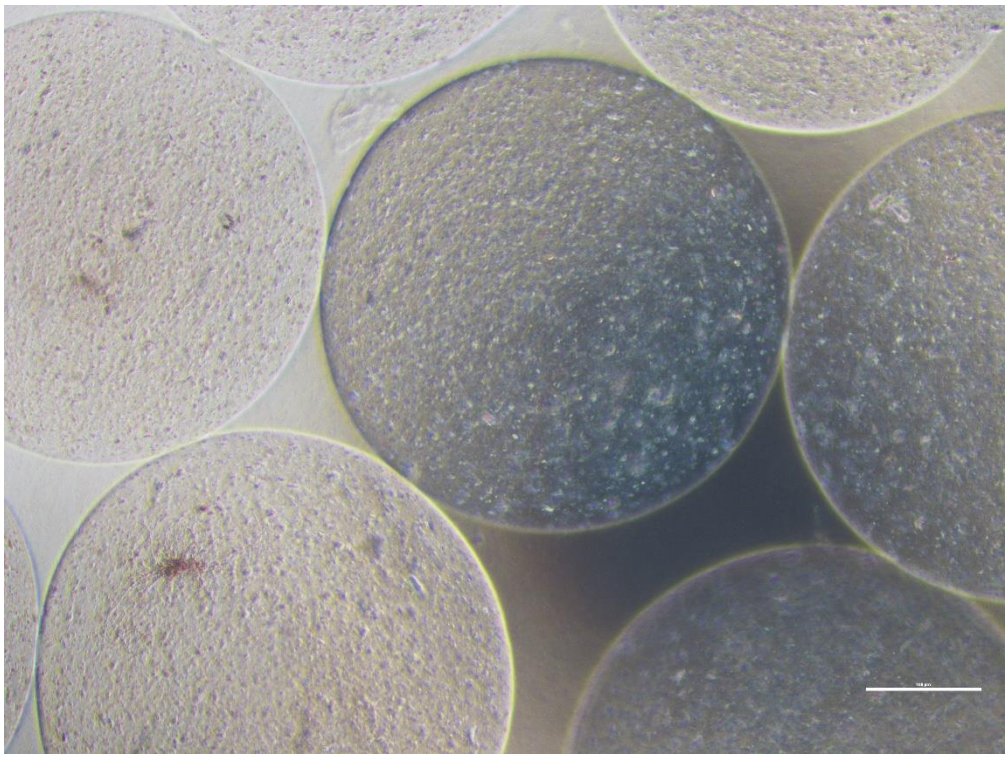

Figure 3. 13 Silk Fibroin (0.25%) incorporated ALG/SER/CS microspheres

Scale bar: 100 μm.

According to the experiments with acetic acid in polymer solution incorporating silk fibroin, the size of microspheres again considerably decreased (Figure 3.14).

After all these parameters were optimized, the average size for ALG/SER/CS microspheres with SF was determined to be about 222 μm in wet state.

It can be seen clearly in Figure 3.14 that produced microspheres had spherical shape and homogenous size distribution.

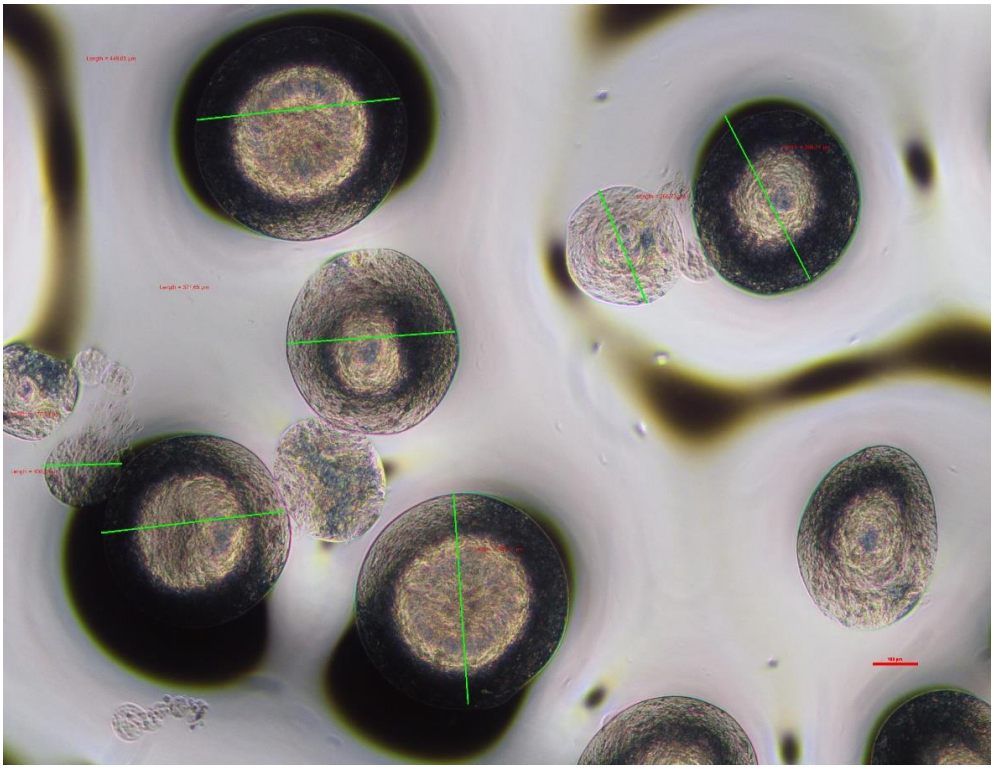

Figure 3. 14. ALG/SER/CS microspheres prepared with addition of AA(10X)

Scale bar: 100 μm.

The microspheres were collected either in EtOH or in distilled water. According to the Aramwit et al. (2012) alcohol changes the structure of proteins and induces β-sheet formation for fibroin. Moreover, Gimenes et al. (2007) studied that ethanol increases the aggregated strands (β-sheet); both its random coil and α-helix components decrease in the secondary structure. The water solubility decreases because of increasing this more organized form (Silva et al., 2014). The shapes were better in pure EtOH. The materials used in this study were water-soluble so when electrosprayed the droplets on water, ionic crosslinking was less efficient.

Acetic acid was added and it caused to increase the dielectric constant of solution and because of this, solution could be dropped at small voltages like 10-12 kV. However, the sizes were 600-950 µm. When the applied voltage was higher, droplets accelerated and their diameter decreased, however sometimes the beads lost their shapes at high voltage (Figure 3.15). The electrospraying machine capacity was 0-30 kV. Hence, voltage of electrospraying was changed several times and finally at 17 kV, the intended sizes were achieved. At 17 kV the shape of microspheres with AA were obtained to be around 200 -400 µm.

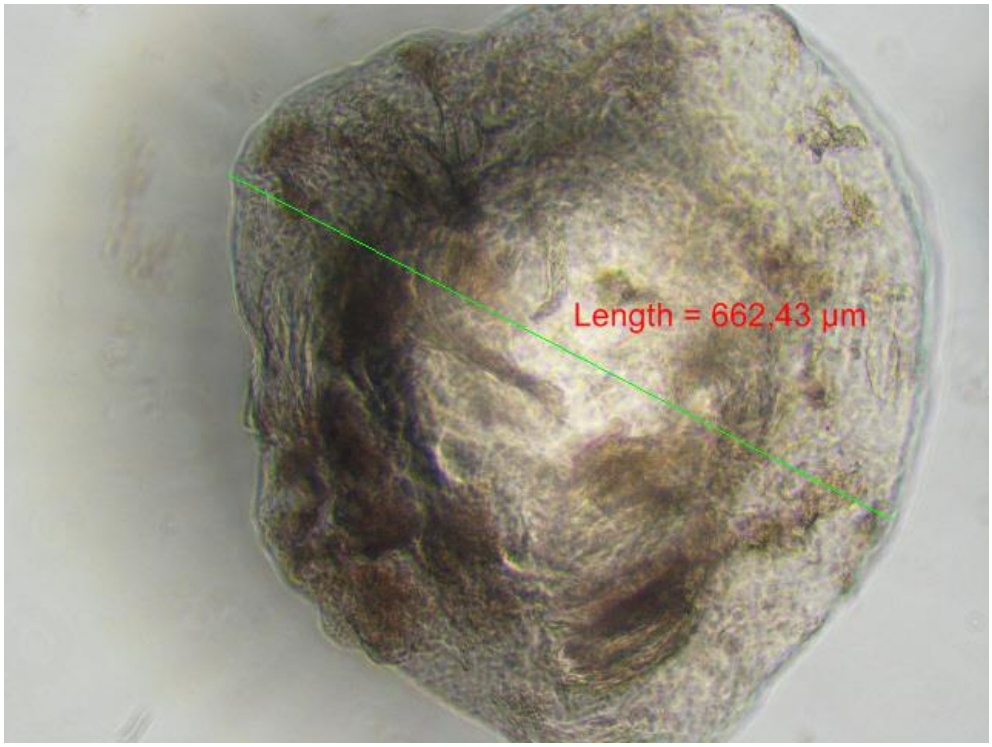

Figure 3. 15 Light microscopy images of ALG/SER/CS microspheres electrosprayed at 19-30 kV. Magnification (10X)

Increase in flow rate means increase in dripping frequency too. The different flow rates were tested and 2 -4 ml/h was chosen as optimum for this solution composition. In the final formulation (1% (40 mg) Alginate, 1 ml 2% sericin, 4 mg CS, 1 ml 0.5 M AA and 3 ml dH_2O) shapes of spheres after electrospraying were very smooth and it was suitable for injection and cell growth on it. (Figure 3.16).

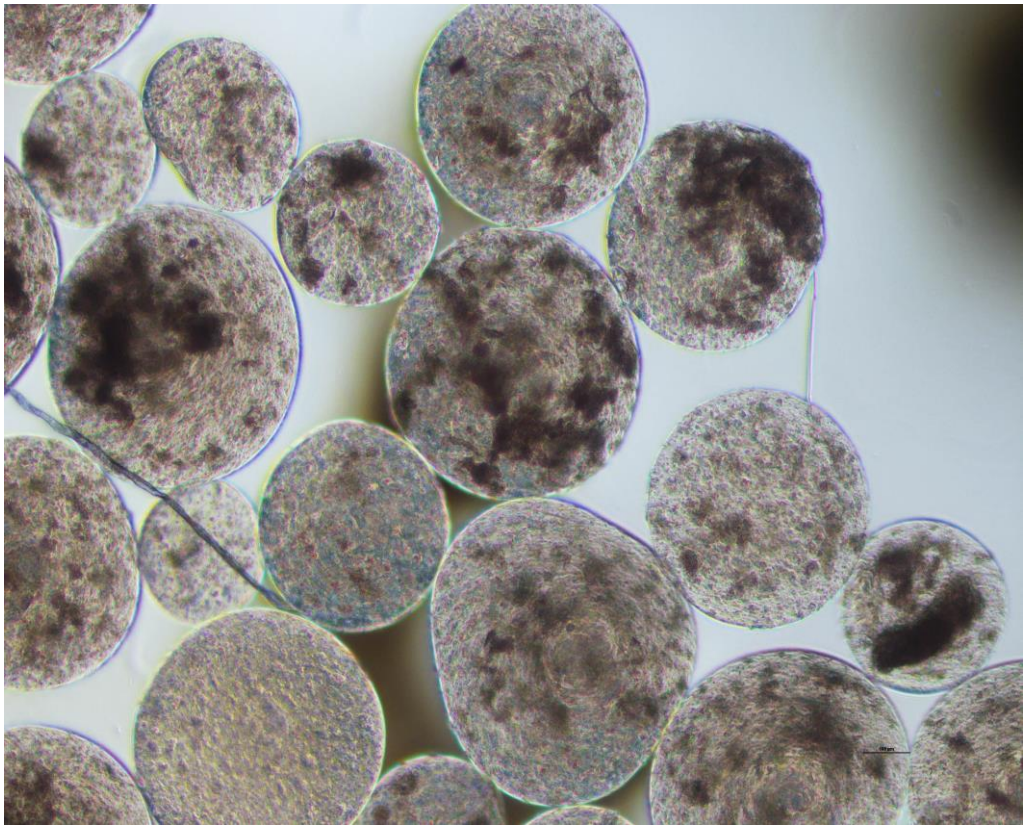

Figure 3. 16 ALG/SER/CS with 0.25% SF and addition of AA

Carboxyl groups of alginate were crosslinked with $CaCl_2$. Addition of $CaCl_2$ causes decreasing in particle sizes of microspheres. However, the $CaCl_2$ concentration and the size should be optimized. Different concentrations were tried in pure EtOH and at last 1.2 % $CaCl_2$ was chosen.

Effect of Applied Electrical Voltage

SEM examinations revealed influences of electrical voltage on microsphere morphology. When the applied voltage was increased from 10 (Figure 3.17) to 17 kV, microsphere was achieved and number of microspheres was increased too. After optimization studies during electrospraying, parameters were set as 17 kV, 2 – 4 ml/hr flow rate, and 12 cm distance (Figure 3.18).

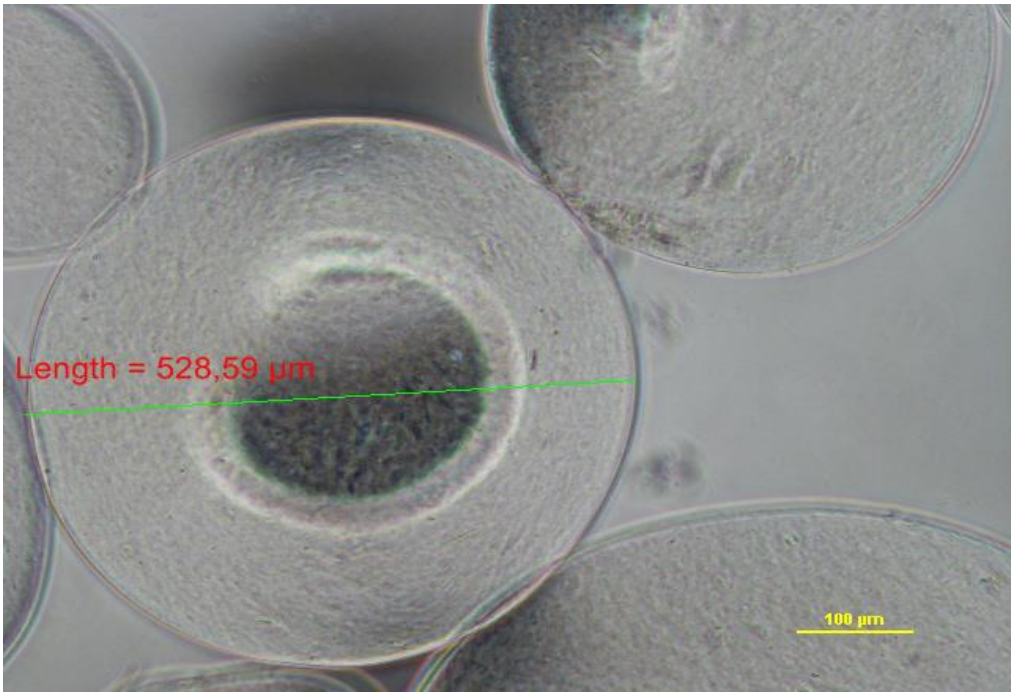

Figure 3. 17 Uncoated ALG/SER/CS microspheres prepared at 10-12 kV (10X) Scale bar: 100 µm

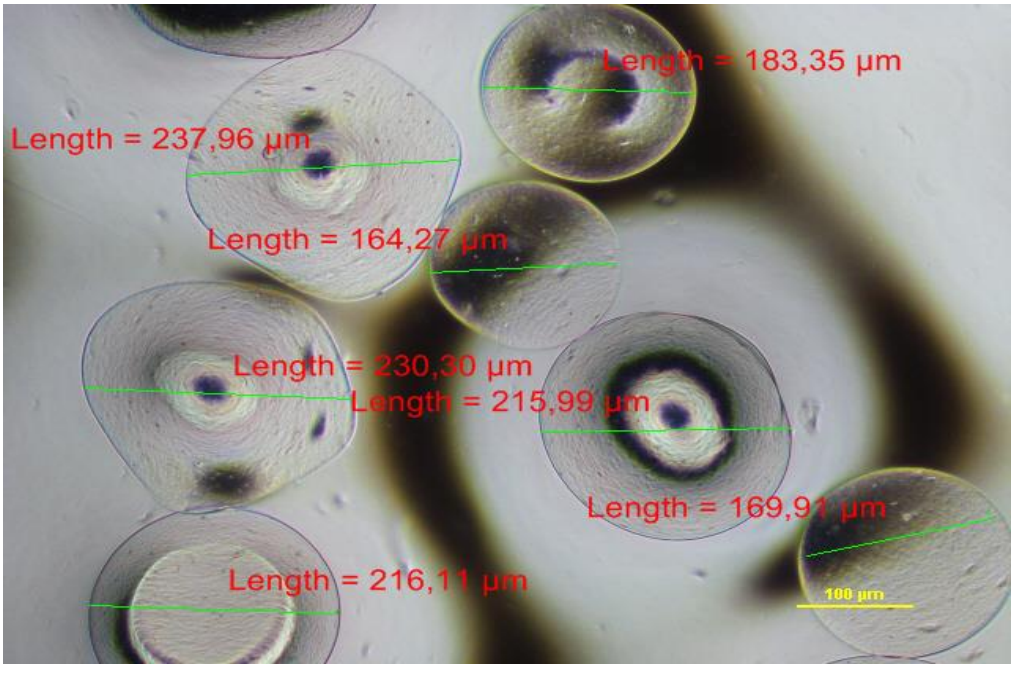

Figure 3. 18 SF incorparated (0.25%) ALG/SER/CS microspheres prepared with AA at 17 kV (10X) Scale bar: 100 µm.

Effect of Polymer Composition

In electrospraying, besides the applied voltage, polymer concentration also plays an important role; above the threshold value fiber formation start to occur. At low polymer concentrations bead formation can be started. So, higher concentrations cause higher surface tension values that decrease the bead formation probability (Fantini et al., 2006). It was investigated in literature that this concentration region aims to produce microspheres (Deitzel et al., 2000).

The stiffness of microspheres (Bian et al., 2011) is also important. This means that the polymer concentration should be regulated carefully. It should not be too low, at the same time to obtain the required stiffness for the microspheres.

3.2 Characterization Studies of Microspheres

Designing cell carier system is a challenging task because it requires tissue/application specific architecture, porosity, degradation, mechanical and surface properties, besides biocompatibility characters (Hutmatcher et al., 2004).

3. 2.1 Morphology of Microspheres

Morphology of microspheres is important to supply a good appropriate environment for cells conceived to grown on them (Vasir et al., 2003). The advantageous properties of 3D microspheres are their high surface to volume ratio, appropriate sizes for non-invasive application as injections and compatible shape that will not cause mechanical damage on soft/delicate tissues. SEM analysis was done to examine the sizes, morphology and surface structure of microspheres. From SEM images (Figure 3.19) it was observed that, microspheres were crumpled because of the drying steps before SEM analysis. There were flake like irregularities on the microspheres suggesting that they both collapsed and lost their spherical shape or their outer coat giving spherical contour has dissolved and lost. SEM images of fibroin encapsulated and coated microspheres (Figure 3.19) had similar average diameter.

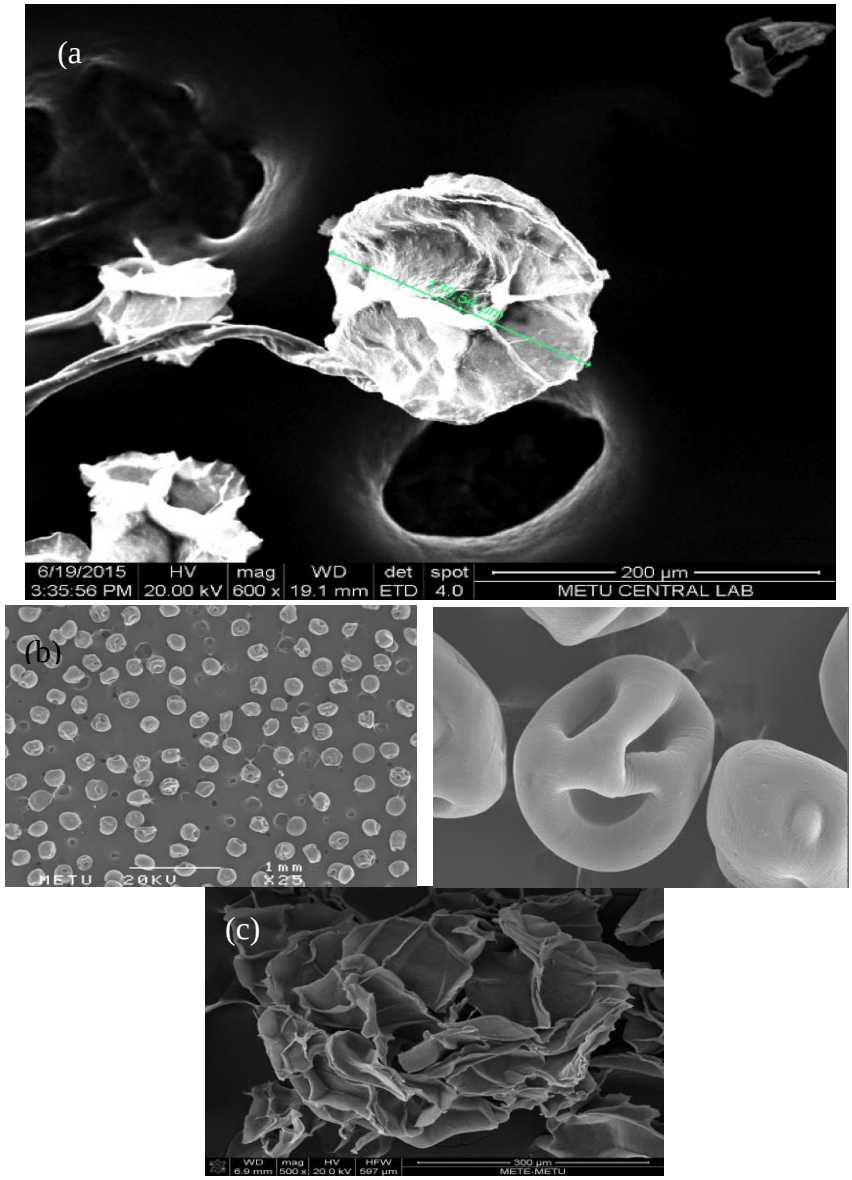

Figure 3. 19 SEM images of electrosprayed (a) ALG/SER/CS incorporated with 0.25% Fibroin (and (b) ALG/SER/CS without fibroin and (c) ALG/SER without fibroin

The shapes changed during drying process of microspheres in freeze dryer. They collapsed because of pressure and loss of inner solvent during lyophilization.

3.2.2 Degradation Studies

Degradation studies of microspheres were done for ten days. The groups were ALG/SER/CS incorporated with silk fibroin or without silk fibroin. Degradation studies indicated that ALG/SER/CS with silk fibroin were more stable than the group with no silk fibroin. According to degradation studies A and B, which did not include SF, degraded more. Moreover SF coated microspheres did not degrade at pH 7.4 for 60 days (Table 3.2).

Table 3. 2 Percentage of microspheres that maintained their structural stability after incubation in water at 37ºC

	A	B	C	D
Day 1	100 %	100%	100%	100%
Day 7	37%	61%	82%	90%
Day 10	32%	41&	80%	87%

A: ALG/SER **B:** ALG/SER/CS **C**: ALG/SER/CS silk fibroin coating

D: ALG/SER/CS incorporate with silk fibroin

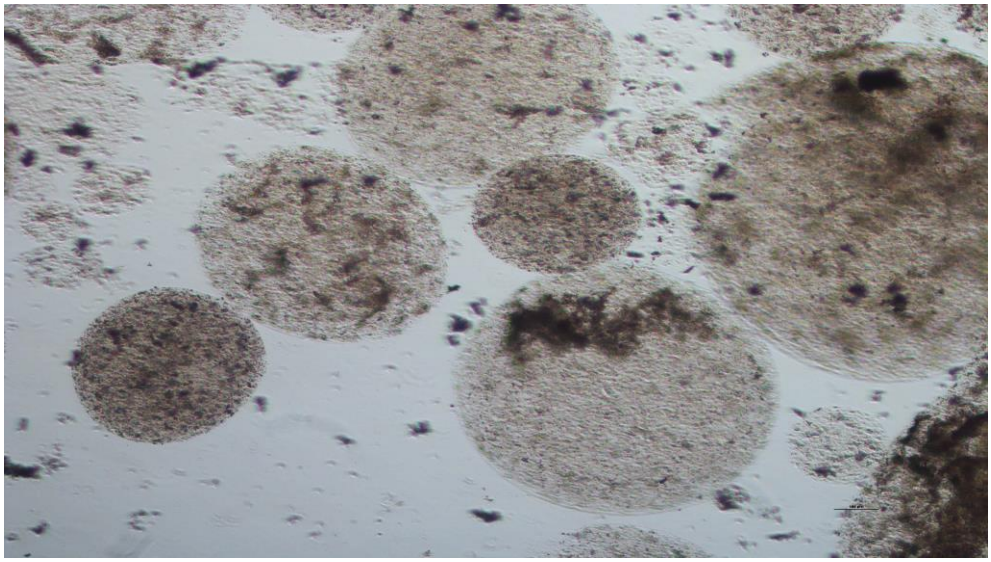

Figure 3.20 ALG/SER/CS with SF microspheres after 60 days of incubation in water.

3.2.3 Histograms and Size Distribution

Histograms of size distribution of microspheres showed a homogenous distribution (Figure 3.21) Most of the microspheres were within size rage of 200 -400µm.

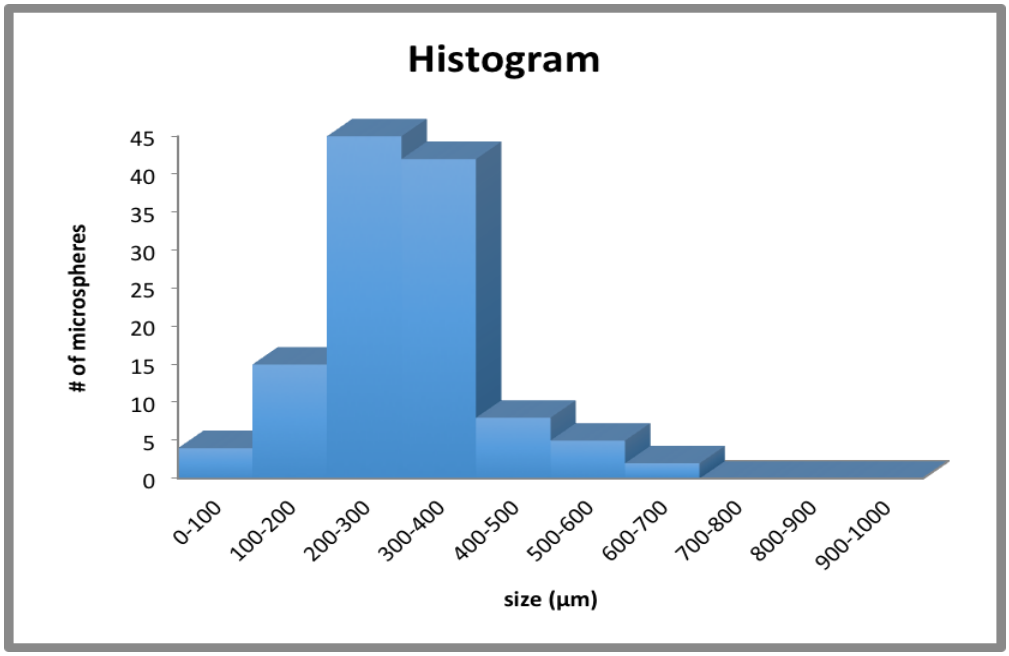

Figure 3. 21 Histogram of size distribution of ALG/SER/CS microspheres with SF

3.2.4 Chemical Properties of Microspheres

Polymeric microspheres are cross-linked for strengthen their chemical, degradation, stiffness and thermal properties (Nair et al., 2007). Fourier transform infrared (FT-IR) spectroscopy with the attenuated total reflectance (ATR) technique is fast method for surface sensitive method to consider whether the crosslinking method and encapsulation method were successful or not. Also the specific bonds were formed and could be observed with FT-IR (Wang et al., 2007).

FTIR-ATR spectra of ALG/SER and ALG/SER/CS microspheres with or without Fibroin were studied in order to investigate alginate, sericin, chondroitin sulfate and fibroin in microspheres (Figure 3.22).

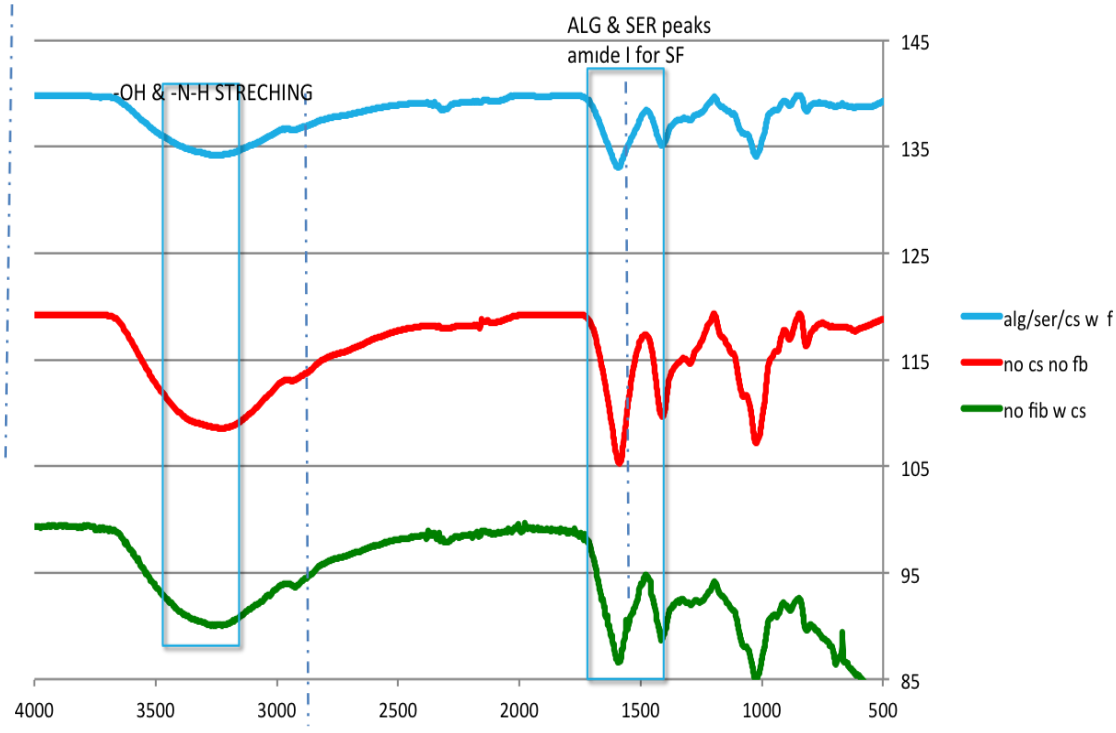

Figure 3. 22 ALG/SER/CS with SF encapsulation

The IR spectra confirm the –OH and N–H stretching foundation at 3300-3550 cm^{-1} in which –OH stretching vibrations and also vibration at 3435cm^{-1} in which the –OH stretching vibration are come together by N-H stretching.

The peak at 1636 cm^{-1} shows the amide bonds that demonstrate the existence of the free carboxyl group. Additionally at 1253 cm^{-1}, the vibration of stretching vibrations of the bands at 1413 and 1380 cm^{-1} occurred due to the coupling of the C–O stretch vibration. At around 1650 cm^{-1} there were 2 peaks, one of them ALG and one of them SER. Also C=O at 1700 cm^{-1} showed that alginate which is polysaccharide (Jaya et al., 2010). The peak at 1253 cm^{-1} shows the stretching vibrations of S O bond (SO_4^{2-}), since it is the characteristic absorption peak of chondroitin sulfate (Sui et al., 2007).

The presence of C=O (amide I) and (N-H) (amide I) groups in microspheres were probably related with silk fibroin and sericin which are protein.

FTIR-ATR showed that these alterations modified the physicochemical characteristics of ALG/SER microspheres however they could not be sufficient for complete dissolution. From FTIR spectra of microspheres, spectra absorptions at 3300 -3500 shows single bonds between hydrogen and oxygen atoms; and between water molecules and strong H bonding (Ling et al., 2013).

Characteristic absorbance bands in a typical polysaccharides (ALG) spectrum, are; at 3400 cm^{-1} O-H stretching, 2900 cm^{-1} C-H stretching, and 1700 cm^{-1} C=O stretching (Nejatzadeh-Barandozi et al., 2012).

In FTIR spectra, the absorption band at 1655 was observed which was the C=O stretching region and was the N-H bonding of amide I, as characteristic for fibroin (Cheng et al., 2010). In the study of Lammel et al., the amide I absorbance wavenumber was given as 1650 cm^{-1} (Lammel et al., 2010).

Two strong bands at approximately 2920 and 2850 cm^{-1} were assigned to the stretching vibrations of CH_2 groups (Heredia-Guarerro et al., 2014).

According to fibroin content, when fibroin increased, the peaks became more smooth and flat. A can be seen from graph the area under the curve at this particular wavenumber increases with the increase in the SF amount of microspheres.

FTIR-ATR spectra of microspheres were studied in order to determine cross-linking of Alginate (Figure 3. 22). Peaks at 1500-1600 cm^{-1} showed that the C=O stretching region which is characteristic for ALG (Wang et al., 2015).

In FTIR-ATR spectra of cross-linked microspheres, the peaks at 1645 cm^{-1} it was observed that peaks at 1645 cm^{-1} was lost because of O–C–O bonds breakage during crosslinking. Moreover, peak at 700-760 cm^{-1} in the FTIR spectra of the microspheres showed the presence of crosslinking.

3.2.5 Resistance of Microspheres to Centrifugal Force

The resistance properties of microspheres were investigated for understanding their rupture strength against a centrifugal force, besides to understand the effects of Sf incorporation or coating on stiffness and flexibility of the microspheres.

The microspheres were exposed to the centrifugal forces at 1000 G by centrifugation for 10 min.

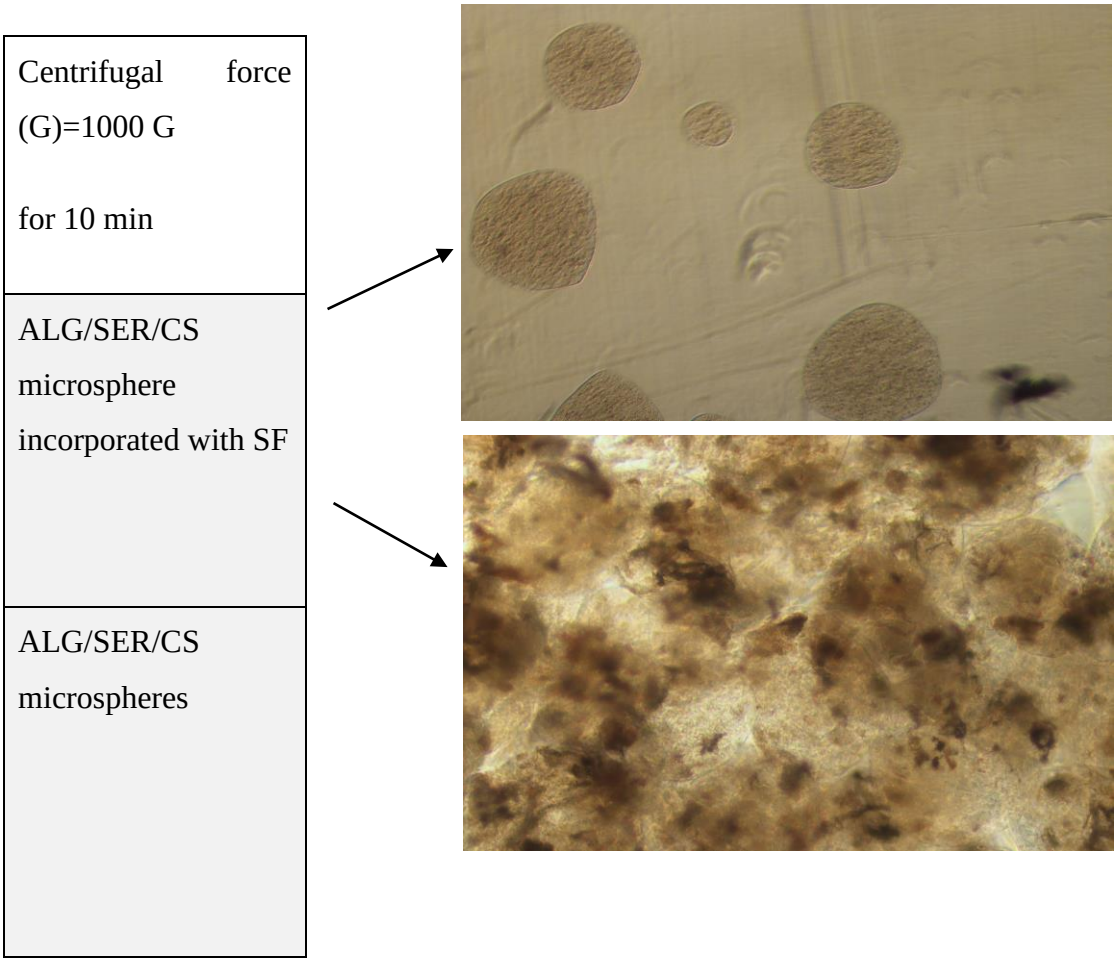

Figure 3. 23 Light microscopy images of (a) ALG/SER/CS microsphere incorporated with SF, (b) ALG/SER/CS microspheres

As a result the encapsulated microspheres were resistant to centrifugal forces. However other type of microspheres, which were not encapsulated, were disrupted.

3.2.6 CS Release from Microspheres

SF incorporated ALG/SER/CS microspheres had the highest CS release into cell culture media among all groups (Figure 3.24).

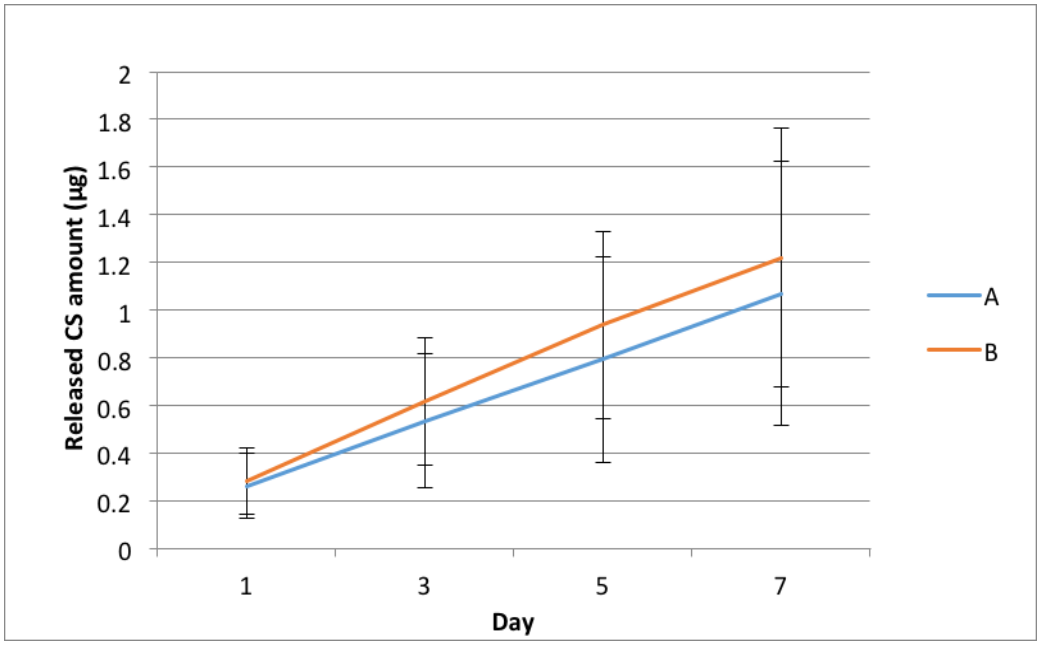

Figure 3. 24 CS release profiles of ALG/SER/CS microspheres (a) incorporated with SF (b) without SF incorporation.

When the amounts of CS released from the microspheres, no significant difference was observed between ALG/SER/CS with SF incorporation and without SF incorporation. Hence, the release amounts of CS were similar. So the usage of silk fibroin to make our microspheres stiffer did not affect the release of chondroprotective and chondrogenic agent CS.

3.2.7. SER Release from Microspheres

The concentration of alginate solution affects the release of silk sericin. There may be ionic interaction between alginate and sericin so the concentration can change some characters like ionic binding and release properties.

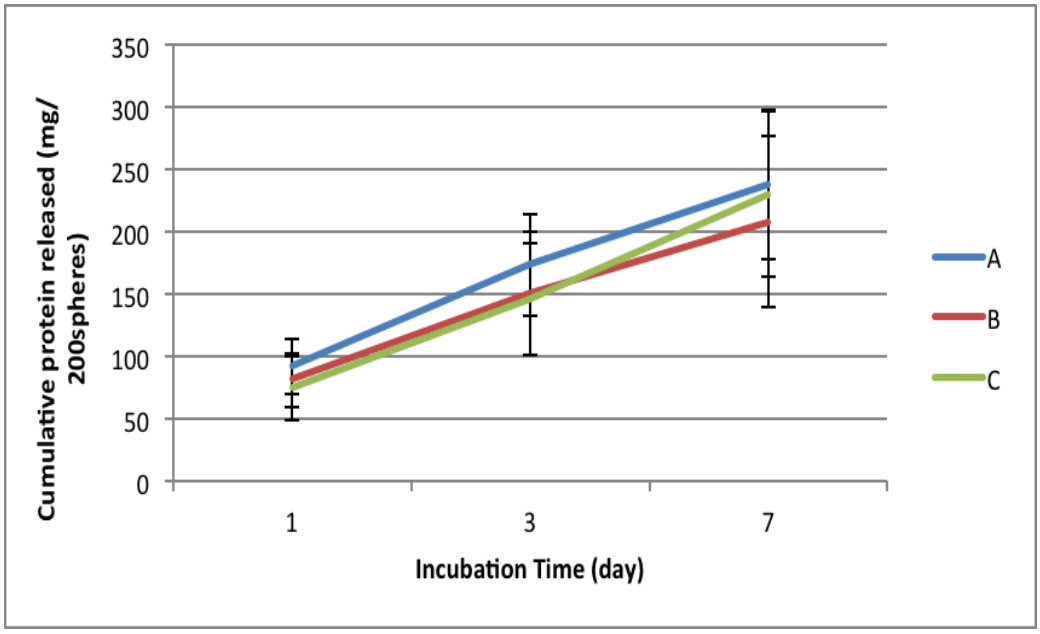

Figure 3. 25 (a) Large (b) middle (c) small microspheres

3.3 Cell Culture Studies

3.3.1 Cell Viability Measurements

First of all the microspheres were successfully sterilized by UV irradiation for 30 minutes (Umeki et al., 2010).

Cell viability studies were first done with L-929 to test the cytocompatibility of the microspheres. L-929 is mouse cell line and is a fibroblast-like cell line cloned from strain L. They reach at confluency within 3 days (Figure 3.25).

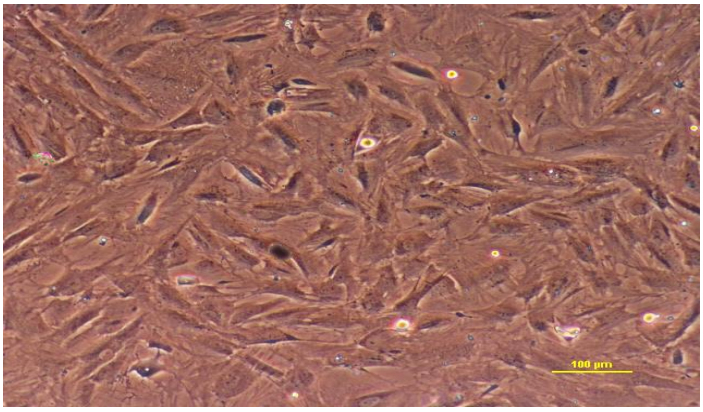

Figure 3. 26 Phase contrast microscopy image of L-929 cells in confluency (4X). Scale bar: 100 µm.

At different time periods, Presto Blue cell viability assay was conducted to study the attachment and proliferation of the cells on electrosprayed microspheres. In preliminary studies before Presto Blue assay, calculations showed that there was 226 microspheres within 100 µL of suspension. The same amount were put into each well. Initial cell seeding density was 50 000/cm^2. They were incubated for 1, 3, 5, and 7 days (Figure 3. 27) Cells plated on polystyrene culture wells (TCPS) served as controls.

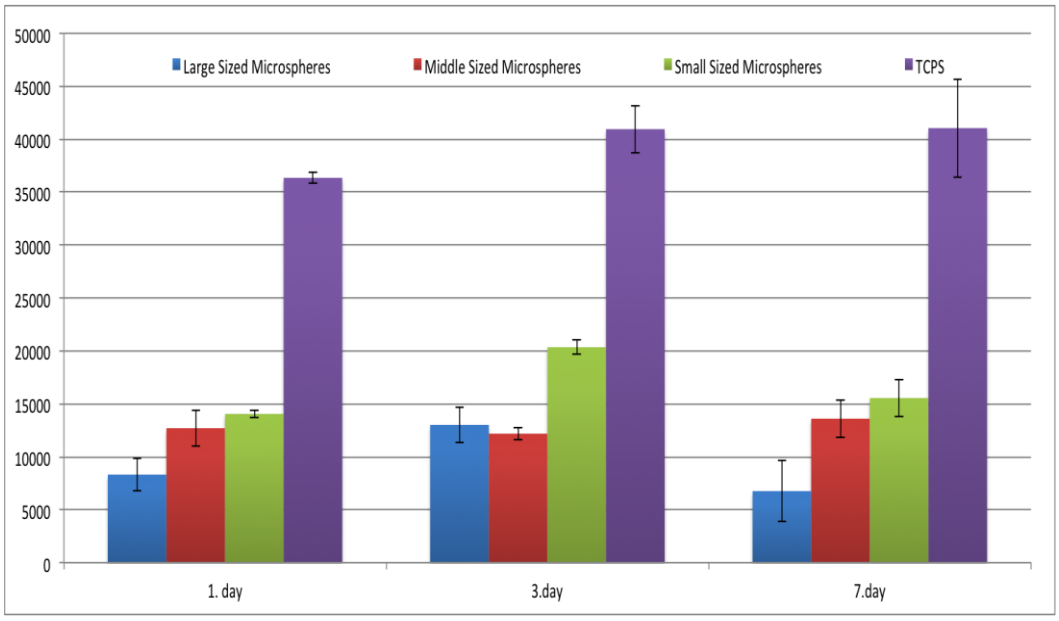

Figure 3. 27 Presto Blue assay results of L-929 seeded on microspheres (n=4).

L-929 cells seeded on TCPS had similar reduction levels for 3th and 7th days. On the test wells proliferation of L-929 increased less than TCPS and then it decreased slightly and protected its start level. Moreover, in the examining final optimized formulation on chondrocyte viability, chondrogenic ATDC5 cells were used. ATDC5 cells were seeded at a cell seeding density of 30 000/cm^2. Increasing reduction percentages showed that the cells seeded on microspheres proliferated (Figure 3.28).

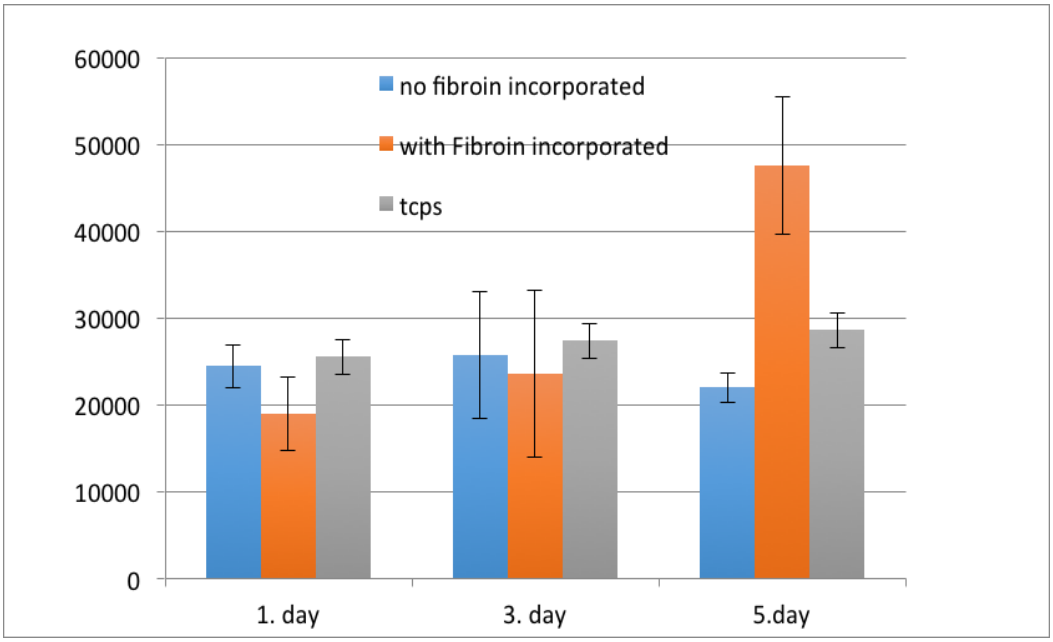

Figure 3. 28 Presto blue assay results of ATDC5 cells seeded on silk fibroin incorporated and unincorporated microspheres (n=4). First group was ALG/SER/CS and second group was ALG/SER/CS with 0.25% SF incorporation.

After incubation with ATDC5 cells, SF incorporated ALG/SER/CS microspheres and ALG/SER/CS microspheres without SF showed better cell attachment at 1st day, although the result was not statistically significant. The results were comparable for both groups at 3rd day in terms of cell growth. However, SF incorporated group had better cell growth with statistically significant difference at 5th day, even being better than TCPS control. L-929 has doubling time 32 h and ATDC5 proliferated with a doubling time of 16 h.

The difference in cell viability was probably related with presence of silk fibroin that supports the cell growth by enhancing scaffold characteristics of the microspheres. Cell culture study showed that microspheres were cytocompatible.

CHAPTER 4

CONCLUSION

In order to produce injectable microcarrier systems for cartilage tissue engineering, ALG/SER/CS with fibroin incorporated based microspheres were prepared by electrospraying technique. In thesis, ALG/SER/CS with Fibroin incorporated microspheres with average size of 192±46 μm were produced successfully. SF coated ALG microspheres showed significantly higher cell attachment and proliferation than uncoated microspheres; also the mechanical properties were significantly improved compared to microspheres without SF. When degradation rates were compared, microspheres without SF showed much faster degradation than both SF incorporated and coated groups.

The microspheres showed high cell carrying capacity and cells viability according to the conducted cell culture and viability studies with L929 and ATDC5 cells. Increased cytocompatibility and viability of cells with SF incorporation suggest that these microspheres can be used safely for cartilage tissue engineering purposes. Yet, the developed injectable systems need further evaluation with *in vivo* experiments.

REFERENCES

Aad, G., Abbott, B., Abdallah, J., Abdelalim, A. A., Abdesselam, A., Abdinov, O. & Alon, R. (2010). The ATLAS simulation infrastructure. The European Physical Journal C, 70(3), 823-874.

Aida, T. M., Yamagata, T., Watanabe, M., & Smith, R. L. (2010). Depolymerization of sodium alginate under hydrothermal conditions. Carbohydrate Polymers, 80(1), 296-302.

Anderson, J. M., Rodriguez, A., & Chang, D. T. (2008, April). Foreign body reaction to biomaterials. In Seminars in immunology (Vol. 20, No. 2, pp. 86-100). Academic Press.

Arya, N., Chakraborty, S., Dube, N., & Katti, D. S. (2009). Electrospraying: A facile technique for synthesis of chitosan- based micro/nanospheres for drug delivery applications. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 88(1), 17-31.

Bassleer, C. T., Combal, J. P. A., Bougaret, S., & Malaise, M. (1998). Effects of chondroitin sulfate and interleukin-1β on human articular chondrocytes cultivated in clusters. Osteoarthritis and Cartilage, 6(3), 196-204.

Bernstein, H. S. (Ed.). (2011). Tissue engineering in regenerative medicine. Springer Science & Business Media.

Bhardwaj, N., & Kundu, S. C. (2010). Electrospinning: a fascinating fiber fabrication technique. Biotechnology advances, 28(3), 325-347.

Bhosale, A. M., & Richardson, J. B. (2008). Articular cartilage: structure, injuries and review of management. British medical bulletin, 87(1), 77-95.

Bian, L., Zhai, D. Y., Tous, E., Rai, R., Mauck, R. L., & Burdick, J. A. (2011). Enhanced MSC chondrogenesis following delivery of TGF-β3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo. Biomaterials, 32(27), 6425-6434.

Bidarra, S. J., Barrias, C. C., & Granja, P. L. (2014). Injectable alginate hydrogels for cell delivery in tissue engineering. Acta biomaterialia, 10(4), 1646-1662.

Bouhadir, K. H., Lee, K. Y., Alsberg, E., Damm, K. L., Anderson, K. W., & Mooney, D. J. (2001). Degradation of partially oxidized alginate and its potential application for tissue engineering. Biotechnology progress, 17(5), 945-950.

Bowersock, T. L., Hogenesch, H., Suckow, M., Porter, R. E., Jackson, R., Park, H., & Park, K. (1996). Oral vaccination with alginate microsphere systems. Journal of controlled release, 39(2), 209-220.

Brandt, K. D., Smith, G. N., & Simon, L. S. (2000). Intraarticular injection of hyaluronan as treatment for knee osteoarthritis. Arthritis Rheum, 43(6), 1192-1203.

Brittberg, M., Tallheden, T., Sjögren-Jansson, E., Lindahl, A., & Peterson, L. (2001). Autologous chondrocytes used for articular cartilage repair: an update. Clinical orthopaedics and related research, 391, S337-S348.

Caplan, Arnold I. "Cartilage." Scientific American 251.4 (1984).

Chen, X., Shao, Z., Marinkovic, N. S., Miller, L. M., Zhou, P., & Chance, M. R. (2001). Conformation transition kinetics of regenerated Bombyx mori silk fibroin membrane monitored by time-resolved FTIR spectroscopy. Biophysical chemistry, 89(1), 25-34.

Chretien, C., & Chaumeil, J. C. (2005). Release of a macromolecular drug from alginate-impregnated microspheres. International journal of pharmaceutics, 304(1), 18-28.

Cram, P., Lu, X., Kates, S. L., Singh, J. A., Li, Y., & Wolf, B. R. (2012). Total knee arthroplasty volume, utilization, and outcomes among Medicare beneficiaries, 1991-2010. Jama, 308(12), 1227-1236.

Deitzel, J. M., Kleinmeyer, J., Harris, D. E. A., & Tan, N. B. (2001). The effect of processing variables on the morphology of electrospun nanofibers and textiles. Polymer, 42(1), 261-272.

Dinescu, S., Gălăţeanu, B., Albu, M., Lungu, A., Radu, E., Hermenean, A., & Costache, M. (2013). Biocompatibility assessment of novel collagen-sericin scaffolds improved with hyaluronic acid and chondroitin sulfate for cartilage regeneration. BioMed research international, 2013.

Doppalapudi, S., Jain, A., Khan, W., & Domb, A. J. (2014). Biodegradable polymers—an overview. Polymers for AdvanceTechnologies, 25(5), 427-435.

Dorotka, R., Bindreiter, U., Macfelda, K., Windberger, U., & Nehrer, S. (2005). Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair. Osteoarthritis and cartilage, 13(8), 655-664.

Draget, K. I., Skjåk-Bræk, G., & Smidsrød, O. (1997). Alginate based new materials. International journal of biological macromolecules, 21(1), 47-55.

Draget, K. I., Smidsrød, O., & Skjåk- Bræk, G. (2005). Alginates from algae. Biopolymers Online.

Ertesvåg, H., Valla, S., & Skjåk-Bræk, G. (1996). Genetics and biosynthesis of alginates. Carbohydr Eur, 14, 14-18.

Eyrich, D., Brandl, F., Appel, B., Wiese, H., Maier, G., Wenzel, M. & Blunk, T. (2007). Long-term stable fibrin gels for cartilage engineering. Biomaterials, 28(1), 55-65.

Fantini, D., Zanetti, M., & Costa, L. (2006). Polystyrene microspheres and nanospheres produced by electrospray. Macromolecular rapid communications, 27(23), 2038-2042.

Felson, D. T. (1990, December). The epidemiology of knee osteoarthritis: results from the Framingham Osteoarthritis Study. In Seminars in arthritis and rheumatism (Vol. 20, No. 3, pp. 42-50). WB Saunders.

Gåserød, O., Sannes, A., & Skjåk-Bræk, G. (1999). Microcapsules of alginate–chitosan. II. A study of capsule stability and permeability. Biomaterials, 20(8), 773-783.

Goh, C. H., Heng, P. W. S., & Chan, L. W. (2012). Alginates as a useful natural polymer for microencapsulation and therapeutic applications. Carbohydrate Polymers, 88(1), 1-12.

Goldring, M. B., & Otero, M. (2011). Inflammation in osteoarthritis. Current opinion in rheumatology, 23(5), 471.

Goldring, S. R. (2008). The role of bone in osteoarthritis pathogenesis. Rheumatic Disease Clinics of North America, 34(3), 561-571.

Gombotz, W. R., & Wee, S. F. (2012). Protein release from alginate matrices. Advanced drug delivery reviews, 64, 194-205.

Gordon, N. F., Scott, C. B., & Levine, B. D. (1997). Comparison of single versus multiple lifestyle interventions: are the antihypertensive effects of exercise training and diet-induced weight loss additive?. The American journal of cardiology, 79(6), 763-767.

Gossec, L., Paternotte, S., Bingham, C. O., Clegg, D. O., Coste, P., Conaghan, P. G. & Dougados, M. (2011). OARSI/OMERACT initiative to define states of severity and indication for joint replacement in hip and knee osteoarthritis. An OMERACT 10 Special Interest Group. The Journal of rheumatology, 38(8), 1765-1769.

Griffith, L. G., & Naughton, G. (2002). Tissue engineering--current challenges and expanding opportunities. Science, 295(5557), 1009-1014.

Hadipour-Goudarzi, E., Montazer, M., Latifi, M., & Aghaji, A. A. G. (2014). Electrospinning of chitosan/sericin/PVA nanofibers incorporated with in situ synthesis of nano silver. Carbohydrate polymers, 113, 231-239.

Hall, A. C., Urban, J. P. G., & Gehl, K. A. (1991). The effects of hydrostatic pressure on matrix synthesis in articular cartilage. Journal of orthopaedic research, 9(1), 1-10.

Hazeri, N., Tavanai, H., & Moradi, A. R. (2012). Production and properties of electrosprayed sericin nanopowder. Science and Technology of Advanced Materials, 13(3), 035010.

Henrotin, Y., Mathy, M., Sanchez, C., & Lambert, C. (2010). Chondroitin sulfate in the treatment of osteoarthritis: from in vitro studies to clinical recommendations. Therapeutic advances in musculoskeletal disease, 2(6), 335-348.

Heredia-Guerrero, J. A., Benítez, J. J., Domínguez, E., Bayer, I. S., Cingolani, R., Athanassiou, A., & Heredia, A. (2014). Infrared and Raman spectroscopic features of plant cuticles: a review. Frontiers in plant science, 5.

Hofmann, S., Knecht, S., Langer, R., Kaplan, D. L., Vunjak-Novakovic, G., Merkle, H. P., & Meinel, L. (2006). Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. Tissue engineering, 12(10), 2729-2738.

Hollister, S. J. (2005). Porous scaffold design for tissue engineering. Nature materials, 4(7), 518-524.

Hootman, J. M., Helmick, C. G., & Brady, T. J. (2012). A public health approach to addressing arthritis in older adults: the most common cause of disability. American journal of public health, 102(3), 426-433.

HOU, J. M., LIU, G. Q., & SHEN, J. J. (2012). Characteristics of Sodium Alginate and Its Application in Water Treatment. Development and Application of Materials, 3, 019.

James, C. B., & Uhl, T. L. (2001). A review of articular cartilage pathology and the use of glucosamine sulfate. Journal of athletic training, 36(4), 413.

Jordan, K. M., Arden, N. K., Doherty, M., Bannwarth, B., Bijlsma, J. W. J., Dieppe, P. & Dougados, M. (2003). EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). Annals of the rheumatic diseases, 62(12), 1145-1155.

Keawkorna, W., Limpeanchoba, N., Tiyaboonchaib, W., Pongcharoenc, S., & Sutheerawattananondad, M. (2013). The effect of dietary sericin on rats.

Khandai, M., Chakraborty, S., Sharma, A., Pattnaik, S., Patra, C. N., Dinda, S. C., & Sen, K. K. (2010). Journal of Advanced Pharmaceutical Research. 2010, 1, 48-60. Journal of Advanced Pharmaceutical Research, 1, 48-60.

Kuettner, K. E., Aydelotte, M. B., & Thonar, E. J. (1991). Articular cartilage matrix and structure: a minireview. The Journal of rheumatology. Supplement, 27, 46-48.

Kulkarni, A. R., Soppimath, K. S., & Aminabhavi, T. M. (1999). Controlled release of diclofenac sodium from sodium alginate beads crosslinked with glutaraldehyde. Pharmaceutica Acta Helvetiae, 74(1), 29-36.

Kundu, S. C., Dash, B. C., Dash, R., & Kaplan, D. L. (2008). Natural protective glue protein, sericin bioengineered by silkworms: potential for biomedical and biotechnological applications. Progress in Polymer Science, 33(10), 998-1012.

Kuo, C. K., & Ma, P. X. (2001). Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: part 1. Structure, gelation rate and mechanical properties. Biomaterials, 22(6), 511-521.

Lee, C. T., Kung, P. H., & Lee, Y. D. (2005). Preparation of poly (vinyl alcohol)-chondroitin sulfate hydrogel as matrices in tissue engineering. Carbohydrate Polymers, 61(3), 348-354.

Lee, C. T., Kung, P. H., & Lee, Y. D. (2005). Preparation of poly (vinyl alcohol)-chondroitin sulfate hydrogel as matrices in tissue engineering. Carbohydrate Polymers, 61(3), 348-354.

Lee, E. S., Park, K. H., Kang, D., Park, I. S., Min, H. Y., Lee, D. H. & Na, K. (2007). Protein complexed with chondroitin sulfate in poly (lactide-co-glycolide) microspheres. Biomaterials, 28(17), 2754-2762.

Lee, J. E., Kim, K. E., Kwon, I. C., Ahn, H. J., Lee, S. H., Cho, H. & Lee, M. C. (2004). Effects of the controlled-released TGF-β1 from chitosan microspheres on chondrocytes cultured in a collagen/chitosan/glycosaminoglycan scaffold. Biomaterials, 25(18), 4163-4173.

Lee, K. Y., & Mooney, D. J. (2001). Hydrogels for tissue engineering. Chemical reviews, 101(7), 1869-1880.

Lee, K. Y., & Mooney, D. J. (2012). Alginate: properties and biomedical applications. Progress in polymer science, 37(1), 106-126.

Lian-Xia, Z. H. P. D., Si-Jia, W. X. Y. M., HE, Y. M. Y. Y. L., & Liang-Jun, W. Z. (2011). Preparation and Characterization of Composite Hydrogel Beads Containing Sericin and Sodium Alginate. Science of Sericulture, 3, 013.

Lippiello, L. (2003). Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis and cartilage, 11(5), 335-342.

Lorenz, H., & Richter, W. (2006). Osteoarthritis: cellular and molecular changes in degenerating cartilage. Progress in histochemistry and cytochemistry, 40(3), 135-163.

LU, S. Z., LI, M. Z., & BAI, L. A Hydroxyapatite Silk Fibroin Porous Composite Using Ultrasonic Vibration Method.

Madeira, C., Santhagunam, A., Salgueiro, J. B., & Cabral, J. M. (2015). Advanced cell therapies for articular cartilage regeneration. Trends in biotechnology, 33(1), 35-42.

Manjula, R., Dasan, K. P., & Joseph, M. A. SF/Poly (Ethylene-Co-Vinyl Acetate) Blends For Controlled Drug Release.

Mikos, A. G., Bao, Y., Cima, L. G., Ingber, D. E., Vacanti, J. P., & Langer, R. (1993). Preparation of poly (glycolic acid) bonded fiber structures for cell attachment and transplantation. Journal of biomedical materials research, 27(2), 183-189.

Nair, L. S., & Laurencin, C. T. (2007). Biodegradable polymers as biomaterials. Progress in polymer science, 32(8), 762-798.

Nayak, S., Dey, S., & Kundu, S. C. (2014). Silk sericin–alginate–chitosan microcapsules: Hepatocytes encapsulation for enhanced cellular functions. International journal of biological macromolecules, 65, 258-266.

Nejatzadeh-Barandozi, F., & Enferadi, S. T. (2012). FT-IR study of the polysaccharides isolated from the skin juice, gel juice, and flower of Aloe vera tissues affected by fertilizer treatment. Organic and medicinal chemistry letters, 2(1), 1-9.

Orive, G., Gascón, A. R., Hernández, R. M., Igartua, M., & Pedraz, J. L. (2003). Cell microencapsulation technology for biomedical purposes: novel insights and challenges. Trends in pharmacological sciences, 24(5), 207-210.

Ouyang, H. W., Goh, J. C., Thambyah, A., Teoh, S. H., & Lee, E. H. (2003). Knitted poly-lactide-co-glycolide scaffold loaded with bone marrow stromal cells in repair and regeneration of rabbit Achilles tendon. Tissue engineering, 9(3), 431-439.

Padamwar, M. N., & Pawar, A. P. (2004). Silk sericin and its applications: A review. Journal of Scientific and Industrial Research, 63(4), 323-329.

Painter, T. J., Smidsrød, O., & Haug, A. (1968). A computer study of the changes in composition-distribution occurring during random depolymerisation of a binary linear heteropolysaccharide. Acta Chem Scand, 22, 1637-1648.

Pham, Q. P., Sharma, U., & Mikos, A. G. (2006). Electrospinning of polymeric nanofibers for tissue engineering applications: a review. Tissue engineering, 12(5), 1197-1211.

Pieper, J. S., Oosterhof, A., Dijkstra, P. J., Veerkamp, J. H., & Van Kuppevelt, T. H. (1999). Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. Biomaterials, 20(9), 847-858.

Pongjanyakul, T., & Puttipipatkhachorn, S. (2007). Xanthan–alginate composite gel beads: molecular interaction and in vitro characterization. International journal of pharmaceutics, 331(1), 61-71.

Prajapati, B. G., & Patel, M. (2010). A technology update: Electro spray technology. International Journal of Pharmaceutical Sciences Review and Research, 11-13.

Quinn, T. M., Häuselmann, H. J., Shintani, N., & Hunziker, E. B. (2013). Cell and matrix morphology in articular cartilage from adult human knee and ankle joints suggests depth-associated adaptations to biomechanical and anatomical roles. Osteoarthritis and Cartilage, 21(12), 1904-1912.

Ravi, M., Paramesh, V., Kaviya, S. R., Anuradha, E., & Solomon, F. D. (2015). 3D cell culture systems: advantages and applications. Journal of cellular physiology, 230(1), 16-26.

Ringe, J., Burmester, G. R., & Sittinger, M. (2012). Regenerative medicine in rheumatic disease—progress in tissue engineering. Nature Reviews Rheumatology, 8(8), 493-498.

Rogina, A. (2014). Electrospinning process: Versatile preparation method for biodegradable and natural polymers and biocomposite systems applied in tissue engineering and drug delivery. Applied surface science, 296, 221-230.

Sakaguchi, Y., Sekiya, I., Yagishita, K., & Muneta, T. (2005). Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis & Rheumatism, 52(8), 2521-2529.

Scott, C. (2013). Enlightening Results. BioProcess International, 11, 8.

Scott, D. L., Berry, H., Capell, H., Coppock, J., Daymond, T., Doyle, D. V. & Wotjulewski, J. (2000). The long- term effects of non- steroidal anti- inflammatory drugs in osteoarthritis of the knee: a randomized placebo- controlled trial. Rheumatology, 39(10), 1095-1101

Servoli, E., Maniglio, D., Motta, A., Predazzer, R., & Migliaresi, C. (2005). Surface properties of silk fibroin films and their interaction with fibroblasts. Macromolecular bioscience, 5(12), 1175-1183.

Shalumon, K. T., & Chen, J. P. (2015). Scaffold-based Drug Delivery for Cartilage Tissue Regeneration. Current pharmaceutical design, 21(15), 1979-1990.

Singh, A., Goel, S. C., Gupta, K. K., Kumar, M., Arun, G. R., Patil, H. & Jha, S. (2014). The role of stem cells in osteoarthritis An experimental study in rabbits. Bone and Joint Research, 3(2), 32-37.

Sinha, V. R., & Trehan, A. (2003). Biodegradable microspheres for protein delivery. Journal of Controlled Release, 90(3), 261-280.

Sinusas, K. (2012). Osteoarthritis: diagnosis and treatment. American family physician, 85(1), 49-56.

Smidsrød, O., & Skja, G. (1990). Alginate as immobilization matrix for cells. Trends in biotechnology, 8, 71-78.

Sokolsky-Papkov, M., Agashi, K., Olaye, A., Shakesheff, K., & Domb, A. J. (2007). Polymer carriers for drug delivery in tissue engineering. Advanced drug delivery reviews, 59(4), 187-206.

Spector, T. D., & MacGregor, A. J. (2004). Risk factors for osteoarthritis: genetics. Osteoarthritis and cartilage, 12, 39-44.

Strand, B. L., Gåserød, O., Kulseng, B., Espevik, T., & Skjåk-Bræk, G. (2002). Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties. Journal of microencapsulation, 19(5), 615-630.

Sui, W., Huang, L., Wang, J., & Bo, Q. (2008). Preparation and properties of chitosan chondroitin sulfate complex microcapsules. Colloids and Surfaces B: Biointerfaces, 65(1), 69-73.

Temenoff, J. S., & Mikos, A. G. (2000). Review: tissue engineering for regeneration of articular cartilage. Biomaterials, 21(5), 431-440.

Tønnesen, H. H., & Karlsen, J. (2002). Alginate in drug delivery systems. Drug development and industrial pharmacy, 28(6), 621-630.

Ulery, B. D., Nair, L. S., & Laurencin, C. T. (2011). Biomedical applications of biodegradable polymers. Journal of polymer science Part B: polymer physics, 49(12), 832-864.

Vasir, J. K., Tambwekar, K., & Garg, S. (2003). Bioadhesive microspheres as a controlled drug delivery system. International Journal of Pharmaceutics, 255(1), 13-32.

Venugopal, J., Low, S., Choon, A. T., & Ramakrishna, S. (2008). Interaction of cells and nanofiber scaffolds in tissue engineering. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 84(1), 34-48.

Venugopal, J., Zhang, Y. Z., & Ramakrishna, S. (2005). Fabrication of modified and functionalized polycaprolactone nanofibre scaffolds for vascular tissue engineering. Nanotechnology, 16(10), 2138.

Wang, C. T., Lin, J., Chang, C. J., Lin, Y. T., & Hou, S. M. (2004). Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. The Journal of Bone & Joint Surgery, 86(3), 538-545.

Wang, X., Wenk, E., Matsumoto, A., Meinel, L., Li, C., & Kaplan, D. L. (2007). Silk microspheres for encapsulation and controlled release. Journal of Controlled Release, 117(3), 360-370.

Wang, X., Yucel, T., Lu, Q., Hu, X., & Kaplan, D. L. (2010). Silk nanospheres and microspheres from silk/pva blend films for drug delivery. Biomaterials, 31(6), 1025-1035.

Wang, Y., Blasioli, D. J., Kim, H. J., Kim, H. S., & Kaplan, D. L. (2006). Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. Biomaterials, 27(25), 4434-4442.

Wang, Y., Kim, U. J., Blasioli, D. J., Kim, H. J., & Kaplan, D. L. (2005). In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials, 26(34), 7082-7094.

Wang, Y., Yuan, M., Guo, Q. Y., Lu, S. B., & Peng, J. (2015). Mesenchymal stem cells for treating articular cartilage defects and osteoarthritis. Cell Transplantation.

Wang, Z., Zhang, Y., Zhang, J., Huang, L., Liu, J., Li, Y. & Wang, L. (2014). Exploring natural silk protein sericin for regenerative medicine: an injectable, photoluminescent, cell-adhesive 3D hydrogel. Scientific reports, 4.

Wantanasiri, P., Ratanavaraporn, J., Yamdech, R., & Aramwit, P. (2014). Fabrication of silk sericin/alginate microparticles by electrohydrodynamic spraying technique for the controlled release of silk sericin. Journal of Electrostatics, 72(1), 22-27.

Williams, C. G., Kim, T. K., Taboas, A., Malik, A., Manson, P., & Elisseeff, J. (2003). In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue engineering, 9(4), 679-688.

Wolfstadt, J. I., Cole, B. J., Ogilvie-Harris, D. J., Viswanathan, S., & Chahal, J. (2015). Current Concepts The Role of Mesenchymal Stem Cells in the Management of Knee Osteoarthritis. Sports Health: A Multidisciplinary Approach, 7(1), 38-44.

Wray, L. S., Hu, X., Gallego, J., Georgakoudi, I., Omenetto, F. G., Schmidt, D., & Kaplan, D. L. (2011). Effect of processing on silk- based biomaterials: Reproducibility and biocompatibility. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 99(1), 89-101.

Xie, J., Ng, W. J., Lee, L. Y., & Wang, C. H. (2008). Encapsulation of protein drugs in biodegradable microparticles by co-axial electrospray. Journal of Colloid and Interface Science, 317(2), 469-476.

Xu, Y., & Hanna, M. A. (2006). Electrospray encapsulation of water-soluble protein with polylactide: Effects of formulations on morphology, encapsulation efficiency and release profile of particles. International Journal of Pharmaceutics, 320(1), 30-36.

Yao, R., Zhang, R., Lin, F., & Luan, J. (2012). Injectable cell/hydrogel microspheres induce the formation of fat lobule-like micro tissues and vascularized adipose tissue regeneration. Biofabrication, 4(4), 045003.

Yeom, C. K., & Lee, K. H. (1998). Characterization of sodium alginate membrane crosslinked with glutaraldehyde in pervaporation separation. Journal of applied polymer science, 67(2), 209-219.

Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., & Sekiya, I. (2007). Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. Cell and tissue research, 327(3), 449-462.

Zaher, W., Isa, A., & Kassem, M. (2015). Skeletal Stem Cells for Bone and Cartilage Tissue Regeneration. In A Tissue Regeneration Approach to Bone and Cartilage Repair (pp. 53-63). Springer International Publishing.

Zhang, L., Hu, J., & Athanasiou, K. A. (2009). The role of tissue engineering in articular cartilage repair and regeneration. Critical Reviews™ in Biomedical Engineering, 37(1-2).

Zheng, M. H., King, E., Kirilak, Y., Huang, L., Papadimitriou, J. M., Wood, D. J., & Xu, J. (2004). Molecular characterization of chondrocytes in autologous chondrocyte implantation. International journal of molecular medicine, 13(5), 623-628.

APPENDIX A

GAG CALIBRATION CURVE

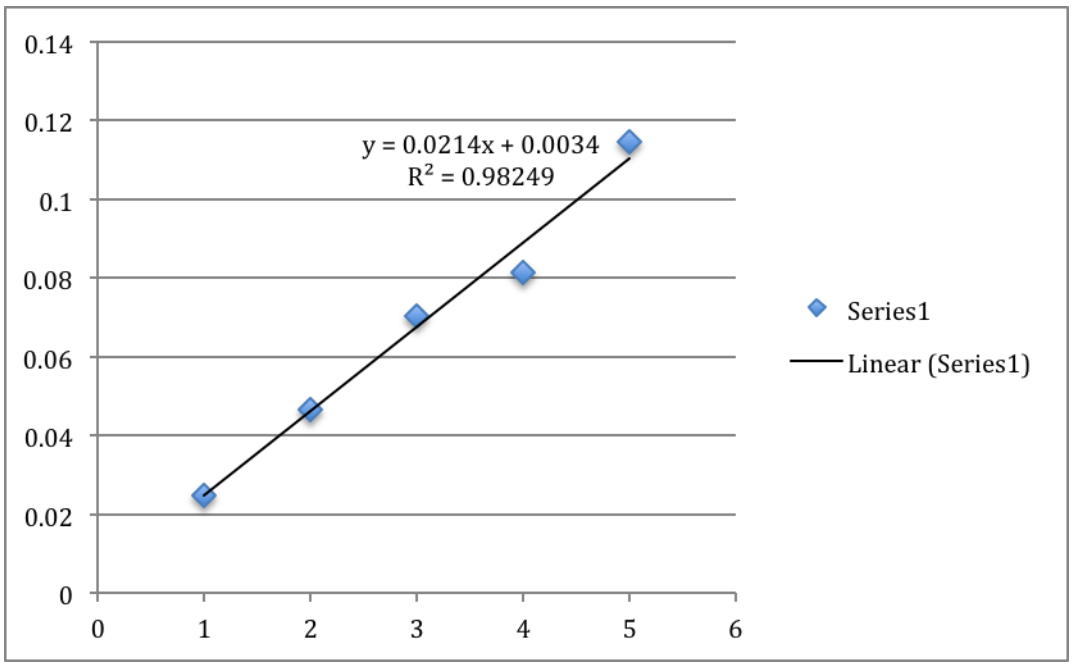

Figure A.1 Calibration curve for sGAG assay

APPENDIX B

BCA CALIBRATION CURVE

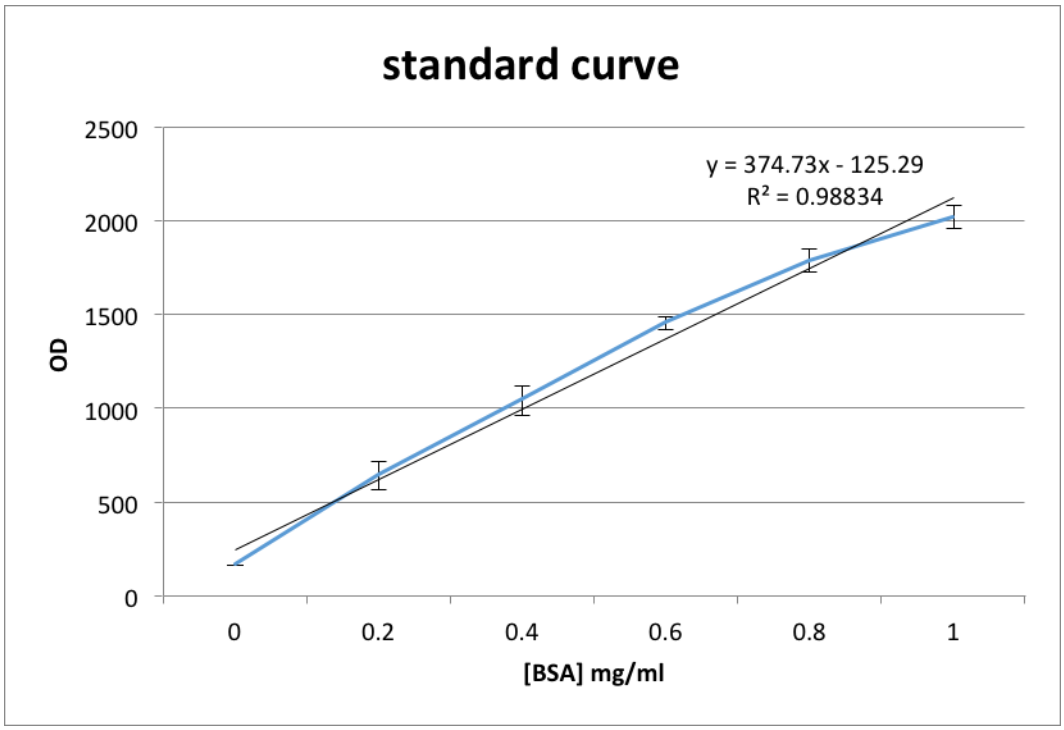

Figure B.1 Calibration curve for BCA assay

APPENDIX C

CELL NUMBER CALIBRATION CURVE FOR VIABILITY ASSAY

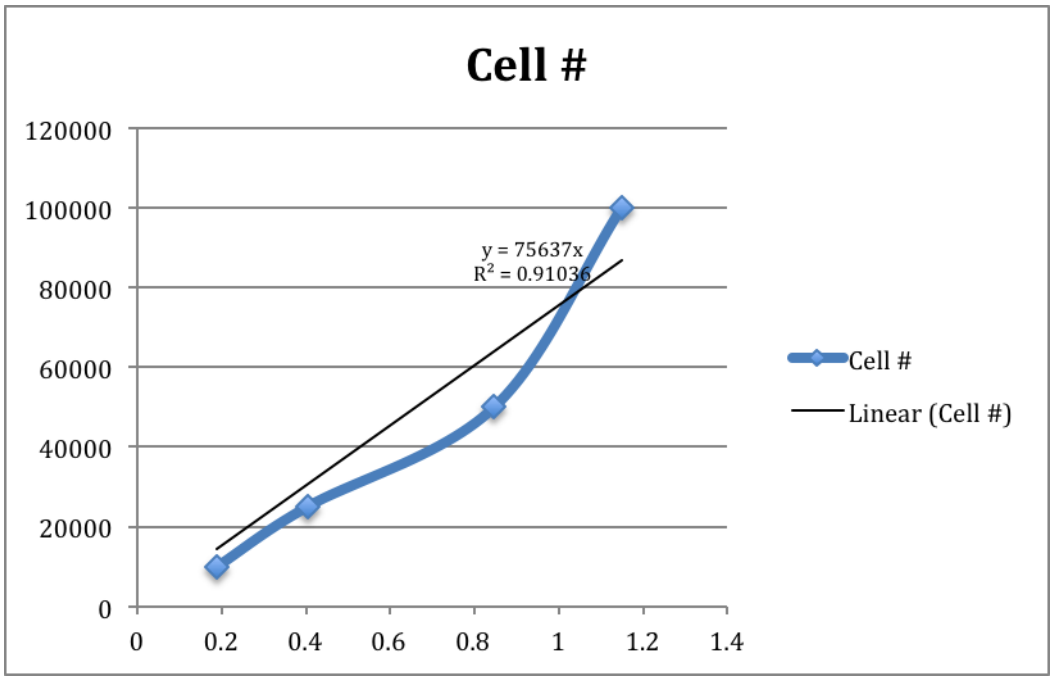

Figure C.1 Calibration curve for L-929 cells for Presto Blue viability assay.

APPENDIX D

CELL NUMBER CALIBRATION CURVE FOR VIABILITY ASSAY

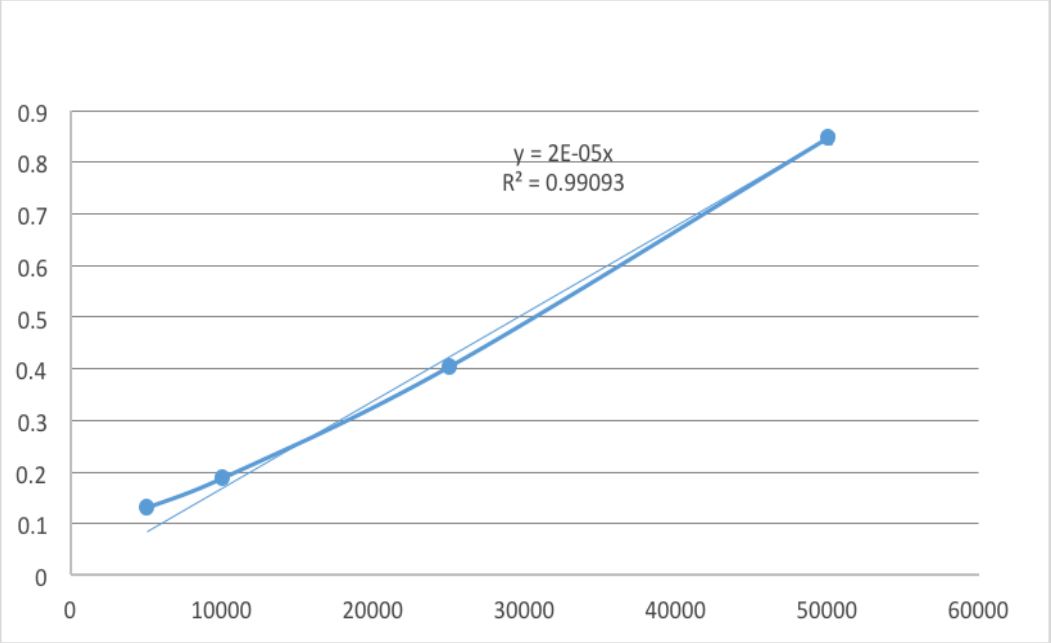

Figure D.1 Calibration curve for ATDC5 cells for Presto Blue viability assay.