Development of Novel Strategies for Cartilage Tissue Engineering

THESIS

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CERTIFICATE

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List abbreviations and symbols

Extracellular matrix (ECM)

Osteoarthritis (OA)

Proteoglycans (PG)

Metalloproteinases (MMPs)

Prostaglandins (PGEF2)

Non steroidal anti-inflammatory drugs (NSAIDs)

Chondroitin sulfate (CS)

Insulin growth factor (IGF-1)

TGF-beta (transforming growth factor beta)

Bone morphogenetic protein (BMP-2)

Glycosaminoglycans (GAG's)

Chitosan-Gelatin-Chondroitin Sulfate (CGC)

Poly-glycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA)

Nanoparticles (Nps)

Gold nanoparticles (AuNps)

PBS (Phosphate buffer saline)

rpm(revolutions per minute).

Dulbecco's modified Eagle medium (DMEM)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

Dimethyl sulfoxide (DMSO)

DMMB (Dimethyl methylene blue)

Fourier transform infrared Spectroscopy (FTIR*)*

Drug delivery system (DDS)

Ascorbic acid (AA)

Degree centrigrade (ºC)

Cyclooxygenase (COX) inhibitors

Ultra Violet Visible (UV –Vis spectra)
Nanoparticles (NPs)

Gold nanoparticles (AuNps)

Tetrachloroauric acid ($HAuCL_4$)

X-ray diffraction (XRD)

Transmission electron microscopy (TEM).

Anterior cruciate ligament (ACL)

Interleukin (IL) 1

MSCs (Mesenchymal stem cell)

Hoechst(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate)

PFA (paraformaldehyde)

pDAB (para dimethyl amino benzaldehyde)

TIMP-1 (Tissue inhibitors of metalloproteinases

Abstract

Osteoarthritis is a world-wide problem causing morbidity and mortality which further leads to social and economical consequences. Self repair capacity of articular cartilage is limited due to its avascular and aneural nature, which calls for an efficient repair strategy. Furthermore small articular cartilage lesions if left untreated can lead to severe osteoarthritis and subsequent disability. Considering these factors there is urgent need to develop novel strategies for its repair. Tissue engineering has emerged as a potent field to repair the damaged tissue like bone, cartilage, skin, muscle, liver, heart etc. Major focus of this research work is in the development of novel strategies for the repair of damaged cartilage due to alarming prevalence of cartilage associated diseases in general population and more so in younger population.

Scaffold, cells and growth stimulating factors are generally considered as triad components of tissue engineering. Our primary aim was to synthesize the novel cryogel scaffold using polymers like chitosan, gelatin, and chondroitin sulfate. To enhance the fabrication efficacy we fabricated these scaffolds by employing a contemporary technology called "cryogelation". Major advantage of using this technology is that it results in the fabrication of an extensively porous architecture which to some extent mimics the native cartilage and ECM combination. Furthermore, as this scaffold is fabricated using natural polymers it can be implanted at the site of cartilage defect without eliciting any major immune rejection reactions. After scaffold was synthesized it was further characterized by different physical and mechanical techniques in order to check the whether scaffold has suitable physical, chemical and mechanical properties. To analyze the physical properties of novel chitosan-gelatin-chondroitin sulfate (CGC) scaffolds they were characterized by rheology, scanning

electron microscopy (SEM), and mechanical assay. Primary goat chondrocytes were used for *in vitro* characterization by performing the tests like MTT, SEM GAG and collagen assay. From the above analysis we concluded that CGC cryogel possess large and interconnected pores as observed by SEM and supported by flow rate values respectively. The most important parameter which is vital for cartilage tissue engineering scaffolds is mechanical strength, and the results confirmed that synthesized scaffold showed optimum mechanical strength, thus could be used for implantation and load bearing studies. Rheological studies confirmed the elastic nature of the scaffold. *In vitro* studies did not show any cytotoxic effects on the seeded chondrocytes indicating that these matrices are biocompatible. However, there was not much proliferation observed in CGC as compared to the samples where CS was supplied exogenously. Similar results were also obtained in GAG and collagen assays. These results provided us an insight for our next step of work which was focused on the incorporation of bioactive molecules to fortify the scaffold. As already discussed tissue engineering field does not only requires the scaffold matrices to fill the tissue void or to provide structural support, or cells that have the ability to form tissues within body but the other major component is bioactive molecules. So next part of our research work was focused on the exploring the utility of appropriate bioactive component to complement already synthesized scaffold.

Main focus of this approach was the synthesis of efficient delivery modes for chondroitin sulfate. To accomplish this objective, chondroitin sulfate was delivered to chondrocytes by different modes like incorporation in scaffold during synthesis, incorporated in microparticles for sustained release (CGS) and further these drug loaded spheres were incorporated in scaffold for slower release of drug so that it can be accessible to

chondrocytes for longer period (CGM). So this study gave newer insights into the efficient and novel delivery modes for CS which has already shown many benefits in treating OA. So here different modes were synthesized and their corresponding effects were analyzed by SEM and *in vitro* release study. Furthermore, primary goat chondrocytes were used for *in vitro* testing of all modes using MTT, SEM, GAG, Collagen assays. The novel delivery mode synthesized CGS and CGM which is known as third generation of biomaterial (cells with scaffold with bioactive components) showed enhanced matrix production and cell proliferation. These systems i.e. CGS and CGM being the latest biomaterials models hold an immense potential for treatment of OA.

Next part of this research work was focused on exploring the potential of ascorbic acid in cartilage repair due to its multipurpose role as an antioxidant, immunostimulant, anti-inflammatory and antibacterial agent. So here in our work we have tried to encapsulate the ascorbic acid (AA) molecule inside the gelatin spheres and then further embedded this in the scaffold. After synthesis the *in vitro* release profile was studied and then further all delivery modes synthesized were studied by MTT, SEM, GAG, Collagen assay. We observed that when AA was encapsulated inside microspheres they all supported better cell proliferation and increase matrix production than control where AA was supplied exogenously in free form. This shows that our approach to encapsulate AA inside microspheres could provide us sustained release of AA together with increase in GAG and collagen production so it works better than free form of drug. Results indicate that encapsulating AA increases the process efficacy and thus can be further explored for cartilage regeneration applications. Some nutritional supplements such as collaflex and retinoic acid were also studied. Collaflex drug also showed increase in matrix production when combined with scaffold and for longer time

duration. Retinoic acid effect was also studied but there was not much increase in extracellular matrix production.

Our next approach focused on nano therapeutics, nanotechnology which could be strategically implemented in new developing drug delivery systems. Moreover the highly efficient drug delivery based on nanomaterials, could potentially reduce the drug dose needed to achieve therapeutic benefit, which will lower the cost or reduce side effects of drugs. In this study we synthesized gold nanoparticles and characterized them by TEM, XRD, Uv-Vis. After this study AuNps were combined with CS and the bioactivity of AuNps-CS (combination of gold nanoparticles- chondroitin sulfate) was evaluated using goat chondrocytes. Analysis was conducted using various cell culture assays like MTT, GAG, collagen and hoescht staining. It was observed that AuNps formation was confirmed by wine color formation and XRD peaks which is characteristic peak for AuNps. UV-Vis spectra also confirmed the stability of AuNps. Then the combination in different concentration when analyzed by UV-Vis it was observed that there was not much shift in wavelength when combination was used underlying that combination is not making much change in spectral properties. Then further FTIR, Zeta potential results also supported the stability of (AuNps-CS). So overall after these analyses it was clear that this combination can be used for further *in vitro* studies to check its effect on chondrocytes. Results indicated that AuNps-CS supported the growth of chondrocytes and enhanced the production of extracellular matrix components, GAG and collagen, as compared to when CS or AuNps used independently. These results suggest that AuNps can act as carriers of CS, which is a promising approach, and can be further evaluated for osteoarthritis model systems.

INTRODUCTION AND LITERATURE REVIEW

Chapter 1

Structural Details of the knee Joint, Cartilage and Preface to Cartilage Tissue Engineering

1. Introduction

The knee is one of the most composite and largest joint inside the body also known as tibia femoral joint formed by three bones femur, tibia, and patella. Femur which is known as the longest and strongest bone functions to support the weight of the body and allows movement of lower extremity. Patella is located in front of the femur on the frontal surface of the knee, it helps in knee extension. Tibia is a long bone of the lower leg which helps in movement and locomotion. Between the tibia and femur, meniscus is found layer of tough fibro cartilage which acts as a shock absorber. Ligaments surround joint capsule and its major role is to hold the bones in proper position as shown in Figure 1.1. The large muscles in the thigh region help in the movement of knee. The cartilage found in the knee bears the same load of bone (Astur *et al*., 2012). The human tibia has many features which contribute to its strength, the composite nature results in good mechanical properties such as collagenous matrix which provides it optimum tensile strength, calcium phosphate gives good compressive strength and its elastic nature due to elastin contributes to its mechanical toughness. Overall knee plays a role in the movement like walking, running, swimming etc.

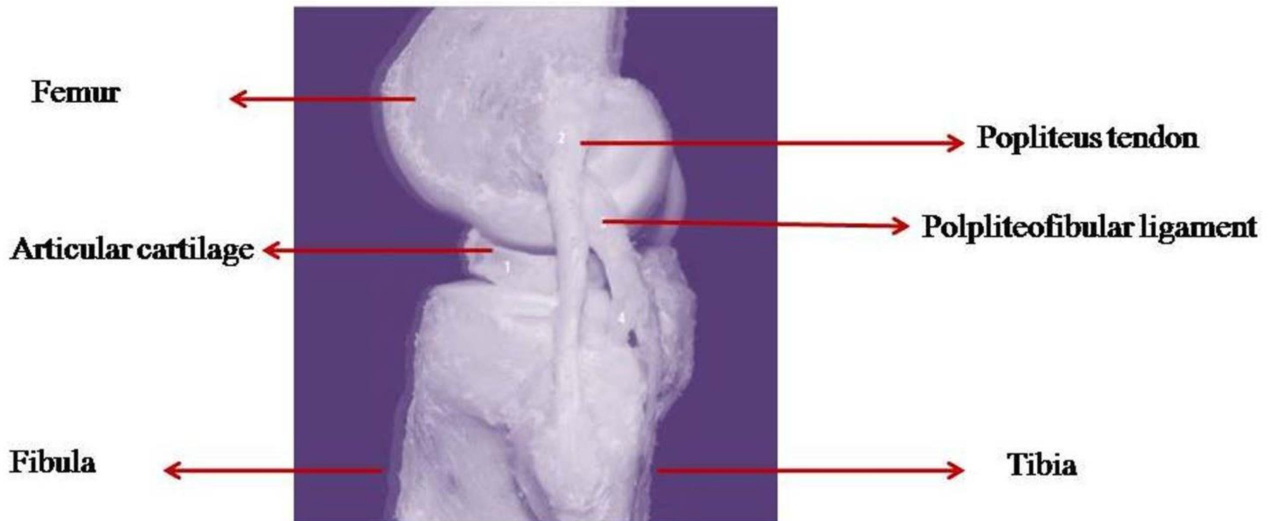


Figure 1.1) Diagram showing the anatomy of knee: femur (thigh bone), tibia (shin bone), fibula (calf bone) (Astur et al., 2012)

In joint region such as knee a well specialized white connective tissue is present known as articular cartilage. Articular cartilage basically functions as a cushion between the bones to allow frictionless movement of the knee. Articular cartilage is composed of chondrocytes, extracellular matrix (ECM) and water (Mort and Billington, 2001). Articular injury is a common disorder of the knee, it gets frequently lost due to wear and tear, thus altering its basic metabolic function, resulting in a degenerative joint disease called osteoarthritis (OA), as shown in Figure 1.2 (Sharma and Rathore, 2015). The degradation of proteoglycans is a premature and reversible process, while the collapse of the collagen network in articular cartilage via collagenase leads to irreversible damage of the fibrillar network. In US alone 27 million cases of cartilage damage are reported per year and in India 80% of the total population by the age of 65 years has osteoarthritis (Memon and Qunilan, 2012).

The major cause of OA may be age, obesity, heredity factor or sport related injury which is the major concern as too much physical stress can result in micro trauma or degeneration of articular cartilage, hence osteoarthritis is a worldwide problem causing morbidity and

mortality (Malemud, 2013). The major limitation for regeneration of cartilage is that it does not possess lymphatic, neural and vascular network, so it has limited self repair capacity (Mobasheri *et al.*, 2009). Moreover, there is also an absence of local progenitor cells, which can otherwise play role in tissue homeostasis, by differentiating into chondrocyte like cell type and formation of proteoglycans. The other limitation can be the defect size. Injuries of particular volume can be healed on their own, while injuries greater than critical size cannot regenerate due to limited joint movement and pain (Ulrich-Vinther *et al.,* 2003; Natarajan *et al.,* 2015). If a small articular cartilage defect is left non-treated it can lead to severe osteoarthritis and subsequent disability. Due to such peculiar characteristics, treatment of the damaged cartilage is a difficult task. Considering these factors there is urgent and important requirement to develop strategies for its repair (Reddi, 1998).

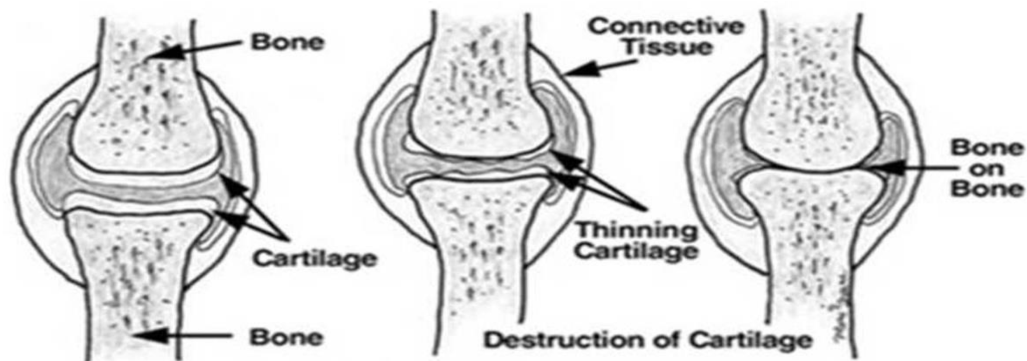


Figure 1.2) Osteoarthritic joint, showing normal joint and the diseased condition (Sharma et al., 2015)

1 a) Statistics

OA is common cause of disability in older adults. Osteoarthritis (OA) is estimated to affect 630 million people worldwide. From 2013- 2015, an estimated 54.4 million US adults

(22.7%) annually had ever been told by a doctor that they had some form of arthritis, rheumatoid arthritis, osteoarthritis , gout, lupus, or fibromyalgia. The most common form of arthritis is osteoarthritis (Barbour KE *et al*., 2013-2015). Osteoarthritis (OA) is the most prevalent form of arthritis in India, affecting over 15 million adults every year. India may become the osteoarthritis capital of the world with over 60 million cases of the disease by 2025, doctors say. Osteoarthritis is the second most common rheumatologic problem and it is the common frequent joint disease with a prevalence of 22% to 39% in India. OA is more common in women than men. OA of the knee is a major cause of mobility impairment, particularly among females. OA was estimated to be the 10th leading cause of nonfatal burden (Chandra prakash pal *et al.,* 2016).

According to the United Nations, by 2050 people aged over 60 will account for more than 20% of the world's population. Means that by 2050, 130 million people will suffer from OA worldwide (Bellamy, et al., 2006)

In the last few decades, Indians in the age-group of 30 to 50 years are falling prey to osteoarthritis and it continues to have serious impact on the lives of elderly people," said Mudit Khanna, consultant Orthopedics at Wockhardt Hospital. Come 2025 and India is likely to notice an endemic of osteoarthritis with about 80 percent of the 65 and above population in the country suffering with wear and tear of joints. Forty percent of these people are likely to suffer from severe osteoarthritis, which will disable them from daily activities."The reason behind the onset of this endemic is said to be increasing longevity of Indians. By 2020, the number of 65 and above population in India is likely to be about 177 million, whereas India had 100 million people in this age group in 2010. People in India are generally averse towards surgery, fearing the potential negative consequences from undergoing surgery. Add

to that the approximately INR 2,00,000 price tag of a total knee replacement, and it is very easy for an Indian to get swayed away from the idea of undergoing surgery. In conclusion, unless significant steps are taken to control the rate at which osteoarthritis is affecting Indians, and also to educate the public about treatments for this disease, we could see a large section of the population suffering from severe disabilities in the near future (decision resource group) (Singh, A. K et al., 2014).

1.1 Composition and structure of articular cartilage

Articular cartilage lacks blood vessels, nerves or lymphatic. It mainly consists of chondrocytes and extracellular matrix. Superficial zone, transitional zone, middle or deep zone, calcified cartilage zone are four zone of articular cartilage. Each zone has a different composition of the matrix, cellular, metabolic and mechanical properties, as shown in Figure 1.3 (García *et al.,* 2013). The zone which has high concentration of collagen and low concentration of proteoglycans forms the superficial zone, which is the thinnest layer. Alteration in this zone disturbs mechanical properties that can lead to osteoarthritis. The transitional zone consists of large diameter collagen fibers with a high content of aggrecan. Middle zone has the highest concentrations of proteoglycans with low cell density. Calcified cartilage zone contains cells in small volume embedded in a calcified matrix and this zone has low metabolic activity, they synthesize collagen type X.

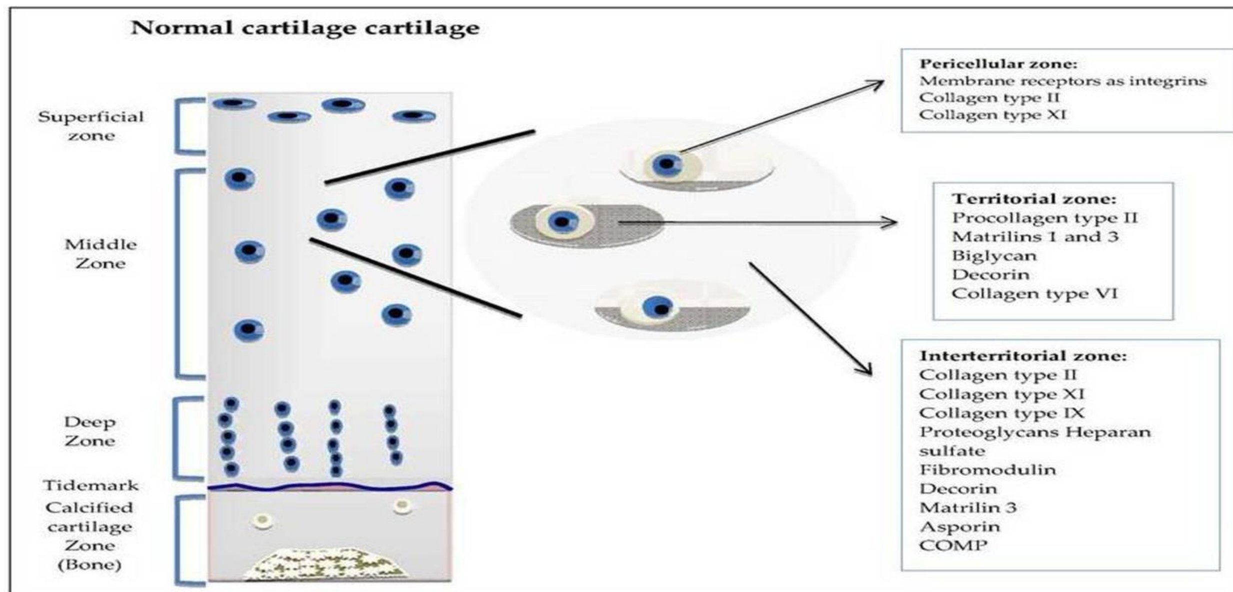


Figure 1.3) Ultrastructure of articular cartilage, showing different zones (Zaira et al., 2013)

Based on the matrix, cartilage is divided into three areas, territorial, inter-territorial and pericellular matrices. Based on distance from cells, the territorial region is farthest from chondrocytes and composed of less organized collagen fibrils. Inter territorial is largest in diameter and farther from chondrocytes. Pericellular matrix protects chondrocytes from a differential mechanical load and consists of collagen type VI, proteoglycans and non collagenous protein (Alexopoulos *et al.,* 2005).

Articular cartilage in spite of being few millimeters thickness has extraordinary mechanical strength with lasting durability. Its different organization and composition gives joints the surface that has little friction along with more lubrication and shock absorption, it also supports the knee to bear bulky cyclic loads through people's life span (Martin and Buckwalter, 2002).

Chondrocytes: It's the only cell type present with in articular cartilage, ranging from 10-30 µm or more in size. They are present all over the tissue but forms less than 10% of overall volume of the matrix. It is isolated from its neighboring cells by cytoplasm and lacks vascular system and relies on diffusion for nutrient transport. Chondrocytes are mainly distinguished by their normally round or polygonal shape and flattened or discoid at tissue boundaries. Moreover based on zone position their shape and size varies. Chondrocytes are typically metabolic cells that have a functionality of synthesis and turnover of the matrix (collagen, glycoprotein, proteoglycans and hyaluronan) (Stockwell, 1979). Chondrocytes originate from mesoderm or neural crest. The construction of cellular condensation by the site of formation of skeletal elements is the first major sign of expression of the differentiated state. The condensation process triggers chondrogenesis helps in expression of tissue specific molecules and other cartilage specific proteins. SOX9 plays a role in activating collagen gene. Sox9 becomes evident and proceeds with an expression of type 2a collagen and alternatively spliced form of collagen 2 which is synthesized by immature chondrocytes or chondroblasts. Aggrecan and cartilage oligomeric matrix protein which are degradation products of articular cartilage and one of the important diagnostic markers for OA, as shown in Figure 1.4 (Hiraki and Mizuta, 2001; Hall and Miyake, 2000; Roach, 1997; Zhong *et al.*, 2015).

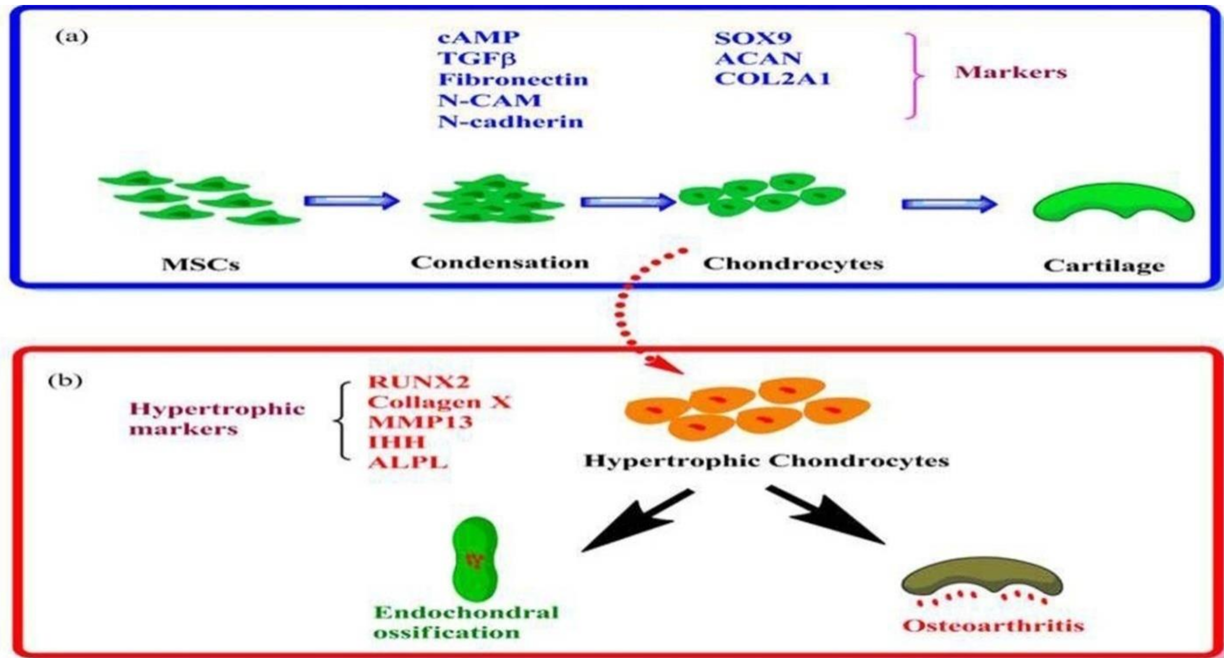


Figure 1.4) Chondrogenesis of MSCs (mesenchymal stem cell) and hypertrophic differentiation (a) Cell contact and MSCs condensation initiate chondrogenesis process b) Chondrocyte undergo chondrogenesis with expression of hypertrophic markers, endochondral ossification leads to mineralization of matrix and apoptotic death of chondrocytes (Leilei Zhong et al., 2015)

Extracellular matrix: A normal adult cartilage is made up of approximately 90-95% ECM (extracellular matrix). Different type of collagens and proteoglycans are the major macromolecules of cartilage, as shown in Figure 1.5 (Gao *et al.,* 2014). After the injury the cartilage is replaced by fibrocartilage which is low in proteogylcans and contains collagen type 1(Schneevoigt *et al*., 2016).

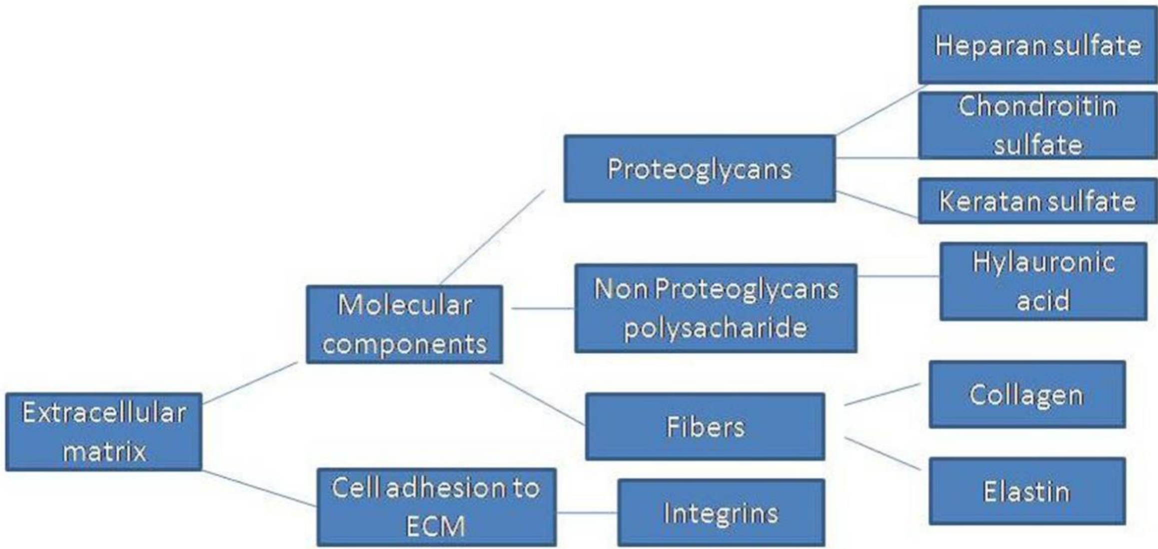


Figure 1.5) Schematic representation of extracellular matrix components present in articular cartilage region (Yue Gao et al., 2014)

Water: Water is the most abundant component of articular cartilage, contributing up to 80% of its wet weight. In normal articular cartilage approximately 30% of this water is present in the intra fibrillar space within the collagen, with small percentage contained in the intracellular space. The water concentration decreases from 80% at the superficial zone of articular cartilage to 65% at the deep zone. The remaining amount of water is present in pore space of articular cartilage. Salts of sodium, calcium, chloride and potassium are found in extracellular tissue fluid to maintain balance. The major source of supply of nutrients to the cell is through flow of water across articular surface. Compressive strength is mainly maintained by water and proteoglycans. (Torzilli, 1985).

Proteoglycans (PGs): About 10-15% of the wet weight of articular cartilage is composed of proteoglycans. Proteoglycans (PG) are the complex molecule containing a protein core covalently bounded to glycosaminoglycans molecule's known as PG aggrecan molecule.

There are a variety of proteoglycans required for restoring cartilage function such as aggrecan, decorin, biglycan, fibromodulin, and lumican. PGs bind to hyaluronan in the presence of link protein to form aggrecan. Aggrecan contributes to major part of cartilage matrix. There are more than 300 associated aggrecan molecules in large aggregate. There are also small non-aggregating proteoglycans which have shorter protein cores as compared to aggrecan, as shown in Figure 1.6. These small proteoglycans affect the activity of cytokine in cartilage by binding to transforming growth factor beta. In addition biglycan and decorin are also present in association with collagen fibrils (García *et al.,* 2013).

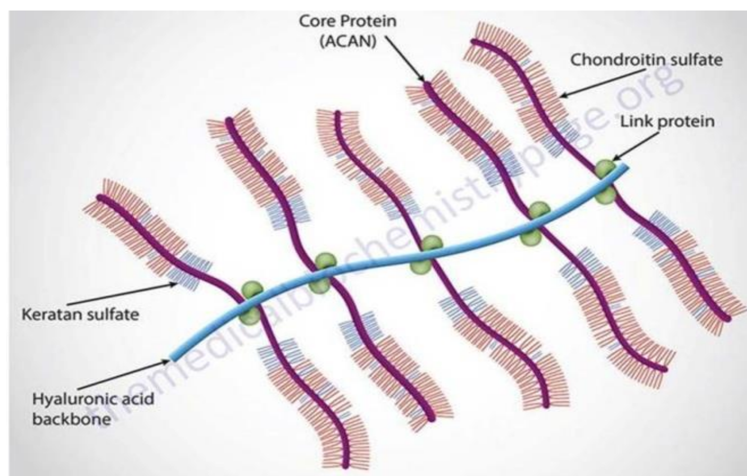


Figure 1.6) Structure of an aggrecan-type complex proteoglycan (Michael W King, 2016)

Collagen is found uniformly distributed throughout the cartilage. It contributes to 60 % of dry weight of cartilage. The most common sources for collagen have been bovine (cow), porcine (pig), and marine (fish), as shown in Figure 1.7. Collagen consists of triple helix structure. Collagen major function is to provide tensile strength to articular cartilage. The major collagen is type 2 (90-95%) with a minor contribution by type V, VI, IX, X and XI. The basic structure of collagen constitutes of three polypeptide chains (alpha) wound in a

triple helix. Collagen type II, IX, XI provides tensile stiffness and strength to tissue and trap large proteoglycans acting as a meshwork. Collagen is degraded by collagenase using simple concept of breaking it into smaller fragments. Articular cartilage can also express collagenase and the defining role of this enzyme in prognosis of osteoarthritis (Silva, 2014).

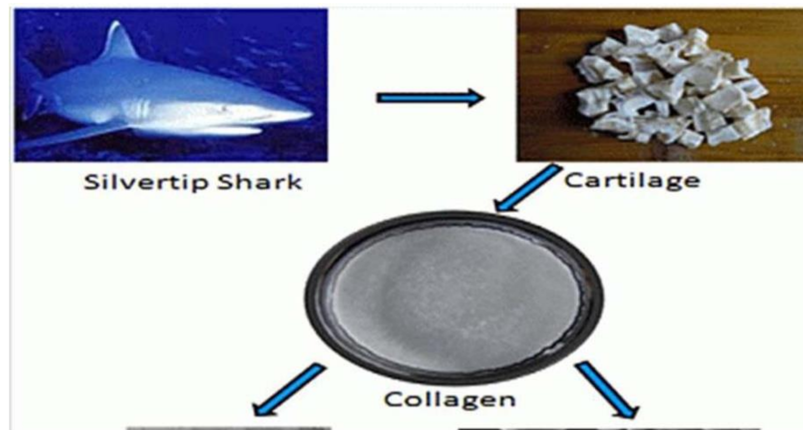


Figure 1.7) Figure showing the source of collagen and role in cartilage (Silva et al., 2014)

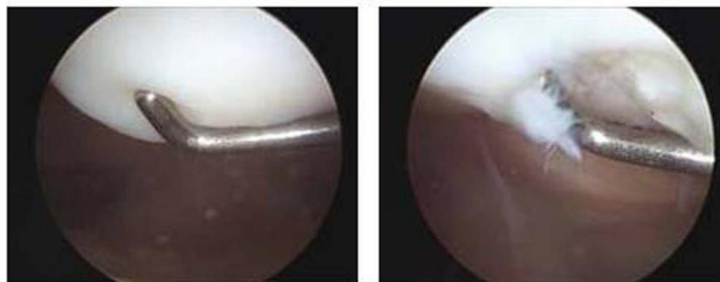
1.2 Injuries to articular cartilage

There are different types of injuries that occur in the knee region. ***Chondral*** may occur as a result of twist while bending the knee due to which hyaline cartilage gets damaged. ***Osteochondral*** where bone underlying also gets damaged. ***Meniscal***: menisci which are pads of cartilage tissue, which help in shock absorption, lubrication, improve knee stability. Damage to it leads to popping along with joint line pain. These are associated with anterior cruciate ligament (ACL) injury. ***Knee bursitis*** bursa acts as a shock absorber when bursa (fluid filled pouch) in the knee is irritated, inflamed or infected. ***Knee joint dislocation*** where the knee cap can dislocate to one side of the knee. It is painful but can be treated by popping

it back into place. ***Knee fractures*** where top of tibia gets collapsed due to sudden knee injury. Genetic variability, patient factor, smoking and degree of injury might affect the progression of disease.

The injuries can be alienated into two forms; partial thickness defect and full thickness defect. In partial thickness defect damage is only on the surface but in the case of full thickness defect, it disturbs the sub-chondral bone below cartilage tissue. In case of partial thickness defect there is no further access to cells like progenitor or blood from bone marrow so it doesn't have self repair mechanism. In full thickness defect, opposite cells from bone marrow are accessible and help in healing process for injury as shown in figure 1.8. The healing is started by formation of fibrin clot and migration of mesenchymal stem cell from bone marrow to defect site thus after one week, the repair is observed. However the mechanical properties of regenerated tissue are low due to fibrous tissue formation (Kakarlapudi and Bickerstaff 2000).

The common symptoms of chondral injury are swelling, local pain and locking as bones are no longer moving over a smooth frictionless surface. Moreover if, the injuries remain untreated, it can lead to conditions of premature end stage arthritis (Bhosale and Richardson 2008). The factors that determine the response to articular cartilage varies with the type of defect, the extent of injury, condition of cartilage and joint at instance of injury, age, structure and function of repair response (James and Uhl 2001).



A **B**

Figure 1.8) A) soft articular cartilage underneath knee cap B) cartilage damage (Eric et al.)

1.3 Etiopathogenesis of Osteoarthritis

This event is divided into following three stages

Stage1: Chondrocytes metabolism is affected with metalloproteinase (collagenase, stromelysin) production increase leads to the destruction of cartilage matrix. Other protease inhibitors like TIMP 1, 2 also increases. ***Stage2:*** The erosion of cartilage surface along with fibrillation occurs with the release of proteoglycans and collagen fragments into synovial fluid. ***Stage3:*** Chronic inflammation response increase due to breakdown products of cartilage like production of metalloproteinase, Cytokines can further produce more metalloproteinase (Hogenmiller and Lozada, 2006).

1.4 Biology of osteoarthritis: *Change in the matrix*

OA affects all joint component and destruction starts with change in articular cartilage color from white color to yellow or brownish. Fibrillation and fissures formation starts in the beginning, further leading to decrease in proteoglycan content.

As the cartilage destruction continues bone and synovium remodeling leads to further degeneration. The other changes are also observed like lower biomechanical properties, increase in hydration as proteoglycan structure loosens and uncurls allowing more space for water. ***Cellular changes***: It is divided into proliferation, catabolism and cell death. The proliferation begins with chondrons swelling (space where chondrocytes are present). Interleukin upregulation then initiates destruction of fibrillar collagen environment. TNF-

alpha and interleukin–beta can initiate aggrecanase and decrease matrix integrity. Release of TGF-beta occurs from matrix with destruction of collagen network. In osteoarthritis cartilage, chondrocytes increases their anabolic activity to repair the damage matrix. The hallmark of osteoarthritis cartilage degeneration is net loss of proteoglycan. The degradative enzyme like metalloproteinases (MMPs), namely, MMPs 1, 2, 3, serine and cysteine proteinases are synthesized by articular cartilage chondrocytes. MMP-3 functions to cleave telopeptide of type II and type IX collagens. Other degradative enzymes are aggrecanase 1 and 2, protein and mRNA present in articular cartilage. Other enzymes like cysteine peptidase, primarily cathepsins are also found.

F spondin, neuronal extracellular matrix glycoprotein are the other factors found to play a key role in OA progression. Prostaglandins (PGEF2) is a pro-inflammatory mediator and leads to collagen degradation and MMP13 activation. COMP production also gets increased which is also involved in OA. Primarily the increase in TGF- beta 1 shows the limited healing response, trying to maintain homeostasis (Mobasheri *et al*., 2012).

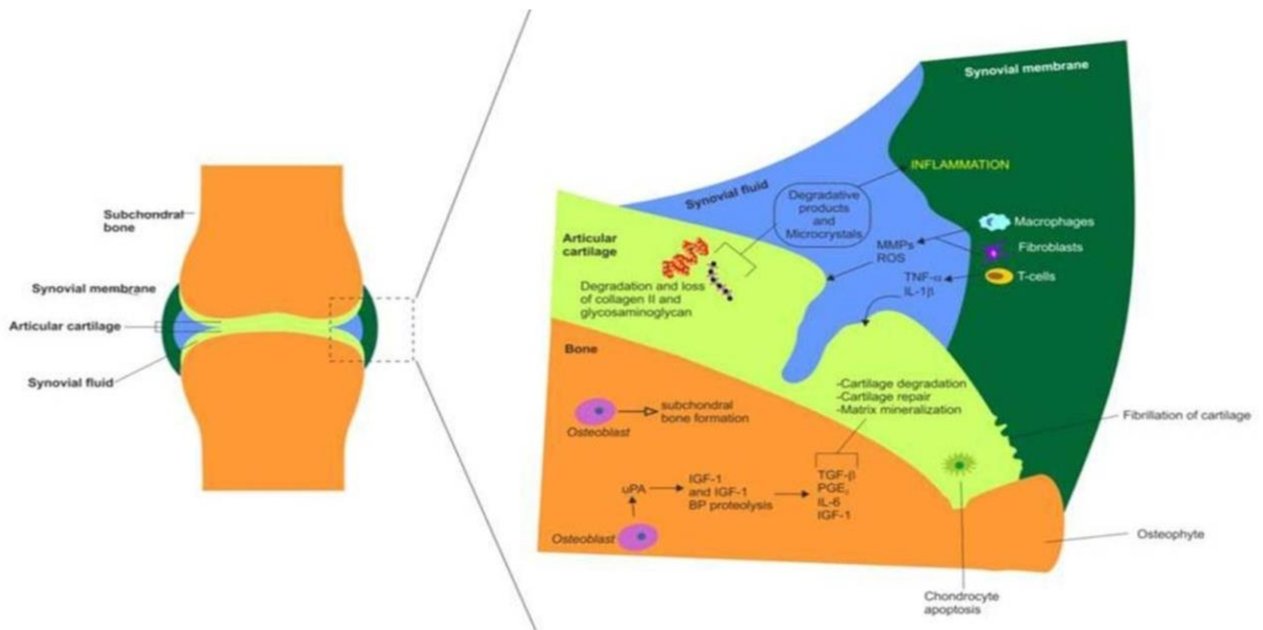


Figure 1.9) Summary of the major synovial, chondral and subchondral changes observed in OA (Mobasheri et al., 2012)

1.5 Conventional treatment technique for osteoarthritis

Microfracture surgery: Due to traumatic injuries articular cartilage damage can occur which can lead to cartilage lesions. They are classified into normal cartilage (grade 0), softening (grade 1), fibrillations (grade 2), fissuring (grade 3), and exposed subchondral bone (stage 4). There are surgical techniques involved to repair the injury; the least invasive one is micro fracture as it doesn't affect the surrounding anatomy of the knee. Basically, the loose cartilage part is eliminated and finally the calcified cartilage removed to the bony base. Bleeding occurs which develops a clot finally forming new fibro cartilage base, as shown in Figure 1.10. Current study reveals that cartilage produced with this doesn't fill the chondral defect fully and fibrocartilage is formed and thus there are chances for its breakdown under high conditions (Seo *et al.,* 2011).

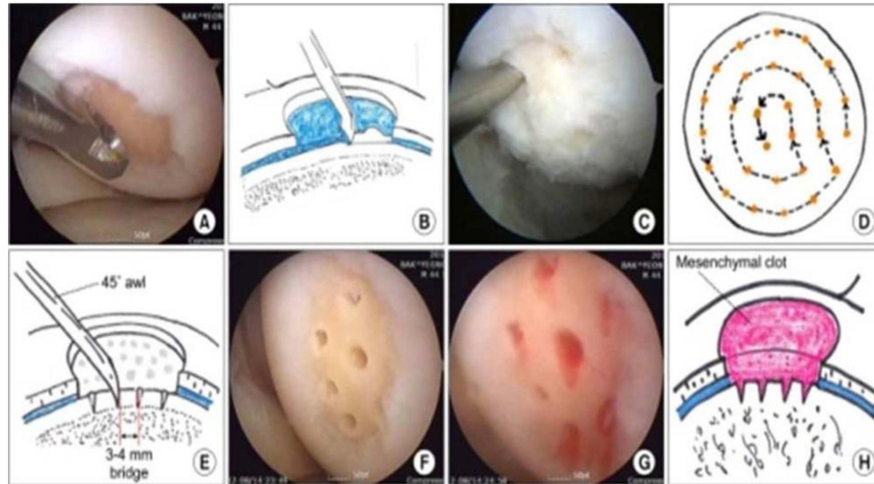


Figure 1.10) A) Unstable cartilage flap B) debride the calcified cartilage C) puncture by awl D) microfracture performed from periphery to centre E) 3-4mm penetration F) Arthroscopic picture G) Mesenchymal blood egress H) Mesenchymal clot in the defect (Seo et al., 2011)

Drilling: In this technique, small holes are drilled in the bone at cartilage damage site. The aim is to provide bone marrow derived stem cells to the site of cartilage damage. Stem cells can differentiate into cartilage cells, basically drilling creates blood clot scaffold on which injected stem cells can be placed and thus normal cartilage forms, as shown in Figure 1.11. During drilling bone temperature is increased which can cause implant failure and screw loosening (Siclari *et al.,* 2014).

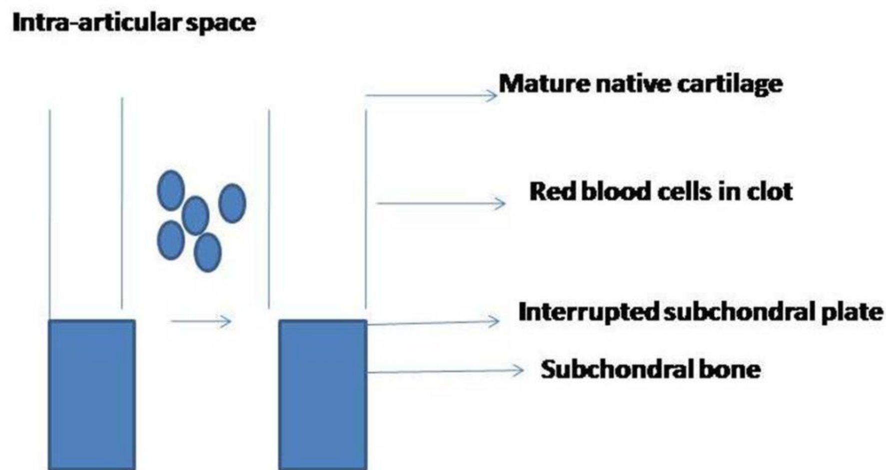


Figure 1.11) Subchondral drilling

Autografts: Amos Matisko *et al.,* 2012 studies show that OA patients with lesions of more than 2 cm^2 diameter have cell and tissue transplantation. For osteochondral defects (up to 3 cm^2 diameter) autografts are reserved. Allograft are reserved for larger defects where cartilage tissue plugs are removed from the non-weight bearing region of the joint further implanted on injury location, as shown in Figure 1.12. This leads to healthy native cartilage formation at defect site. Major limitation is donor site morbidity, as well as joint discomfort (Sanghvi *et al.,* 2014).

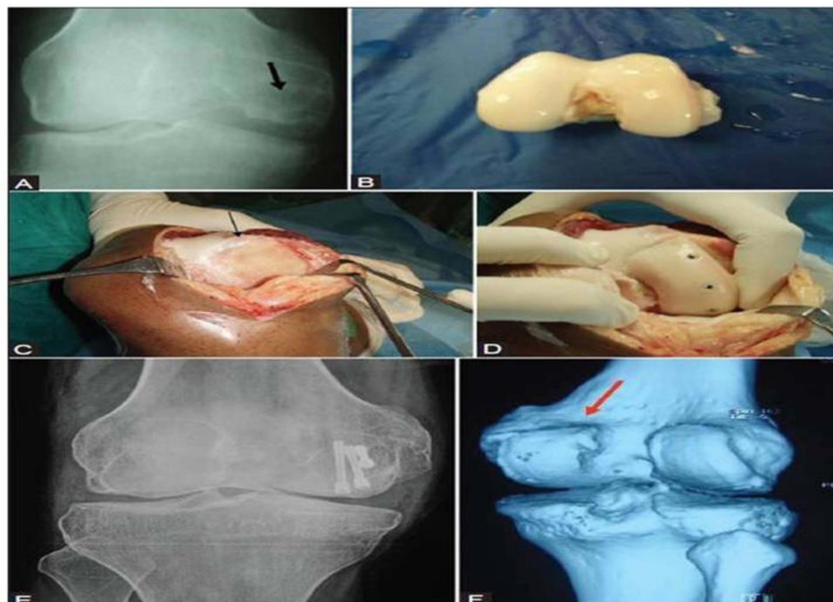


Figure 1.12 A)femoral condyle radiograph with collapse, flattening, and deformation B) Photo of cadaveric allograft (C) expose the articular defect D) osteochondral allograft has been transplanted. (E and F) radiograph shows graft union and topographic (Sanghivi et al. 2014)

Arthroplasty: Derek F Amanatullah *et al.* reported another important and effective method for treatment known as total knee arthroplasty. It is to resurface a knee damaged by arthritis. This is for those who have severe arthritis. In this procedure the surgeon removes the damaged surface of the knee joint. Prosthesis is composed of metal and plastic. Prosthesis is attached to bone with surgical cement. Then the incision is closed and sterile bandage is applied.

The advantage of this technique is that it causes less damage and pain. But the major disadvantages associated are nerve injury, wound healing problems, infections and error in placing prosthetic. Presently over 80,000 surgeries are performed and these can grow up by 170% by 2030. Patients undergoing replacement report low satisfaction with respect to relief of pain after operation (Amanatullah *et al.*, 2015). Currently autologus chondrocyte transplantation emerges as promising approach to enhance cartilage repair (Zhang *et al.*, 2016).

1.6 Pharmacological treatment of osteoarthritis

Current treatment for OA is limited as the first line is to control the symptoms. Currently there are no pharmacological agents capable of retarding the progression of OA. But the line of treatment and the relief will depend on many factors like severity of pain and how much it affects everyday activities. As such, till date, there is no pharmacological treatment that can

cure or prevent OA. They just provide symptomatic relief. Non steroidal anti-inflammatory drugs (NSAIDs) or paracetamol are the primary lines of treatment. But these drugs have some adverse effect like increased incidence of mortality, cardiovascular, renal and gastrointestinal events. Topical analgesics like capsaicin, lidocaine, COX-2 selective and NSAIDs are also used; they also have similar gastrointestinal side effects.

Glucosamine and chondroitin sulfate (CS) are naturally found in connective tissue whereas chondroitin sulfate is found in cartilage of crab and shrimp shells. Both are taken as dietary supplements.

Glucosamine sulfate: Glucosamine derived from ground shells of shellfish, which help in reducing pain and inflammation in arthritis by restoring glycosaminoglycan help cartilage for repair and its formation. The mechanism by which it works is that it reduces prostaglandin E2 production and interferes with nuclear factor Kappa B DNA binding in chondrocytes and synovial cells. It also inhibits gene expression of factors causing OA. Overall, it has been shown that, if glucosamine sulfate is consumed for longer duration, it leads to cartilage damage reduction (Register, 2012).

Chondroitin sulfate: (CS) Chondroitin is naturally present in the body, which forms the major component of cartilage. The main function is to keep cartilage healthy and by absorbing fluid into connective tissue. Moreover they may inhibit those enzymes that break down cartilage. CS has been used as an effective treatment in OA. It also helps in giving elastic properties to cartilage and also reduces swelling in joint as it has anti inflammatory properties. So the underlying benefits of CS are that it reduces the pain, improves functional status, reduces joint swelling and stiffness. CS is mostly administered orally. CS is quickly

absorbed by the gastrointestinal tract. CS has anti-inflammatory property, as it can inhibit many inflammatory intermediates, such as nitric oxide synthase, cyclooxygenase and prostaglandin. The anabolic activity of CS has also been studied; it leads to increase type 2 collagen and proteoglycan synthesis in human chondrocytes. It has an anti-apoptotic effect, as it reduces the sensibility of rabbit chondrocytes to apoptosis. CS also plays a role in cell signaling pathway regulation. It inhibits the activation of MAP kinase pathway, p38 which activates stress signaling, ErK in rabbit chondrocytes intra-articular injection (Henrotin *et al.*, 2010). CS also reduces the production of some pro-inflammatory mediators and proteases, to reduce the cellular death process and improve the anabolic and catabolic balance of extracellular matrix (Raynauld *et al.,* 2016).

Hyaluronan which maintains visco -elasticity is the component of synovial fluid. It decreases the pain as compared to NSAIDs. But it cannot be considered as an effective therapy due to its harmful effects like allergy and toxicity (Grainger and Cicuttini, 2004).

1.6 (a) Product available in the market for OA treatment

Most of the products available in the market are usually drugs for suppressing pain and inflammation rate or some natural remedies.

Drugs: Celebrex, meloxicam, mobic, naproxen, voltaren, aleve, lodine; **Natural remedies**: Dong quai, New Zealand green mussel, natrol, SAMe, Avocado-soybean unsaponifiables **Injections:** Cortisone injections, lubrication injections, hyaluronic injections; **Cream**: Capsaicin; **Surgery:** realigning bones, Knee braces.

1.7 Current treatments

Autologous chondrocyte Implantation (ACI): Currently there has been drastic improvement in ACI. In this process, cells from patient are isolated, cultivated and proliferated and re-implanted underneath a flap. Collagen membrane is usually used as periosteum membrane and several factors are also included, as shown in Figure 1.13. Some products available in market are ACI-Maix, Bio seed C, Cartiform.

The limitation of this technique is that it is expensive, two stage procedure and cells usually get dislocated (Rönn *et al.,* 2011).

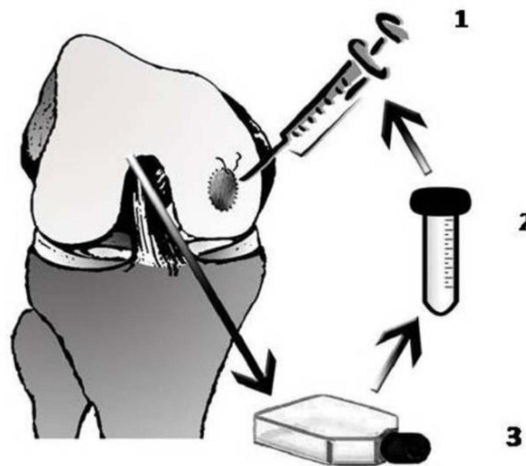


Figure 1.13) Schematic drawing of autologous cartilage implantation (ACI) 1) surgery cartilage harvest is generally performed 2) cells expanded in monolayer 3) cells implanted by injecting them underneath a sutured collagen membrane (Rönn et al., 2011)

Gene therapy for the repair of articular cartilage defects: Another recent technique for cartilage repair is gene therapy. In this technique gene is inserted into the cell so that cell manufactures desired protein which can further alter the behavior of the cell. Basically, most of the molecules are proteins that control cell behavior. For example, insulin growth factor I (IGF I) stimulates chondrocytes for the synthesis of aggrecan and collagen type 2, which are major building blocks of articular cartilage. BMP2 has been found to improve the repair of articular cartilage defects in rabbits. During the last 15 years huge progress has been made in identifying genes that regulate cartilage, but still gene therapy repair for thickness cartilage defects is unsatisfactory. It has been found that growth factor improve cartilage repair by stimulating chondrocyte proliferation and chondrogenesis (Cucchiarini and Madry, 2014).

1.8 Limitation of current treatment

Therapies discussed above are available, but because of limitations such as cell dislocation and biodegradability, they are not ideal for long term applications. They often fail or require additional surgeries. They fail to restore the function of tissue also creating neo tissue.

Due to these disadvantages the tissue engineering field is used to increase cartilage regeneration by combining cells and biomaterial. Tissue engineering is combination of cells and scaffolds but also bioactive factors.

1.9 Cartilage tissue engineering

A promising approach for the repair of articular cartilage defect is cartilage tissue engineering. Due to poor regenerative properties of tissue, it is difficult to repair the articular cartilage. Cartilage tissue engineering has immense potential to repair, regenerate or improve wounded or diseased articular cartilage. The regeneration process involves three basic

components, that is, cells, scaffolds and bioactive factors such as TGF –Beta, IGF-1, as shown in Figure 1.14 (Collin *et al.,* 2016). But there is lack of durable method that can mimic original articular cartilage (Bernhard & Vunjak, 2016).

Growth factors: Transforming growth factor (TGF) are the growth factors that enhance tissue development and enhance matrix properties of neo- cartilage. TGF – beta 1, 2, 3 is involved in regulating different activities such as cell proliferation and differentiation along with chondrocyte phenotype maintenance. In some animal studies, it has been shown that TGF- beta 1 repairs the defect in cartilage. Insulin growth factor (IGF-1) and bone morphogenetic protein (BMP-2) play role in increasing the matrix production of glycosaminoglycan (GAG) and aggrecan. Basic fibroblast growth factor (bFGF) participates in the chondrogenic process. Stevens' studies showed when TGF-beta is activated; it can play a role in intrinsic repair of articular cartilage. *In vivo* studies showed role of growth factors like TGF-beta in the healing of local cartilage defects along with OA (Collin *et al.,* 2016). Some enzymes such as chondroitinase-ABC also help in enhancing functional properties of neo- cartilage as they digest GAGs to remove steric hindrance on collagen assembly (O'brien, 2011).

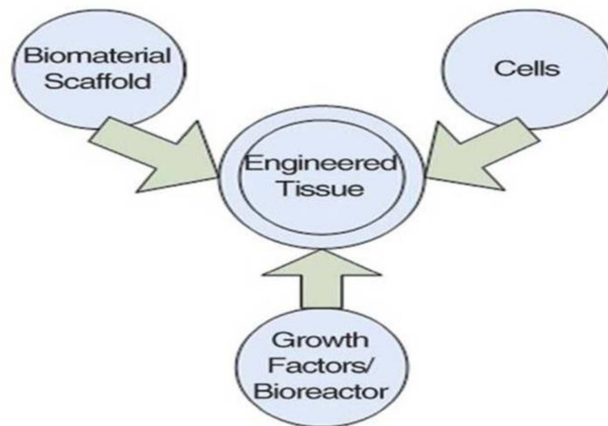


Figure 1.14) Components of tissue engineering (Fergal, 2011)

Cell sources for cartilage tissue engineering: There are mainly two cell sources for cartilage tissue engineering chondrocytes or MSCs. Chondrocytes constitutes 1.7% of the volume of tissue and they are native cell type present in cartilage tissue. Chondrocyte is a distinctive cell type present in articular cartilage tissue and plays a role for its formation and functionality. There is a scope of chondrocyte implantation designed for facial renovation the healing of long segmental tracheal injuries (Lin *et al.,* 2006). Due to some limitation of chondrocytes such as insufficient cell number and slow growth there is another cell source such as mesenchymal stem cells (MSCs) which can differentiate into different type of lineages with high self renewal capacity. Bone marrow and adipose tissue are the source from where MSCs can be obtained (Oussedik *et al.*, 2015). Wakitani and group studied mesenchymal cell based restoration of complete thickness cartilage injury in rabbit. Allogenic MSC showed cartilage healing (Yoshiya and Dhawan, 2015).

1.10 Scaffolds for cartilage defect repair

Scaffolds are used for cartilage defect repair so that they can mimic the native cartilage composition (collagen and glycosaminoglycan). The main role of the scaffold is to provide surface area for cell attachment, increase interaction and extracellular matrix deposition (ECM).

1.11 Properties required for ideal scaffold

Cytocompatibility and tissue compatibility: Scaffold should allow cells to attach, proliferate and differentiate during *in vitro* and *in vivo* implantation, and thus should be biocompatible with cells of cartilage tissues. Collagen fibers are used for peripheral nerve regeneration for proper attachment, proliferation of neural cells (Ma *et al.,* 2014).

Bioactivity**:** Not only should they enable cell adherence but also regulate their different activities. Some biological signals such as cell adhesive ligand can help cell to attach. Scaffold might also play a role as a delivery vehicle or reservoir of bioactive components which will further help in regeneration. This is because apart from scaffold providing the support, the bioactive component will trigger the matrix production leading to the faster regeneration process.

Biodegradability**:** It should be biodegradable so that initially it can help and support the growth of cells, but once new tissue forms it should be able to degrade so that there is no requirement of another surgery to remove scaffold. The use of synthetic polymers reduces the degradation rate so natural scaffold such as chitosan scaffold is used for cartilage strategy (Li and Zhang, 2005).

Porosity and interconnectivity**:** Scaffold should provide the surface for chondrocyte to migrate and exchange of waste and nutrition. Mean pore size (96–150 µm) has been shown to provide cell attachment in bone tissue engineering. However, on the contrary some emphasize the need of larger pore size (300–800 µm) for successful bone growth in scaffolds (Bush and Hall, 2003).

Mechanical property: It is one of other significant aspect of cartilage tissue. The quality is desired so that when the scaffold is implanted it can withstand load in the joint. It also helps in retaining chondrocytes phenotype. Mechanical strength of chitosan scaffolds has shown the applicability in cartilage regeneration (Sahai and Tewari, 2015).

1.12 **Types of material for scaffold**

1) Natural
2) Synthetic

1.13 Scaffold from natural substances

Chitosan: Due to its excellent biocompatibility, chitosan has been widely utilized. Moreover, chitosan can provide appropriate microenvironment for cartilage regeneration by stimulating cell proliferation and repair of tissue through a variety of ways. Chitosan also has excellent biodegradability and can also act as a carrier for many growth factors. Moreover chitosan when cross linked with chondroitin sulfate forms hydro gels that support chondrogenesis.

Agarose: It is a modified form of the agar, a polysaccharide derived from an Asian sea weed. Chondrocytes when seeded on scaffold synthesized from agarose enhances extracellular matrix (ECM) production. But its limitation is its poor biodegradability.

Alginate: It is an anionic polysaccharide. It elicits immune response when implanted which limits its use (Cao *et al.*, 2014).

Collagen: It is widely distributed in the human body. The major advantages are biodegradability, biocompatibility, easy availability and highly versatility. The scaffold built with type 2 collagen is more flexible for cartilage regeneration (Parenteau *et al.,* 2014).

Collagen gels have been tested for chondrocytes and MSCs (Noguchi *et al.,* 1994). Collagen matrices containing glycosaminoglycan are used for gene therapy applications (Samuel *et al.,* 2002). Some better recoveries are shown where chondrocytes and bone morphogenic proteins delivered in collagen fiber scaffold (Frenkel, 2002).

Hyaluronic acid: It is an extracellular matrix molecule with many physical and biological functions. It is a major component of cartilage matrix and induces stem cells to differentiate into chondrocytes. Matrices made of it, have been used as carriers for chondrocytes (Unterman *et al.,* 2012).

Synthetic scaffold: Use of synthetic scaffold has some advantages as they can be modified easily and biodegraded and its mechanical strength can also be easily controlled. Examples are poly-lactic acid (PLA), poly-glycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA). But some of the major disadvantages of them are not much Cytocompatibility for growth of cells and biodegradation may induce an inflammatory response (Woodfield *et al.,* 2004). Both natural and synthetic scaffold can be fabricated into designs of choice using a particular fabrication technology discussed below.

1.14 Fabrication technology

There are different fabrication methodology adapted which can facilitate the cell distribution and growth.

Solvent casting: It is the most simple, easy and inexpensive technique. Here solvent is evaporated to form the scaffold, it is performed by dipping the mold into polymeric solution and allows the solution to evaporate so polymeric membrane layer created. One of major drawback is the toxic solvent used.

Particulate leaching techniques: It is one of the most known techniques. Porogens such as salt wax or sugars are utilized to form pores or channels. Salt particles and polymer solution are poured into a mold and the solvent evaporates and salt crystals are removed to form pores. Porogens can control pore size. But the major drawback is that pore size and interconnected pores are not controlled (Subia *et al*., 2010).

Gas foaming: Foam is substance that is formed by trapping gas, in this gas foaming, the required polymer is made into disc shape by compression modeling using a heated mold. The disc is then kept in a chamber with high pressure of CO_2. The pores are formed by carbon dioxide with formation of sponge like structure. It requires neither organic solvent nor high temperature. It requires high pressure carbon dioxide. The porosity of scaffold depends on the amount of gas dissolved. The major drawback is that it gives non-porous surface and clogged pores and only 10-30% pore's interconnected (Poursamar *et al.,* 2015).

Electro spinning: It's a straight forward, convenient, non-expensive method to fabricate submicron scales polymer fibers. The polymer solution is exposed to the electrostatic field at high voltage so fine polymer flows to target. As the solvent evaporates there is an unstable loop formed that solidifies and collects as an interconnected mesh of fine fibers. But the major limitation is that it cannot be used for scaffold to hold high mechanical strength especially in case of cartilage (Junoh *et al.,* 2015).

Hydrogel: hydrogel is the water-swollen structure of cross linked polymeric chain linked by covalent bonds, formed by linking one or more co monomers. Hydrogen bonds or strong van der Waals forces act. As the solvent used here is water the material has high biocompatibility. For periodontal tissue engineering chitosan –hyaluronic hydrogel scaffold

have been used (Miranda *et al.,* 2015). Recently for bone regeneration, hyaluronic acid hydrogel scaffolds has shown potential (Cui *et al.,* 2015). But one of the major limitations is that it has low mechanical integrity and structural stability (Huglin, 1989).

Some of products available in the market Dexon, Biofix, Dacron, Dexon, Resomer, lupron depot.

1.15 Limitation with conventional fabrication technologies

The conventional fabrication technologies mentioned above have limitations; hence it calls for other alternatives. They cannot control pore size, distribution of pores, interconnectivity of pores, toxic solvents used, cells migrate deep so cannot exchange gaseous and nutrients.

So considering above limitations, there is a need for urgent novel fabrication technology development. Keeping that in mind we tried using cryogelation as fabrication technology which has the potential to overcome the limitation of other mentioned technologies.

1.16 Cryogel

Cryogels are produced via gelation/polymerization at sub-zero temperature. The technique for its synthesis is known as "cryogelation". The cryogels are highly porous, interconnected, and mechanically stable (Lozinsky *et al.,* 2003). The process is simple and can be synthesized by either polymerization or poly condensation. Owing to above properties, cryogels have been used for a variety of applications by labs members of my co-guide (Dr. Ashok Kumar, IIT Kanpur) such as bioreactors, bioseparation, and as polymeric scaffold for tissue engineering. Also, cryogels can be synthesized into different format like monoliths,

discs, beads or sheets. Here the high porosity of gel increases surface area for cell adherence, as shown in Figure 1.15 (Mishra *et al.,* 2013).

1.17 Mechanism of cryogel formation

1) ***Non –frozen liquid microphase formation***: When at sub zero temperature polymer mixture with suitable solvent is kept, solvent freezes to form ice crystals, dissolved solute forms non-frozen liquid microphase.
2) ***Cryoconcentration:*** Due to solvent freezing, liquid phase reduction happens. Gel precursors concentrates in a small volume of non-frozen liquid microphase, and thus causing increased rate of reaction at sub- zero temperature in contrast to the reaction in homogenous solution having the same concentration of precursors but at a temperature above solutions freezing point.
3) ***Interconnected porous network formation***: Polymerization occurs in non frozen liquid microphase. Frozen solvent grows until they meet other crystal. So around polymeric wall these crystal networks are formed. When these crystals melt by thawing, they leave behind well interconnected polymeric network known as cryogel.

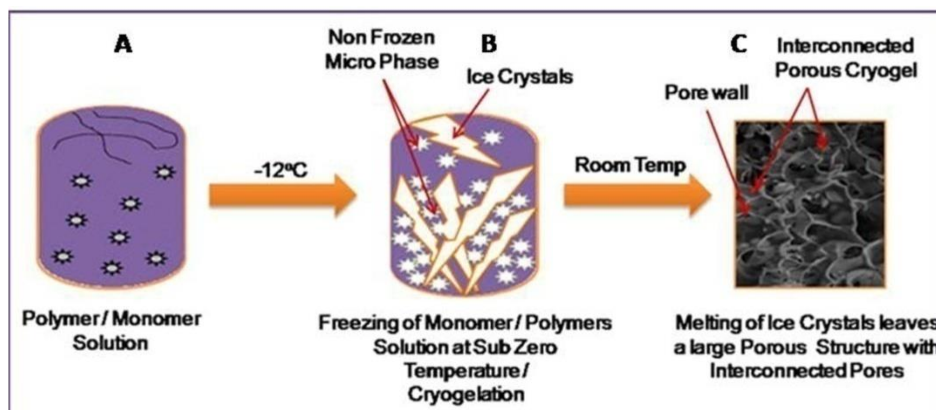


Figure ***1.15) Cryogel mechanism A) showing the polymer/monomer solution with crosslinker B) freezing of solution C) melting of ice crystals showing the formation of interconnected pores(Kumar A. et al, 2010)***

1.18 Applications of cryogels

a) ***Bioseparation:*** Supermacroporous scaffolds are utilized for protein, microbial and mammalian cell separations. Polyacrylamide poly dimethyl acrylamide are used for affinity-based separation of various mammalian cells (blood cells). Stem cells with $CD34^+$ have been developed on cryogel affinity columns. Recently separation of stem cells from umbilical cord blood has been done (Kumar and Srivastava 2010). Major advantage of using cryogel based separation technique is that crude homogenate without any pre treatment can be directly processed on the columns, thereby decreasing cost (Tripathi *et al.*, 2010)

b) ***Bioreactors for production of therapeutics****:* Cryogel bioreactor has been developed for the production of protein therapeutics such as urokinase and a monoclonal antibody. The advantage of this system lies in the fact that it provides an integrated reactor where cells are immobilized over a cryogel based bioreactor and secreted product can be purified using a different cryogel separation column (Jain *et al.,* 2011).

c) ***Cryogel as scaffold for tissue engineering***: Cryogel, due to its properties such as high water uptake capacity, efficient exchange of nutrients and wastes, desirable mechanical properties, interconnected porous network, and biocompatibility, make it an ideal scaffold for engineering of different tissue such as neural (Vishnoi and Kumar, 2013), bone (Mishra and Kumar 2014), skin (Takei *et al.,* 2013), pancreas (Kawazoe *et al.*, 2009) and cartilage (Bhat *et al.*, 2013).

As already mentioned cartilage tissue engineering is not only the combination of scaffold, chondrocytes but also the growth factors or bioactive components. So to deliver these bioactive molecules or growth factors there are some drug vehicles being used.

1.19 Drug delivery vehicles

1) One of the new frontier areas of science is drug delivery technology which involves multidisciplinary scientific approach. This field has grown in recent years. It is rapidly growing with more efforts to try to understand the release mechanism and different polymer to be used (Kumar, 2000).

Basically drug delivery is a method to deliver the pharmaceutical compound to achieve a therapeutic effect in humans so as to benefit health care. Nasal and pulmonary routes are gaining importance for the treatment. Several drug delivery systems are known which includes liposome, pro-liposome, cyclodextrins, gels, microspheres and nanoparticles.

The main advantage of using delivery system as compared to conventional dosage forms

1) Improved efficacy
2) Reduced toxicity
3) Improved patient convenience, reduction of booster dosage.
4) Decrease in fluctuation in steady state levels.
5) Low health cost

Greater flexibility in dosage form design. Thus, all controlled systems help in reducing the effectiveness of drug therapy, their by improving the therapeutic activity of the drugs as compared to side effects (Tiwari *et al.,* 2012).

Advantage of controlled release

a) Deliver drug over extended time duration

b) Specific time during treatment

The most common form of release of the drug is controlled release over extended time duration, it would be useful for the drugs that are quickly metabolized and discarded from the body after administration (Bader, 2014).

1.20 Future of drug delivery system

Particulate system: The biodegradable micro and nanoparticle could be beneficial for administration of the drug that cannot be given orally.

a) Chronopharmacokinetics systems: To counteract natural processes such as bacterial and parasitical growth patterns oral controlled delivery with pulsatile regimen could be used.

b) Combination therapies: to deliver multiple drugs to tumors simultaneously for cancer therapies.

c) Targeted drug delivery: It is a method of delivering medication to the patient so that medication reaches to some parts of the body relative to others. This improves efficacy and reduces side effects

d) ***Sustained release drug delivery systems:*** Drugs have been used to improve health and extend lives. Development of new drug is expensive and time consuming. In the past drugs were delivered by conventional method such as oral ingestion or intravascular injections with major draw backs such as uncontrolled delivery of drug, frequent administration and short half life. But now the practice of drug delivery has changed dramatically with progression of sustained drug delivery which is release of drug in the body slowly over extended time period and it overcomes the drawbacks of

conventional method by controlled delivery, better drug utilization, patient comfort and decreasing local and systemic side effects (Lammers, 2013).

Desired biopharmaceutical characteristics of drug to qualify for controlled drug delivery system

Molecular weight or size: Small molecules can pass and transport across a biological membrane more conveniently.

Solubility: The drug must be present in the form of a solution at the site of the absorption.

Apparent partition coefficient (APC): drugs being absorbed by passive diffusion should have minimal APC.

General absorption mechanism: for oral control delivery system, its absorption mechanism must be by diffusion through the entire GI tract (Bhowmik *et al*., 2012).

1.21 Type of New Drug Carriers Systems

1) ***Immunoconjugates****:* Antibody drug conjugates or immunoconjugates in which drug linked to the antibody. Here, the drug is targeted by means of monoclonal antibodies which avoid non-targeted organ toxicity. But there are some limitations to this delivery system like short shell life, immunogenicity and devoid of efficient interaction.
2) ***Virus****:* Viruses hold possible potential vehicles for drug and gene therapies as they can contaminate specific cells and deliver genomic matter to the nucleus. The use of recombinant virus can improve efficiency for transfection. But certain limitation is to

create viral vectors lacking replication machinery with capability to infect mammalian cells.

3) ***Liposomes, Transferosomes, Ethosomes, Niosomes, Virosomes, Cochleate, and Cubosomes:*** These are phospholipids vehicles formed of a bilayer membrane. Liposomes can act as drug depot injected subcutaneously, but as they are meta stable there is some limitation like poorly controlled release due to content leakage. Ethosomes and transferosomes due to addition of ethanol and surfactants have increased flexibility. Niosomes are novel drug delivery system similar to liposomes, which functions to encapsulate drug used for skin delivery due to their facilitated fusion. Cochleates are lipid composed of natural products, negatively charged phospholipid and divalent cation and are stable particles found to deliver amphotericin B, factor VIII delivery, proteins, and peptides. They are mostly used for oral delivery (Martinho *et al.,* 2011).

4) ***Therapeutic Polymer:*** Polymers are chain of monomers linked in long chain. The main advantage of use of polymer for drug delivery is controlled release system. Here, drugs are conjugated in polymer matrices. Several types of polymers including nano- microparticles and micelles used. (Vilar *et al.,* 2012). In solar cells size controlled sio_2 nanoparticles used as scaffold layers in thin film.

1.22 Microparticles as drug delivery vehicle

Microparticles range between 0.1 and 100 µm. Microparticles are available in different variety of materials including glass, polymers and metals. This system provides delivery of drugs for the longer time period with sustained and controlled release. They are small particles of solid surrounded by a wall of a natural polymer. Microparticles can provide a

constant concentration of drug to target site in a controlled manner. ***Advantages of microparticles*** is that it is very important tool in pharmaceutical area with controlled release of drug offering benefit to diseases like osteoarthritis, rheumatoid arthritis, musculoskeletal disorders, especially in case of degenerative joint therapy which requires long term therapy. Some advantage of using micro-particle as delivery system is listed as following.

1) Delivery of drugs effectively which are sparingly soluble in water.
2) They can be used to give immediate release 80% of the drug in about 10 minutes.
3) Bioavailability of drug is increased
4) Drug can be targeted to specific sites
5) Decrease the drug dose, frequency and toxicity.
6) Can be applied to produce the amorphous drug with desirable properties.
7) Reducing local side effects like gastro intestinal irritation
8) Sustained release with reduce amount of drug.
9) The micro particles are used in immunization, transfection and gene therapy.
10) Parental microparticles can be used to deliver high concentration of water soluble drugs without severe osmotic effects at site of administration
11) Can be stored in dry form or suspension without loss of activity
12) For patients who have difficulties in swallowing solids. Microparticles can be used as dosage
13) Smaller microparticles are needed to prepare for sites like eye, lung, joints (Madhav and Kala, 2011).

1.23 Polymer and other substances used in microparticles preparation

Synthetic and natural polymers can be used as coating materials, based on drug core to be encapsulated.

***Natural or synthetic hydrophilic colloids*:**

Polyacrylic acid, poly acryl methacrylate, gelatin, poly(lactic acid), pectin(poly glycolic acid), waxes(poly hydroxyl butyrate-co-valerate), cellulose derivatives, cellulose acetate phthalate, nitrate, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose,methylcellulose,poly(orthoesters),polyurethanes,poly(ethyleneglycol),poly(ethylene vinyl acetate), polydimethylsiloxane, poly(vinyl acetatephthalate), polyvinyl alcohol, polyvinyl, pyrrolidone. ***Biocompatible polymer:*** *i*ncludes poly (lactic) acid (PLA), poly (glycolic acid) (PLGA). Natural polymers albumin chitin starch, collagen, chitosan, dextrin, gelatin, hyaluronic acid, dextran, fibrinogen, alginic acid and casein (Xu *et al.*, 2009).

1.24 Applications

Microspheres in vaccine delivery: The most important way to deliver the drug in osteoarthritis is *via* intra articular drug treatment, but a major limitation of direction injection is a requirement of booster dosage. Researchers have developed polymers to encapsulate drugs. Such polymers present good biocompatibility and adhesion on articular cartilage. Today many natural or synthetic polymers have been used like albumin, chitosan, silk.

Many drugs incorporated microparticles have been already used for OA. Example being sulforaphane (SFN)–PLGA microspheres. SFN has anti inflammatory property and combination of it with PLGA was used for treating OA. Intraarticular delivery of kartogenin-conjugated chitosan microparticles for cartilage regeneration (Ko *et al.,* 2013; Kang *et al.*, 2014). Major point in the use

of microspheres is that they can be passively targeted to antigen presenting cells.

Controlled release vaccines: New controlled release vaccines are being heavily discovered for AIDs, Hepatitis B, anthrax.

Microspheres in cancer treatment: Main limitation of anticancer drug is a deficiency of selectivity for tumor tissue separate. Microspheres system can be used for site specific action without causing indicative side effects on normal cells.

Ophthalmic drug delivery: Polymer exhibits biological behavior like adhesion, interesting physic-chemical characteristics makes it useful for ocular drug delivery (Parida *et al.,* 2013).

1.25 Microparticles scaffold

There is less feasibility in the approach where bioactive molecule is directly loaded. To overcome these issues embedding these drugs loaded microparticles into scaffold would be more feasible approach. It will help in release of drug for longer time and also scaffold provides as base (Chau *et al.,* 2008).

Celecoxib, whose role has been identified as inhibitor, was encapsulated in microparticles for treatment of OA. (Homar *et al.,* 2007). Microspheres encapsulated with transforming growth factor beta incorporated in chitosan scaffolds were used for cartilage regeneration (Kim *et al.,* 2003).

1.26 Microparticles as a drug delivery tool for OA

In order to increase the duration of action of model drug for OA we developed microparticles as a drug delivery vehicle to deliver drugs like CS and ascorbic. The major advantage of using microparticles is protection of drug, stability and more solubility. The microsphere as DDS has many advantages over traditional method, such as continuous rate of delivery, protection of drug, dosage amount decreased, and efficient delivery (Janssen *et al.,* 2014).

1.27 Nanoparticles as drug delivery vehicle

Structures ranging from 1 to 100 nm are termed as nanoparticles. The main advantage is that nanoparticles are taken up by cells more easily than larger molecules making them suitable to act as carrier for delivery of many bioactive molecules. Liposomes, solid lipids, polymers, dendrimers, silicon or carbon materials, magnetic nanoparticles are some examples of nanocarriers (Zhang *et al.,* 2006). The most important point is conjugation of drug for targeted therapy. The drug to be encapsulated can be either adsorbed or covalently attached to nanocarrier's surface.

The basic goals for research of nano-bio-technologies in drug delivery include

Specific drug delivery and targeting decreased toxicity with therapeutic effect, more safety, biocompatibility, quick development of newer protected medicines. Some of nano vehicle used are albumin, chitosan, gelatin, gold, hydrogels, magnetic iron oxide, polyalkylcyanoacrylate composites, poly (D, L-lactic-co-glycolic) acid (PLGA).

1.28 Advantage of using nanoparticles drug delivery system

1) Sustained and controlled release of drug at the site of localization
2) Encapsulation of drug without any chemical reaction.
3) Release of the drug can be modulated.
4) No wastage of drug and enhanced bioavailability of drug at specific site for longer time duration
5) Improves solubility of poorly water soluble drugs.
6) Lower frequency of administration.
7) Multiple drugs can be delivered to target site (Singh *et al.*, 2011)

The uptake mechanism of nanoparticles varies depending on their properties. Nanoparticles

(Nps) can enter cell via phagocytotic or non- phagocytotic pathways, clathrin mediated pathways or other endocytotic pathways. Knowledge about uptake of drug is crucial for target site (Plajnsek *et al.,* 2012).

1.29 Nanoparticles as therapeutic vehicle for drug delivery for osteoarthritis

In recent news published by American Association of Pharmaceutical Scientists (AAPS), it is demonstrated that using nanoparticles as drug delivery vehicle to deliver osteoarthritic drugs to the knee joint could help increase the retention of drug in the cavity for a longer time and therefore reduce the frequency of injections. Michael group (2011) from Bend research and Pfizer injected nanoparticles in knee cavity and found that 70% of nanoparticles remain in knee cavity after one week whereas in conventional drug treatment the drug disperses in one or two days.

Susan *et al*. have reported mouse model for OA, injected with a novel nanoparticles loaded with a gene inhibitor into knees of affected mice (Janssen *et al.,* 2014). Due to the nanoparticles as a delivery vehicle large amount of gene was delivered to cytoplasm and nucleus of chondrocytes. Here also particles remained in the joint for two weeks compared to only a few days for spherical nanoparticles.

In another study it was shown that for OA chitosan nanoparticles have been used as non-viral gene delivery vectors to transfer the exogenous gene into primary chondrocytes (Lu *et al.,* 2011).

It can be concluded from all these studies that nanoparticles can help in retention of OA drug for longer time. However, its utilization is yet to be fully explored in some areas especially osteoarthritis disease. Local delivery by nanomaterials can limit present side effects of OA drugs. Also there are many OA drugs that are yet to be explored and, therefore straight forward adaptions

to the existing technologies can be used to optimize OA drug delivery and improve therapeutic outcomes.

1.30 Future limitation of nanoparticles for osteoarthritis and further work

Still many polymer and other materials can be tried for synthesizing nanoparticles for osteoarthritis which are biocompatible, non toxic to chondrocytes as not much work has been done in this area. It would be beneficial to understand the mechanism of the drug delivery by nanoparticles and how these particles behave with in the living cells, organs and organism. Many OA drugs are being used that reduce pain but they are not able to retain for longer time duration. So, nanotherapeutics can be used as a potential field for overcoming these limitations. Gold nanoparticles have already shown its potential in cancer therapeutics (Cao and Liz, 2014).

2. Gaps in existing research

Cartilage is an avascular and aneural tissue which has very limited repair capacity. Moreover small articular cartilage defects, if left untreated, can lead to severe osteoarthritis. Conventional repair strategies which are being used for the repair of damaged cartilage tissue are drilling, abrasion, arthroplasty, microfractures, osteochondral graft and allograft. In case of autologous chondrocytes cell suspension is injected at the defective area but the major disadvantage is lack of cell retention. There are some fabrication technologies for scaffolds such as solvent casting, particulate leaching, melt molding, phase-separation, freeze-drying and gas foaming. There are some conventional problems/gap such as clogged pores, non porous surface, toxic solvent, low mechanical integrity (Izadifar *et al.,* 2012). Synthetic and natural polymers are used for fabrication of scaffold. Synthetic polymers show reduced biocompatibility which lead to rejection of cells and

biodegradation which may cause an inflammatory response. The most important factor to be considered in case of cartilage tissue engineering is to construct healthy cartilage having mechanical and load bearing properties matching those of native cartilage. There is dirth of many such construct with good mechanical properties.

There are many drug modalties available in the market which can reduce pain and improve cartilage function. Many of these drug include side effects such as gastro problems, renal effect, kidney also liver problems etc. Their mode of administration whether oral or intra articular can cause short residence time and poor stability. To overcome this problem there is requirement for drug delivery vehicle which can protect the drug properties and release the drug in a controlled manner for longer time.

Some of bioactive factors such vitamin C has role of being an antioxidant. Also it acts as an immunostimulant, anti-inflammatory and antibacterial agent. Despite several formulations containing vitamin C in the market, few are topically effective, Major limitation of these formulations 1) low concentration of vitamin C 2) incomplete absorption and metabolism of different forms 3) AA oxidized to dehydroascorbic acid due to exposure to air, light and high temperature which results in short shelf time of formulations containing vitamin C.

Currently, nanoparticles are widely used for delivery of various bioactive molecules for improved drug efficacy; targeted delivery, reduced toxicity, and increased uptake for various diseases such as cancer, heart, but not much have been explored in case of OA.

3. Objectives

The proposed research aims:

1. Synthesis of novel three dimensional (3D) scaffolds fabricated from natural polymers like

chitosan, gelatin blended with chondroitin sulfate (CGC) using *cryogelation* technology and their characterization.

2. *In vitro* testing of these novel scaffolds with goat chondrocytes in order to study various effects like cell proliferation and extracellular matrix (ECM) production.

3. Encapsulation of drug loaded microspheres into scaffold for treating osteoarthritis.

4. Scope of the work

To overcome the limitation of current repair strategies mentioned in the gap we have used tissue enginnering as a potential field to improve cartilage regeneration by combining cells and biomaterial of 3D construction. Scaffolds are being developed to provide support to chondrocytes which enhance cell attachment and proliferation along with increase in matrix production. Our main focus in this study was to make a scaffold that can mimic native cartilage to close. 3D CGC (Chitosan-Gelatin-Chondroitin sulfate) scaffold are synthesized using cryogelation technology aiming to mimick native cartilage matrix which may help to replenish the loss of damage matrix till the neo tissue forms. Chitosan serves as a structural analog of cartilage specific glycosaminoglycans (GAGs), which regulate chondrocyte function, helps in differentiation and control interaction with the cells. Gelatin is an irreversible hydrolyzed form of collagen having triple helical structure which is broken down into single strand molecules. Chondroitin sulfate (CS) is a major component of cartilage a kind of glycosaminoglycan found in animal connective tissue. In this study we have synthesized scaffolds using cryogelation technology which has several advantages over conventional fabrication techniques. Cryogelation technique provides well interconnected pores where size of pore can be controlled by varying polymer concentration, and the scaffold can be synthesized in different forms like disc, monolith, sheet etc (Singh *et al.,* 2012).

We have used natural polymers CS which is biocompatible and biodegradable. In order to work on mechanical strength of scaffold we have used CS which is also responsible for water retention capacity of cartilage, by binding water to the polar carboxylate group of CS, which further provides resistance to compression leading to increase in mechanical strength.

Another scope of this work is in order to overcome the limitation of traditional drug delivery and to protect potential of OA drugs available in market we have tried using microparticles as one of the types of DDS. The main advantage of the DDS is that microencapsulation technology allows protection of drug from the environment, increases bioavailability to the target site reduce dosage frequency and toxicity of various drugs. Now scaffolds have advanced, from first generation in 1960s and 70s which dealt with mimicking physical properties of replaced tissue, to third generation biomaterial which focused as incorporation of bioactive molecule into it by different modes. So that scaffold could mimic ECM to close. So we have tried encapsulating these drug loaded microparticle scaffold to generate third generation biomaterial.

We have tried encapsulating AA to protect its properties and study its role further along with other bioactive molecule in connection to cartilage repair is slow process.

We have also used nanoparticles to study its role in OA. The most noticeable point is that nanoparticles with optimized physiochemical and biological properties are taken up by cells more easily than larger molecules, they have huge potential as delivery vehicle for bioactive factors. Not much work has been done in field of OA, especially in case of drug delivery. Gold nanoparticles (AuNps) are promising nano carriers for drug delivery due to their unique properties like facile synthesis and functionalization, biocompatibility, ease in cell permeability, and high drug loading capacity.

Chapter 2

Synthesis, Characterization and *in vitro* Studies of Chitosan-Gelatin-Chondroitin Sulfate (CGC) Cryogel Scaffold for Cartilage Tissue Engineering

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2.1 Introduction

The emerging field of tissue engineering has a huge potential to regenerate damaged tissue by combining cells from the body with scaffold as biomaterials, in addition to some bioactive factors (Obrien, 2011). Articular cartilage act as a cushion between the bones to allow movement, but due to wear and tear sometimes articular cartilage gets damage that leads to a condition known as osteoarthritis (OA) (Machado *et al.*, 2015). OA is a common degenerative disorder which leads to pain, swelling, joint stiffness, muscle weakness, deformed joints, cracking, creaking and impaired movement. Age, genetics, past trauma, obesity are the risk factors that can lead towards this disease. Unlike other tissues cartilage is avascular and aneural hence, it has limited self repair capacity and thus once damaged it is difficult to heal (Sinusas, 2012).

There are acute injuries where matrix is damaged without damage to chondrocytes or mechanical damage of chondrocytes and extracellular matrix (impact load injury). Loss of matrix macromolecules leads to mechanical disruption of cartilage surface which can further result in activating some factors like, activated protein complex (APC) which act as a stimulator for MMPs such as aggrecanase that further causes matrix degradation. These factors may lead to degeneration of proteoglycans or suppress proteoglycan synthesis. To

stop this process chondrocytes helps to restore lost matrix components, allowing matrix components to attain its regular composition and function, however if this continues damage caused may become irreversible (James and Uhl 2001). Therefore, there is an urgent demand for medical and surgical intervention to prevent further cartilage damage. A wide variety of technologies and medical products to restore the normal activity to the afflicted joint are available such as total knee arthroplasty and non-implant based procedures (arthroscopic chondroplasty and microfracture) and grafting procedures (autografts and allografts). In total knee arthroplasty, knee joint is replaced with the artificial part but here the limitation is dislocation of implant or infection. Arthroscopic chondroplasty, where the damaged tissue is removed, but the cartilage formed might not be as strong as native cartilage, microfracture works with the removal of damaged cartilage this might also lead to discomfort and pain (Stroh *et al.*, 2011). One of the latest cell based technology is autologous chondrocyte implantation, where cells from patients were isolated and implanted to the defect site with a covering mechanical membrane periosteum. Some of the factors which restrict the application of autologous chondrocyte implantation like the fixation of periosteal flap covering chondrocytes may be insecure, irregular cell distribution, loss of cells into the joint cavity resulting from recurrence of surgery up to 25% to 36% patients (Tuan, 2007). Due to the limitation of some current technologies, tissue engineering has evolved as a potential future approach for restoration of such defects. Scaffold, cells, and growth stimulating factors are generally considered triad components of tissue engineering. Mostly chondrocytes or chondroprogenitor cells are extracted by enzymatic digestion, and then expanded with further seeding of these cells on different biocompatible scaffolds and maintained for graft implantation (Kuo *et al.*, 2006). Scaffolds, typically synthesized from polymeric

biomaterials, provide structural support for cell attachment and tissue development. The basic pre requisite for scaffolds to be engineered for cartilage tissue engineering should be to provide biocompatibility, biorecognition for cells, mechanical properties to withstand load, degradation kinetics, uniform pore size and pore size distribution, large surface area so that cells can penetrate and acquire enough surface to exchange gaseous and nutrients (Carletti *et al.,* 2011).

Basically, there are two types of polymers, synthetic and natural polymers, which are used for fabrication of scaffold. Each one has its own advantage and disadvantage; synthetic polymers are more durable, versatile and can achieve desirable properties. In some cases there is reduced biocompatibility which may lead to rejection of cells; biodegradation may cause an inflammatory response. Natural polymers are biocompatible and biodegradable. Naturally the suitable scaffold for an engineered tissue should mimic the ECM of the target tissue in its native state. But due to the dynamic and complex nature of tissue, it is difficult to mimic ECM in native state exactly. The basic purpose of the scaffold as one of the components of tissue engineering can be to partially mimic native ECM (Cao *et al.,* 2014). The main advantage of the use of synthetic material is that they have the good biomechanical strength and can be modified by changing the composition of the polymer; however the main disadvantage is that these materials are foreign to the body (Mogoşanu and Grumezescu, 2014).

Natural polymers can overcome limitation as they are biocompatible and biodegradable. Natural polymers which provide mechanical strength can be used to overcome the limitation of a natural polymer, as mechanical strength is an important factor in cartilage tissue

engineering (Brittberg, 2008). Natural polymers hold immense potential in tissue engineering for scaffold fabrication as they resemble glycosaminoglycans in ECM (Benders *et al.,* 2013).

In this study, we selected chitosan, gelatin and chondroitin sulfate as the biomaterials of scaffold components, chitosan composed of glucosamine and N- acetyl glucosamine is natural polysaccharide and is a component of cartilage ECM. These components maintain chondrogenic activities and support the expression of cartilage ECM proteins. Chitosan also serves as a structural analog of cartilage specific glycosaminoglycans (GAGs), which helps chondrocyte to differentiate, regulate its function and control interaction with the cells and matrix and may also mimic other biological activities of GAGs like binding to growth factors. The polycationic nature of chitosan helps cells to adhere and also interact with other anionic GAGs. The free amino group may provide a site for bioactive binding and additionally the antimicrobial activity of chitosan could reduce the risk of infection when implanted at the defect site (Elder *et al.*, 2004). Gelatin is an irreversible hydrolyzed form of collagen, where collagen triple helical structure is broken down into single strand molecules. It is non-immunogenic and due to its RGD sequence it retains signaling capacity, and is absorbable (Enrione *et al*., 2010). Chondroitin sulfate (CS) a mucopolysaccharide is a kind of glycosaminoglycan found in animal connective tissue like cartilage. CS helps in intracellular signaling, cell recognition and association of ECM components to cell surface glycoproteins, the process of chondrogenesis for chondrocyte to proliferate and differentiate (Chang *et al*., 2010). CS is responsible for water retention capacity of cartilage, by binding water to the polar carboxylate group of CS, which further provides resistance to compression leading to increase in mechanical strength. CS has anti-inflammatory properties that would help in the healing process (Dinescu *et al.*, 2013). Over the time period many designs and fabrication

techniques have been developed for scaffolds that can be used in cartilage regeneration. The fabrication process for cartilage tissue engineering should be such that it must generate reproducible architecture scaffold, and can also bear the load joint for longer time duration. The fabrication technology can influence different properties of scaffold like mechanical, biocompatibility and biochemical properties. Some of the fabrications technologies are solvent casting, particulate leaching, melt molding, phase-separation, freeze-drying and gas foaming. Since there are some conventional problems (Izadifar *et al.,* 2012), in this study we have prepared the scaffold by using cryogelation technology which has several advantages over conventional fabrication techniques. It provides well interconnected pores, size of pore can be controlled by varying polymer concentration, and the scaffold can be synthesized in different forms like disc, monolith, sheet (Singh *et al.,* 2012).

To overcome the limitation of currently available strategies such as autologous chondrocyte transplantation, tissue engineered tool has been selected which aims to repair and regenerate the disease cartilage using triad components (Scaffold, cells and bioactive factors).

The aim of this study was to synthesize the novel cryogel scaffold using chitosan, gelatin, and chondroitin sulfate; such that it can mimic native cartilage ECM and further this scaffold could be used as an implant in cartilage defect area. Here the scaffold was synthesized by using cryogelation technology and further characterized by different physical and mechanical techniques in order to check whether scaffold has suitable physical, chemical and mechanical properties. Further *in vitro* characterization was done by using MTT, SEM GAG and collagen assay.

2.2 Materials and Methods

Materials: Low viscosity chitosan (M.W 150000, viscosity: 200 mPaS), Chondroitin sulfate(CS), gelatin (cold water fish skin), Dulbecco's modified Eagle medium (DMEM), Collagenase type2, penicillin-streptomycin, nystatin, papain, trypsin-EDTA solution, 1,9 dimethyl methylene blue, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Glutaraldehyde was obtained from S.D. Fine Chemicals Ltd (Mumbai, India). Fetal bovine serum was purchased from Hyclone (UT, USA). All other chemicals used were of analytical grade and were used without any further purification.

2.2.1 Preparation of supermacroporous Chitosan-Gelatin-Chondroitin Sulfate Blend (CGC)

Chitosan-gelatin-chondroitin sulfate (CGC) cryogels were prepared by the cryogelation process. During optimization different concentration range of chitosan, gelatin and chondroitin sulfate were tried. Different combinations were tried CGC 3% (Chitosan 1%, gelatin 1%, CS 1%), CGC 3.01% (Chitosan 1%, gelatin 1%, CS 1.01%), CGC 3.05 (Chitosan 1%, gelatin 1% , CS 1.05 %), CGC 4% (Chitosan 1%, gelatin 2%, CS 1%), CGC 4.01 (Chitosan 1%, gelatin 3%, CS .01%), CGC 4.05(Chitosan 1%, gelatin 3%, CS .05%), CGC 4.1(Chitosan 1%, gelatin 3%, CS 0.1 %), CGC 4.2(Chitosan 1%, gelatin 3%, CS 0.2 %), CGC 4.3 %(Chitosan 1%, gelatin 3%, CS 0.3 %), CGC 4.4(Chitosan 1%, gelatin 3%, CS 0.4%), CGC 4.5 (chitosan 0.8%, gelatin 3.2%, chondroitin 0.5 %) , CGC 5% (chitosan 0.8%, gelatin 3.2%, CS 1%). After optimization for the final fabrication two different polymer ratios were selected by changing the concentration of CS (CGC 4.5% and CGC 5%). LVC (low viscous chitosan, 0.8% w/ v) was dissolved in 6ml (1% aqueous acetic acid solution) and kept on a mechanical rotor for 30 min. CS (0.5% and 1%) was dissolved in 1

ml water. The chitosan and CS solutions were mixed well and then sonicated to obtain a homogenous mixture for 5 min. Gelatin (3.2%) was mixed in 1% aqueous acetic acid (2 ml) on a rocker for about 30 min, this solution was mixed with above chitosan-chondroitin sulfate mixture, then in this mixture 1% v/v glutaraldehyde was added from a stock solution of (25% v/v). The whole mixture in the tube was then vortexed for 5 s for proper mixing of crosslinkers and polymers. As soon as crosslinker was added and mixed the solution was immediately poured into the syringe of capacity of 5 ml and further kept in the liquid cooling bath (cryostat) at -12 ºC. After 12 h incubation, gels were thawed in water at room temperature and removed from plastic syringes. Gels were kept in water and at low speed on shaker to remove the unreacted aldehyde group. CGC 4.5% and CGC 5% were synthesized by above method.

2.2.1 Physical Characterization of Scaffold

2.1.2.1 Flow rate measurement of cryogel

Monolith cryogels were cut in sections of 13 mm in diameter and 5 mm in thickness. The resistance to the flow of the solvent was measured by using peristaltic pump by passing the solvent through the cryogel at varying flow rates. The samples were taken in triplicates. The cryogel was swollen, placed in the syringe mold such that there is no leakage from any side of the syringe. The pump was connected to the opening of the syringe through rubber tubing’s. The liquid was allowed to pass through the cryogel samples and flow resistance was measured at a different flow rate (i.e. 1,2,4,5,6,8,10 ml/min) till the back pressure was observed. The pump was calibrated by running experiment without any column connected (Vishnoi and Kumar, 2013).

2.1.2.2 Swelling kinetics of CGC Cryogel

The scaffolds were cut into the discs of (13 mm diameter and 4 mm length) and were weighed. Additionally, these samples were immersed in phosphate buffer saline (PBS, pH 7.4). Wet weight of the samples were recorded at regular time intervals such as 15, 30, 60, 90 and 120 s till the samples reach to saturated state with PBS. To observe any changes in the behavior of the cryogel cyclic swelling and de-swelling was carried out up to five cycles to further analyze any change in the cryogel. Swelling kinetics was determined using the gravimetric procedure. All the samples were taken in triplicate so to reduce any errors. Swelling ratio was calculated using the formula

Swelling ratio =

$Wt_{-}\ W_{d/}\ W_{d/}$

Where W_t is weight of swollen cryogel

W_d is weight of dry cryogel

Water uptake percentage was calculated as follows:

Wu is water uptake capacity

Wu=100X(Wt-Wg)

Wt is mass at regular time interval

W_g is mass at dry cryogel

We is a mass of water in the swollen gel at a specific temperature and after swelling equilibrium (Jain and Kumar, 2009).

2.1.2.3 Microstructure analysis

The microstructural analysis of the cryogel was done using scanning electron microscopy (SEM, FEI quanta 200). Cryogel samples were diced into pieces of 2 mm diameter and then they were pre-treated with gradients of alcohol (20-100%) for about 15 min in each concentration. The cryogel were dried overnight in vacuum desiccators for better resolution and imaging, after which they were gold coated using gold coater (Vacuum Tech, Bangalore India). Then samples were scanned by SEM at a high voltage of 20 kV and spot size of 3.5 mm under high vacuum. Samples were examined in triplicates.

2.1.2.4 In vitro degradation in aseptic conditions.

The cryogel scaffolds were studied for their degradation rate for a period of about 8 weeks. The cryogel scaffold were weighed and immersed in gradient of ethanol (20 %, 40%, 60 %, 70% and 100%). To 15 ml tubes containing 0.1 M sterile PBS, scaffolds were transferred under sterile conditions. The studies were done at 37 °C under sterile conditions. The samples were then collected at regular intervals and washed with de-ionized water. The samples were dehydrated and weighed to examine the change in the weight of the sample. The degree of degradation was thus determined by the following equation

$\% \text{ D.D} = W_1 - W_F / W_F$

Where W_1 is the dry weight of the sample before incubation and W_F is the dry weight of the sample after incubation. Further, SEM analysis was also done to analyze the change in

morphology (in terms of the collapse of gel walls) of degraded scaffolds (Bhat and Kumar, 2012).

2.1.2.5 Fourier transform infrared spectroscopy (FTIR)

In order to determine the different functional groups in CGC cryogel sample and different cross linking of polymers, FTIR was carried out. Cryogel which was solid sample was grinded into powdered form by freezing in liquid nitrogen and stored in different tubes. FTIR was done by means of Perkin Elmer 1000 paragon spectrophotometer.

2.2.3 Mechanical characterization of CGC Blend

2.2.3.1 Determination of compression stability and elasticity

To determine the mechanical stability CGC cryogels were sliced into specific dimensions of 13mm diameter and 10 mm in length and saturated with 0.1 M PBS (pH 7.4) and were kept in the mechanical analyzer (Zwick /Roell ZO10 Germany). Samples were compressed (strain applied) up to 90% of their original height under a load cell of 0.2 kN with a displacement rate of 1mm/min. Compression modulus was determined by plotting a graph between stress (kPa) and strain (%) (Singh *et al.,* 2011).

2.2.3.2 Rheology measurements

In order to further study their mechanical properties, samples were analyzed by studying the change in flow and deformation. Sections (5mm in height and 13mm diameter) were subjected to 1N force. The storage modulus, loss modulus and phase angle were analyzed to study the rheological behavior of the scaffold. Analysis was done for both wet and dry state.

For drying cryogel samples were stored overnight in desiccators. For wet state analysis, dry sample was placed under pelletier and followed with the addition of water with the help of syringe. The rest of analyses were done same as in case of dry sample (Bhat *et al.,* 2011).

2.2.4 Chondrocytes isolation

Chondrocytes were isolated from 10-12 month old goat. Goat knee was obtained from local abattoir house. To remove external contamination the joint was sprayed with 70% ethanol. The knee joint was opened with the use of sterile blades and cartilage was exposed. Cartilage pieces were scraped gently and collected in a tube containing PBS with 2% antibiotic (penicillin, streptomycin, and gentamicin) and were then diced into small fragments by the sterile blade in petriplate and were further washed thrice in fresh PBS with 2% antibiotic solution. Further to digest the cartilage pieces were treated with trypsin-EDTA (0.25%) by keeping on a rotor mixer for 30-45 min at 37 ºC. After incubation tissue pieces were washed with PBS to remove any further trypsin left. Finally the digestion was carried by using collagenase (0.3% type 2 in plain DMEM) for about 16 h at 37 ° C. After incubation the cell suspension was transferred into a culture flask with DMEM, 10 % FBS, and 1% antibiotic and maintained at 5 % CO_2 and 37 ° C until they attain confluency, as shown in Figure 2.1 (Bhat *et al.*, 2011).

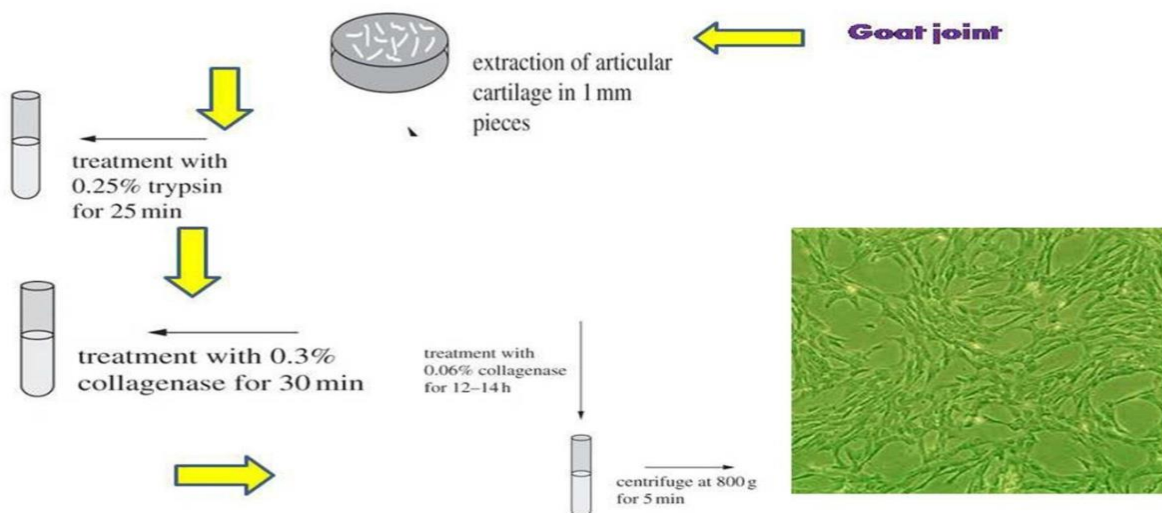

Figure 2.1) Diagrammatic representation of chondrocytes isolation (Bhat et al., 2011)

2.2.5 Seeding of primary goat chondrocytes

Scaffolds of dimensions 13 mm diameter and 2 mm thickness were treated with ethanol gradient (20% 40% 60% 80% 100% v/v) for about 15 min in each concentration followed by UV sterilization for half an hour. After that, they were washed in PBS for 15 min and then incubated in DMEM media overnight. When chondrocytes cultured in flask reached confluency they were further sub-cultured and expanded in tissue culture flask. Then chondrocytes from the second passage (P2) were detached from culture flask by the 0.25 % trypsin treatment, chondrocytes from second passage (P2) were used for seeding because if passage number is increased further it can alter chondrocyte phenotype. Then cells with P2 were seeded at a cell density of 1X10 ^{5} cells/ml on the scaffold. Seeded scaffolds were maintained at 37 ° C with 5% CO_2.

2.2.6 Determination of *in vitro* cell viability of chondrocytes (MTT)

In order to assess the synthesized cryogel scaffold for its viability or cytotoxicity towards the seeded chondrocytes, 4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT)

assay was performed. In this assay insoluble purple formazan is formed by oxidation of MTT solution by an enzyme present in mitochondria of cells. Scaffolds saturated with DMEM overnight were then seeded with cells at a density of $1x10^5$ cells/ml in 24 well plate (tissue culture treated). Then media was removed and 500 µl of 0.5 mg/ml MTT solution was added in to each well. As a control, chondrocytes were directly treated on 24 well plates (non-treated) with same density. Another parameter that was also kept was CG EX (where chondroitin sulfate was supplied at regular time period on chitosan-gelatin scaffold while changing media). After incubation, MTT was removed and 1.5 ml of DMSO was added and incubated for 15 min. The formation of purple formazan product was measured spectrophotometrically at 570 nm by using DMSO as blank. The assay was done for a period of six weeks (Singh *et al.,* 2013).

2.2.7 Microscopic analysis of scaffold seeded with chondrocytes

Chondrocytes cultivated on the scaffolds were analyzed by SEM on 7th, 14th, 21st, and 28th day. The cells on the scaffold were fixed using 2.5% glutaraldehyde for 6 h and washed in PBS to remove glutaraldehyde and scaffolds were washed with ethanol gradient 20 to 100 % for about 15 min each. Then samples were dried in desiccator maintaining aseptic conditions and mounted on aluminum stubs and gold coated for SEM analysis. Samples were then analyzed by SEM with constant voltage 20 kV and high pressure.

2.2.8 Glycosaminoglycan (GAG) Estimation

GAG estimation was done by using the dimethyl methylene blue (DMMB) method, in order to determine the GAG secreted by chondrocytes on scaffolds. Scaffold seeded with cells were washed with PBS and stored at -20ºC. Samples were dried by lyophilization and dry

weight was noted. Samples were then subjected to papain digestion overnight. The supernatant was collected and was mixed with DMMB and absorbance was measured at 540 nm with scaffold without cells as blank. The assay was done for every six weeks.

The GAG was calculated using the following formula.

GAG content (%) = [(chondroitin- 4- sulfate (µg ml $^{-1}$) x dilution factor x papain volume) / (dry weight of sample (g) x 10^6)] x 100 (Bhat *et al.,* 2013).

2.2.9 Collagen estimation

Hydroxyproline assay was used for the estimation of collagen production. The cell seeded scaffold were frozen at -20 ºC followed by lyophilization and its dry weight was measured and papain was used to digest the scaffolds. The supernatant (250 µl) from the digest was mixed with 250 µl concentrated HCL and kept in oven overnight at 120 ºC. Then contents were transferred into glass scintillation tubes and kept at 90 º C until brown residue formed at the bottom, these tubes were cooled to room temperature followed by addition of 1ml sodium phosphate buffer. Mixture was allowed to react with chloramine T solution at room temperature for 20 min followed by addition of 250µl of pDAB and incubated at 60 º C for 30 min. Samples were cooled and measured spectrophotometrically at 540 nm. Scaffold without cells treated in the same way was used as blank. To determine hydroxyproline concentration, which tells us about collagen content hydroxyproline standard curve was used. The total collagen content was determined by the following equation: (Bhat *et al.*, 2013).

Collagen content (%) = [(hydroxyproline (µg ml $^{-1}$) x dilution factor x proportion hydrolysed) / (dry weight of sample x 0.143x 106)] x 100. The experiments were carried out in triplicates and data are presented as the average of all samples and p value was calculated.

2.3 Results and Discussion

2.3.1 Synthesis of CGC Scaffold

Different polymers i.e. chitosan, gelatin and chondroitin sulfate were mixed in different combinations and crosslinked using glutaraldehyde. Various combinations mentioned in materials and methods, 2.2.1 were tried. Out of those combinations CGC 3%, CGC 3.01%, CGC 3.05%, CGC 4% , CGC 4.01 , CGC 4.05, CGC 4.1, CGC 4.2, CGC 4.3 %, CGC 4.4 could not be used for further studies because optimum shaped cryogel scaffold could not be obtained, scaffold formed were shrunken cracks were found on the surface, moreover they were not able to regain its shape. Optical images of few combinations (CGC 3 %, CGC 3.05) CGC 4.1%, CGC 4.01%, (A,B,C,D) respectively, shown in Figure 2.2 (1). The scaffolds which were able to retain its proper cryogel shape were CGC 4.5% and CGC 5%, as shown in Figure 2.2(2)). The most important prerequisite to be considered in case of cartilage tissue engineering is mechanical strength to engineer healthy cartilage, the construction must have high mechanical properties to bear the load. It also helps in retaining chondrocytes phenotype. So both CGC 4.5 % and CGC 5 % were characterized on the basis of mechanical test. Among them CGC 5% showed increase in mechanical strength (50 kPa at 20-40% deformation) as compared to CGC 4.5 % , Figure 2.3 (3). Moreover the flow rate of both the scaffold was also estimated using peristaltic pump , flow rate determines porous nature of the scaffold what we observed that CGC 5 % showed more flow rate 10 ml min^{-1} at 2 bar pressure as compared to CGC 4.5 % which was 7 ml min^{-1}. So because CGC 5 % showed better scaffold properties for cartilage tissue engineering it was selected for further use. CGC scaffold was synthesized by cryogelation technology at -12 ºC. Scaffolds play a very

pivotal role in tissue engineering by mimicking role of the native tissue, in this research work scaffolds was designed so to mimic natural cartilage. This applicability of these scaffolds widens as polymer selected for the fabrication of cryogel was done on the basis of the native composition of the cartilage. Polymers like chitosan, gelatin and chondroitin sulfate were selected due to similarity with cartilage native tissue. Also this tri-polymer is first novel scaffold synthesized by us, using cryogelation technology. Thus increases the impact of this work. Natural polymer like chitosan was chosen as it is non-toxic, biodegradable and has similarities with GAG which is a component of cartilage ECM (Wissemann and Jacobson, 1985). Gelatin is denatured form of collagen, it helps in attachment and proliferation of chondrocytes due to the presence of Arg-Gly-Asp informative signals (Austin, 2007). The most important factor to be considered in the case of cartilage tissue engineering is mechanical properties, to engineer healthy cartilage; the construct must have mechanical properties matching those of native cartilage so that scaffold should be able to bear the load. In this work the incorporation of chondroitin sulfate was done to increase the mechanical strength of the scaffold. Moreover, CS reduces the degradation of GAG and collagen that are an important component of cartilage ECM (Sechriest, 2000). Glutaraldehyde is the most common and convenient crosslinking agent, less expensive and with faster reaction time; it suppresses the immunogenicity of the implant (Kirk *et al.,* 2013). Glutaraldehyde enhances the mechanical stability of the scaffold, but is considered toxic to the cell. We overcame this limitation of the chemical by using an optimized concentration which doesn’t cause toxicity. Here we synthesized the scaffold using cryogelation technology, which helped the chondrocytes to grow and proliferate well by allowing the flow of nutrients and exchange of gasses due to well interconnected pores.

The crosslink's possible from this combination are

1) NH_2^+(Chitosan) + OSO3(-)(chondroitin sulfate) can form polyelectrolyte complex
2) Chitosan - (NH_2^+) + (CHO-) -Glutraldehyde -(CHO) + NH_2^+(Chitosan)
3) Gelatin – (NH_2^+) + (CHO-) -Glutraldehyde -(CHO) + NH_2^+(Chitosan)
4) Gelatin (COO-) + NH_2^+ (Chitosan)
5) Gelatin (COO-) + Gelatin (NH_2^+)
6) NH_2^+ (Chitosan) + COO^-(Chondroitin sulfate) form polyelectrolyte complex.

Apart from the above mentioned links various other cross linkages are also possible.

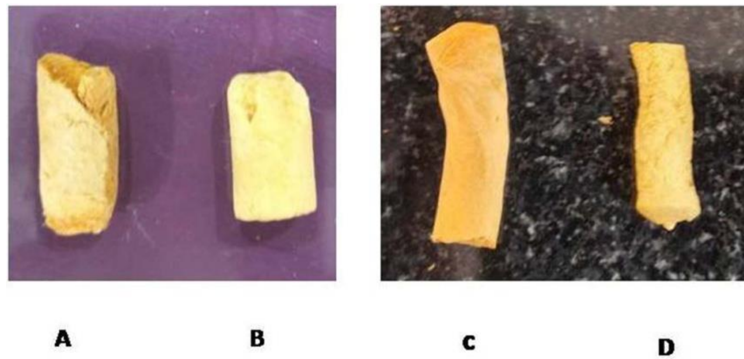

Figure 2.2(1) Optical image of CGC(chitosan-gelatin-chondroitin sulfate) scaffold for optimization A) CGC 3 % B) CGC 3.05% C) 4.1 % D) 4.01

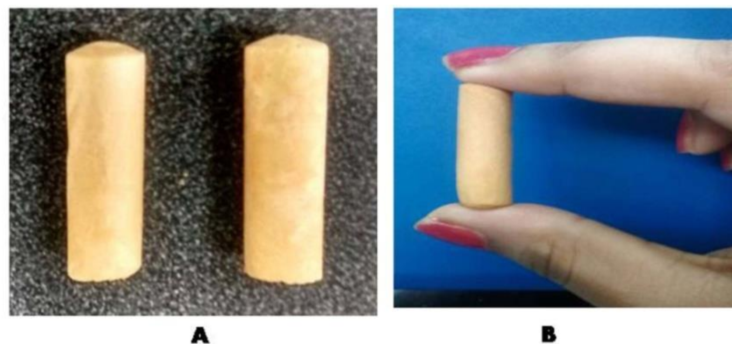

Figure 2.2(2) Optical image of CGC (chitosan-gelatin-chondroitin sulfate), scaffold synthesized A) CGC 4.5% B) CGC 5%

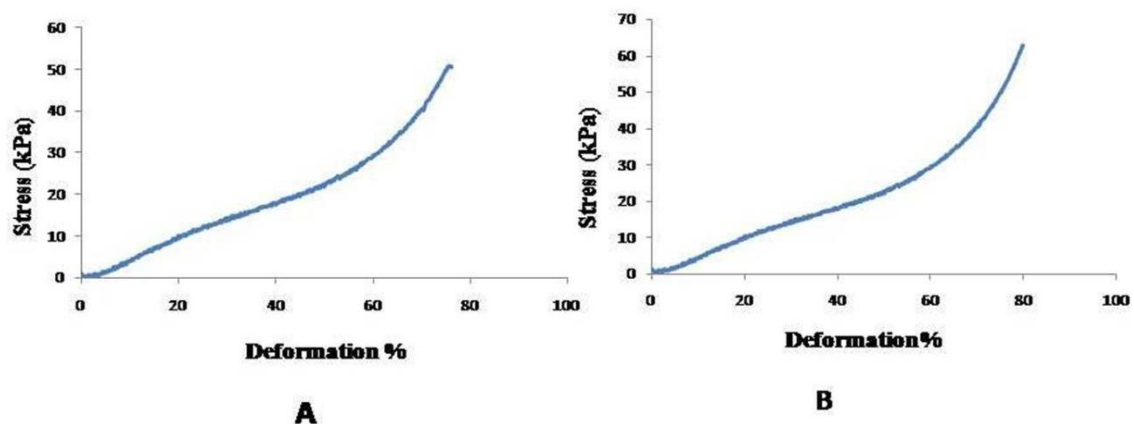

Figure 2.2(3) Stress-strain curve of CGC (chitosan-gelatin-chondroitin sulfate) 4.5 (A), CGC 5 % (B)

2.3.2 Physical characterization of chitosan-gelatin-chondroitin Sulfate

Determination of flow characteristics

2.3.2.1 Flow rate

Flow rate is defined by amount of solvent passing through matrix in unit time and is measured by allowing the liquid to travel through the matrix in unit time with no back pressure. This study of the material depicts the porous nature of the matrix. In cartilage tissue area usually the scaffold seeded with chondrocytes are subjected to repeat mechanical loading in a mechanical bioreactor, which increases the secretion of extracellular matrix following an increase in mechanical properties of neo-tissue. So for the above condition to be sustained scaffold should have well interconnected porous network which will help in nutrient exchange and waste exchange. This will further prevent the cell death when seeded at high density. Moreover, it is important to know about the flow rate of the scaffold. The

flow rate of the scaffold was determined to be 10 ml min^{-1} at 2 bar pressure. Our scaffold shows formation of well interconnected pores which may help the chondrocytes to proliferate with unhindered growth. These well interconnected pores facilitate the exchange of nutrients and gases which in turn increase the chondrocytes proliferation. Well interconnected pores are formed due to cryogelation technique. Also its known that pore size influences cell and tissue interaction, more cell proliferation occurs if the pore size is large with bigger pore size, the cells can migrate easily and help in exchange of nutrients and waste material (Jain *et al.,* 2009; Oh, *et al.,* 2007)

2.3.2.2 Swelling kinetics of CGC blend

In order to determine the solvent or water uptake capacity of CGC scaffold its swelling kinetics was studied. The gravimetric method was performed for this experiment. In order to study the water retention capacity of scaffold synthesized, its swelling kinetics was studied. It is the rate at which gel swells in liquid. This analysis is an important parameter as native cartilage is in aqueous environment so it is important to study how the scaffold behaves in water. All *in vitro* studies are done in aqueous environment. Factors like monomer concentration, cross linking density and temperature etc. may affect swelling behavior of scaffold. The swelling ratio was also calculated, and the scaffold showed good swelling ratio of 9.35 with good pores and high surface area and thus cells can grow very well and attach 3D behavior.

Scaffold swelled up to 90 percent within 1 min 20 sec which depicts high water uptake capacity as shown in Figure 2.3. This high water uptake capacity may be due to large interconnected pores of scaffold. Swelling and de-swelling were carried out up to five cycles with the same sample. As kinetics obtained from the graph showed almost a constant

behavior from first cycle to fifth cycle which depicts the stability of scaffold with repeatedly being subjected to swelling or de swelling.

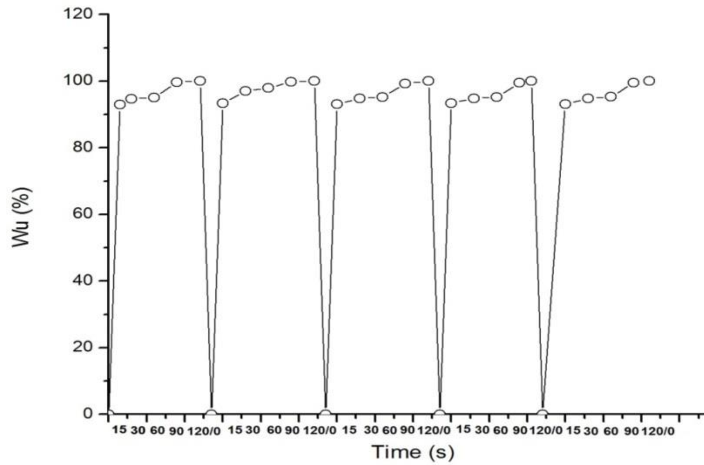

Figure 2.3) cyclic swelling and deswelling behavior of CGC (chitosan-gelatin-chondroitin sulfate) 5% scaffold

2.3.2.3 SEM analysis of CGC scaffold

Morphology of chitosan-gelatin-chondroitin sulfate was determined by using different optical tools like scanning electron microscopy (SEM). SEM showed the pore size of the newly synthesized scaffold was in range of 40-135 µm, as shown in Figure 2.4. The macroporous structure was generated within scaffold by uniformly arranged interconnected pores formed during cryogelation. The presence of large and interconnected pores would be an added advantage for chondrocyte to migrate as chondrocyte dimension are 10 times lower than pore diameter in this case. Pore size of cryogel matrices can be modified by varying concentration of polymers or monomers or by altering the temperature of synthesis. In this case all factors were optimized to obtain larger pores. The presence of large pores also modulates the efficient gaseous and nutrient exchange which can further help cells to grow and proliferate well on the scaffold.

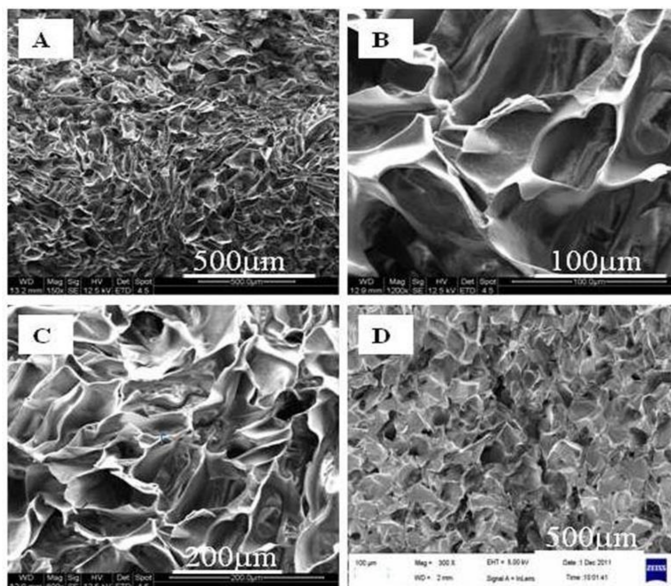

Figure 2.4) Scanning electron microscopy (SEM) images showing microstructure of chitosan-gelatin-chondroitin sulfate(a)150Xmagnification(b)1200Xmagnification(c)600X magnification (d)300X magnification

2.3.2.4 Degradation rate of scaffold

The aim of tissue engineering is the regeneration of tissue with synchronized degradation of matrix used for the cells at a rate which can match the development of neo-tissue. The implanted cells face the variation in the boundary during surface erosion and new surface exposure which may alter the cellular process.

Degradation of the scaffold is one of its desirable properties. The degradation of CGC scaffold was examined under aseptic mode to examine degradation pattern. The degradation rate of the scaffold in 0.1 M PBS was 13-15% till the end of interval (8 weeks). This rate of degradation shows that the scaffold will start to degrade at a time point which is in agreement

with the formation of a neo-tissue (Bhat *et al.,* 2013). This increases the application of CGC scaffold for *in vivo* usage as CGC scaffold degrades at regulated pace and there will be no need to remove scaffold by another surgery. This slow rate of degradation can be attributed to higher crosslinking in CGC scaffold, which is ideal in case of cartilage repair as it is slow. Also previous studies have suggested that gelatin degrades quickly but if crosslinked with chitosan the rate of degradation decreases (Zhuang *et al.,* 2007).

Furthermore to study degradation rate SEM analysis was also done which showed cracks on the walls of the gel showing the biodegradable property of CGC scaffold, as shown in Figure 2.5. Additionally as scaffold is composed of natural polymers, so products will not impose any immune rejection issues.

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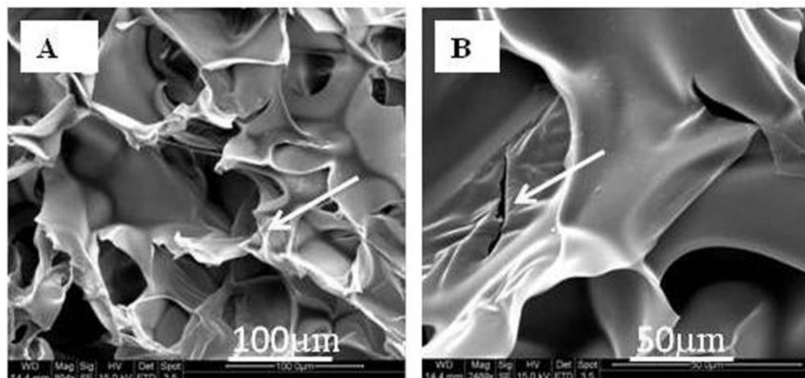


Figure 2.5) Degraded scaffold showing the formation of cracks on the wall of cryogel (A and B) (pointed by white arrow)

2.3.2.5 Fourier transform infrared spectroscopy (FTIR)

A peak around 3300-3500 cm^{-1} confirms the presence of chitosan owing to the NH group stretching in the polysaccharide and peak around 3388.22 cm^{-1} (C) confirms the presence of chitosan in the scaffold, as shown in Figure 2.6.

Glycosidic linkage in the polymer shown by peak around 1069.51 cm^{-1} (B) (Bhat *et al.*, 2011) or chondroitin sulfate the peculiar peak at 1253 cm^{-1}, corresponds to stretching vibration of s=o bond which is characteristics peak of chondroitin sulfate so this turns weaker and shifts to 1243 cm^{-1} (A) which indicate crosslinking of sulfate group of chondroitin with chitosan (Amrutkar and Gattani, 2009).

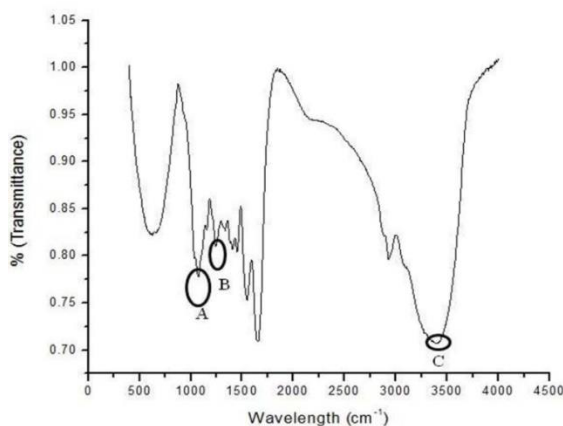


Figure 2.6) FTIR (Fourier transform infrared spectroscopy) spectra of CGC(chitosan-gelatin-chondroitin sulfate) scaffold showing the presence of various peaks which pertains to the corresponding bonds (A, B, C)

2.3.3 Mechanical characterization of CGC Blend

2.3.3.1 Determination of compression stability and elasticity

Mechanical strength is a significant requirement for cartilage repair strategies. The mechanical strength of scaffold is desired so as to ensure that scaffold synthesized can withstand load when it is implanted in the joint as tissue replacement. So mechanical stability is a critical parameter in designing a scaffold for cartilage tissue engineering, since cartilage is main load bearing tissue experiencing a varying degree of mechanical stimulus so that mechanical stability should be at par with native cartilage. By alteration in porosity the

mechanical strength of scaffold can be changed, the successful scaffold should posse balanced mechanical properties with defined porosity, providing a chronological transition in which regenerated tissue assumes function as the scaffold degrades. Mechanical load bearing capacity is not only important for joint movement but high mechanical strength helps chondrocytes to attach on the scaffold and maintain its phenotype. Mechanical stimuli can induce modulation in cartilage matrix either in catabolic or anabolic manner; also forces applied on the construct can transduce chondrocyte microenvironment and regulate gene expression (Callahan *et al.,* 2013). The main reason for selecting chondroitin sulfate as one the component of scaffold was to provide mechanical strength, due to its highly charged sulfate group which induces electrostatic repulsion that provides resistance to compression. Thereby providing high mechanical strength (Stuart and Panitch, 2008). The known matrices with high compressive stiffness helps chondrocytes to proliferate and synthesize more ECM together with phenotype retention (Bryant and Anseth 2002). The CGC scaffold showed good elastic modulus of 50.0 kPa at 20-40% deformation, calculated from the slope of the graph plotted with stress against strain, as shown in Figure 2.7. Scaffold showed elastic behavior till around 60-70% compression of the gel length. CGC scaffold showed no signs of deformation by compression test so showing the applicability of these scaffolds that this scaffold can offer sustainable mechanical strength, which can be utilized as a temporary porous design for the cultivation of chondrocytes in three dimensional environment. So from this study we concluded that high mechanical strength indicates that these matrices are appropriate for *in vivo* cartilage tissue engineering applications.

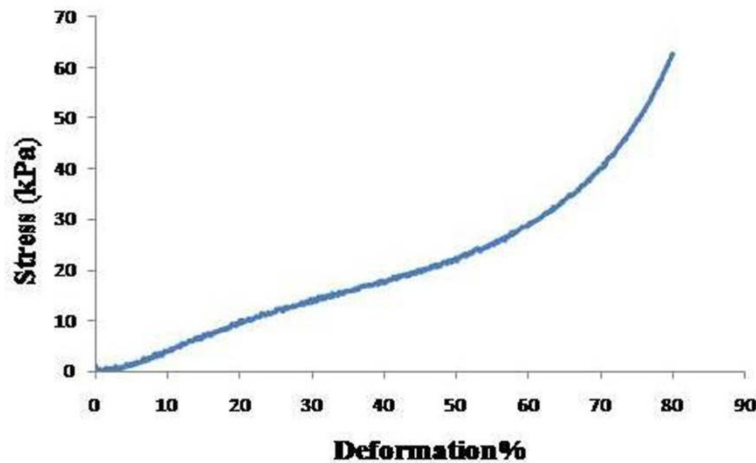
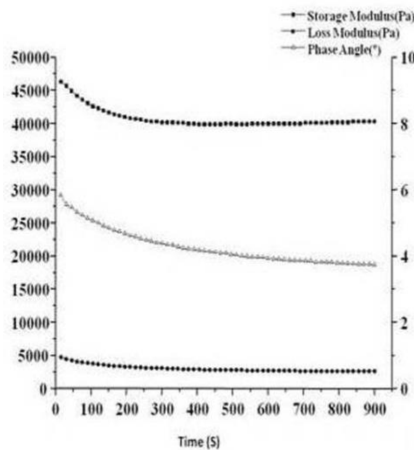


Figure 2.7) Unconfined compression (stress–strain) curve of chitosan–gelatin-chondroitin sulfate cryogel

2.3.3.2 Rheological analyses

The study of flow and deformation of material is defined as Rheology. When any material is subjected to a stress of different sorts (force per area), materials respond in different ways. Either material can show the viscosity or elasticity or there is the third property of material termed as the visco-elastic behavior of material (Engler *et al.*, 2006). Depending on the time required to process the polymer, if polymers are coated, pumped, or sprayed the behavior shown by short processing time material or solid by long processing time (Friedrich *et al.*, 1995). This dual nature (fluid-solid) in rheology term is referred as the viscoelastic behavior of materials. It is used basically in studying multifaceted structure of substances such as body fluid, suspensions, polymers, etc. The flow of these materials cannot be characterized by a single value of viscosity (at a fixed temperature). It is a relationship between the flow/deformation, the performance of material and its internal structure that cannot be defined by classic fluid mechanics or elasticity. Storage modulus G' and loss modulus G"defines the elastic behavior and viscous behavior of polymer (Singh *et al.*, 2010). All these

parameters can be used as excellent tools in material characterization especially for mechanical properties. In the dry state, when material was subjected to shear- stress, the storage modulus of CGC was approximately 46,000 Pa as shown in Figure 2.8 A. On application of external force by varying the phase angle from 4º-6º, the storage modulus of the polymeric material decreased to approximately 40000 Pa and then maintained constant throughout the run. This shows the elastic nature of CGC scaffold and can resist to external forces. Then when CGC scaffold was subjected to such forces in the wet state at 37º C the visco-elastic behavior showed a decrease, as shown in Figure 2.8 B. There was a decrease in storage and loss modulus to 30000 and 2500 Pa respectively and phase and variation in phase angle between 2.5º to 4º. Both the storage and loss modulus decreased approximately to 30000 and 2500 Pa, respectively. This shows that while studying the viscous behavior of CGC scaffold it has less viscous behavior. From the above rheological studies it can be concluded that CGC scaffold has good elastic behavior and therefore has the possible potential to be used in cartilage tissue engineering, as elasticity is an inherent property of native cartilage.



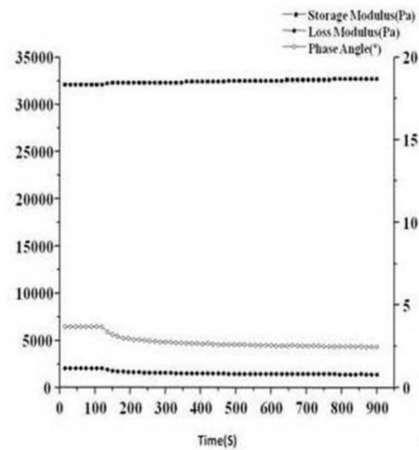


Figure 2.8) Rheological analysis of CGC(Chitosan-gelatin-chondroitin sulfate) scaffold in dry (A) wet (B) with all three parameters storage modulus, loss modulus, phase angle

In vitro studies

2.3.4 To determine the chondrocyte proliferation by MTT assay

Goat cartilage was used as the cell source because it is one of the most frequently used animal models for cartilage studies due to factors such as reasonable thickness of cartilage, resemblance of goat knee anatomy to human knee, ease of use, availability, and cost. Moreover, to examine the suitability of any model system, use of high number of healthy chondrocytes is a better approach, whereas the cell number is limited in case of human systems (Miot *et al.,* 2006). The viability and proliferation of chondrocytes in CGC blend were determined using MTT, a tetrazole (MTT reagent). In this method the viable cells convert tetrazolium salts to an insoluble product, purple formazan which are further solubilized in solvents such as dimethyl sulfoxide (DMSO) and can be quantified by checking optical density by spectrophotometric analysis. An increase in optical density indicates cell adhesion and proliferation in the scaffolds. 2D (where chondrocytes were grown on tissue culture plate) and CG scaffold (chitosan-gelatin) were used as controls. CG EX was another parameter used where chondroitin sulfate was supplied on chitosan- gelatin scaffold externally.

Further when the 2D system was compared with all the scaffolds (3D system), it was observed that CGC blend which we synthesized and CG, CG EX other parameters showed increase in rate of cellular proliferation which means there was no inhibition of growth or cytotoxicity on scaffolds. In 2D there was increased rate of cell proliferation for 21 days after

that it starts dropping down, this may be due the fact that chondrocytes reached confluency in 2D monolayer faster as compared to 3D. Initially on 3D scaffolds chondrocytes need time for proper recognition of biomaterial and for adhesion. After 21 days the cell number in 2D showed a decrease which can be due to toxins built up, as in monolayer there is less surface area available. But in 3D system the proliferation continued for longer time duration, as in 3D system the cells are allowed to grow or interact with their surrounding in all the three dimensions, which is advance method of growing cells as compared to 2D. The 3D system provides more contact space for the cell to adhere and provide space for mechanical inputs, which further required for cell contraction, integrins ligation and even intracellular signaling, as shown in Figure 2.9. When we compare proliferation in CG with that CGC 5%, it was observed that chondrocyte proliferation in CGC 5% is comparable with that of control i.e. CG. It shows that CGC 5% scaffold doesn't elicit any kind of toxic effects on the cells. Moreover the lack of enhancement of chondrocyte proliferation in CGC 5% may be due to inaccessibility of free CS, but there is a considerable increase in proliferation in CG EX. This may be due to free CS supplied exogenously at regular time period. Also it holds because it has been known that chondroitin sulfate is the major GAG present in ECM of the cartilage tissue which supports chondrogenesis (Yamada and Sugahara, 2008). In a study by Ruffell *et al*. it has been shown that chondroitin sulfate helps in cell-cell interaction, mediating cell-cell signaling and recognition through cell surface receptors.

So from the above study we conclude that if CS is available in free form, it can help cells to proliferate better. But CG EX cannot be used as a system for cartilage tissue engineering as CS has to be here provided repeatedly like booster dosage, which can be costly and

inconvenient for long term strategy (Roth and Anderson, 2011). So we thought of system or process which can trap CS in a controlled manner.

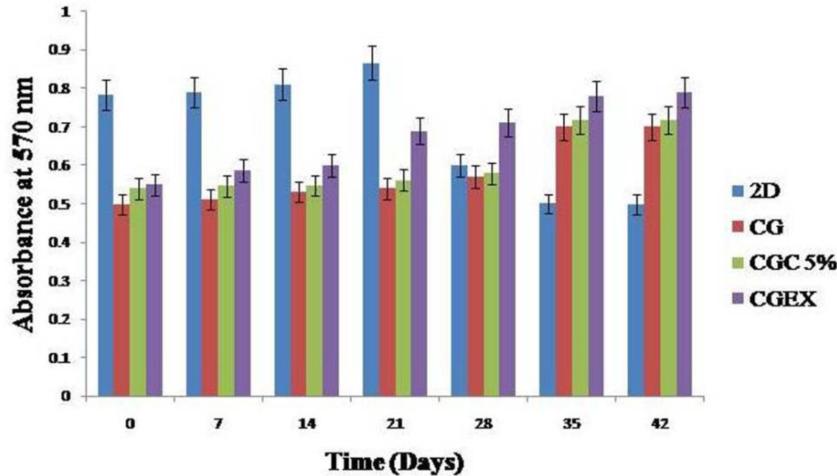


Figure 2.9) In vitro* cell viability *of CG(chitosan-gelatin),CGC(chitosan-gelatin-chondroitin sulfate 5%), 2D , CG EX by MTT assay, cell culture experiment was run for 42 days. The experiment was done in triplicates and p<0.05

2.3.5 Scanning electron microscopy of chondrocytes cultured on scaffold (CGC blend)

When scanning electron microscopy was done of chondrocytes seeded CGC scaffold with CG as control and CG EX as another parameter, we could see the chondrocytes were able to attach on scaffold and maintained round morphology after 1st week of culture, as shown in Figure 2.10. This attachment also signals that chondrocytes were able to identify the scaffold surface as native. This may be attributed to the presence of gelatin which has informative signals which help chondrocytes to attach on CGC and also chondroitin sulfate which helps in phenotype maintenance (Enrione *et al.,* 2013). This suggests that macroporous chitosan-gelatin-chondroitin sulfate cryogels provide the conditions that support cartilage tissue

formation by affecting the cell adhesion and providing the native conditions which helps to sustain chondrocyte phenotype.

The above results also support our MTT observation that scaffolds are supporting chondrocytes attachment and proliferation.

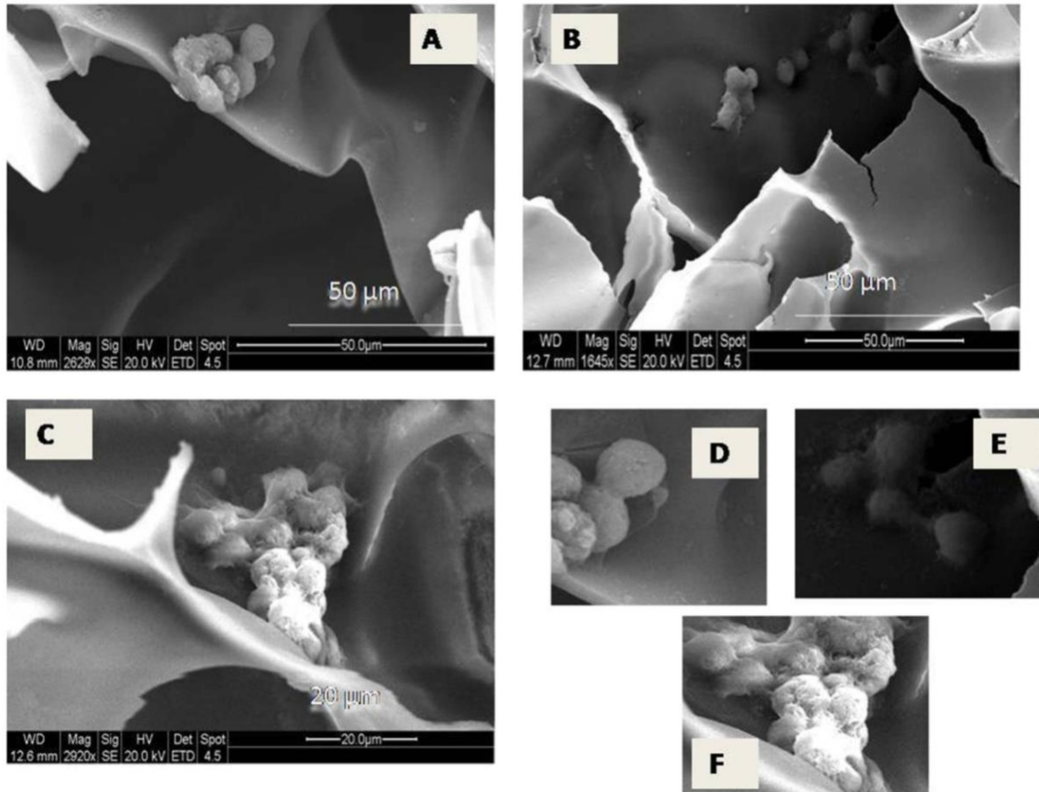


Figure 2.10) SEM images of chondrocytes after 1st week in culture on A) CG, Chitosan gelatin (2629X magnification) B) CGC, chitosan, gelatin, chondroitin sulfate 5% (1645X magnification) C) CGEX (2920X magnification). D, E, F (magnified image) respectively. Showing the attachment of Chondrocytes on all different scaffolds with its maintained phenotype

2.3.6 Glycosaminoglycan analysis on chondrocyte seeded on the scaffold

One of the important components of cartilage matrix is GAG and is the measure of cartilaginous tissue formation (Wang *et al.,* 2013). GAG is estimated by DMMB (Dimethyl

methylene blue) method. We observed increased amount of GAG in CG EX which could be due to continuous supply of CS at regular time interval exogenously. But the major disadvantage of this mode would be continuous supply of CS which will add to the cost, Moreover booster dosage results in many risks and limitations such as reduced drug stability and solubility (Garver *et al.,* 2015). In another observation we noted that as compared to CG (control) the GAG % was comparable with CGC scaffold synthesized, as shown in Figure 2.11. This may be attributed to non-accessibility of CS in CGC to effect that GAG production which has led to a conclusion that our synthesized scaffold needs to be improved. The delivery mode was synthesized using microparticle as a delivery vehicle which would be dealt in next chapter.

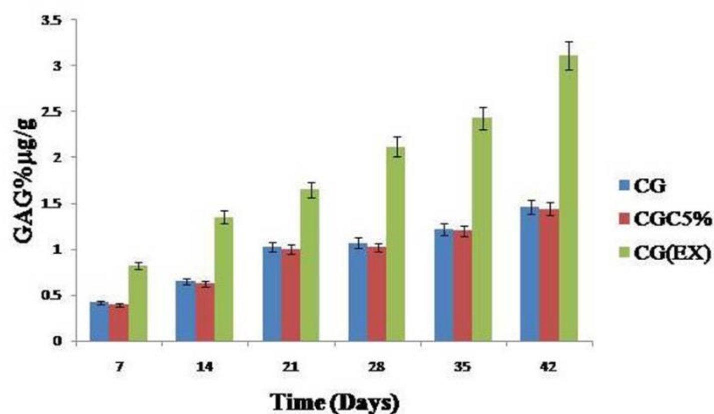


Figure 2.11) GAG (glycosaminoglycan) analysis using DMMB(dimethyl methylene blue) method for 42 days. Sample was done in triplicate and p<0.05

2.3.7 Collagen estimation

Collagen content was estimated using hydroxyproline assay using, hydroxy proline standard which is a post translational product of proline hydroxylation catalyzed by the enzyme prolyl hydroxylase. As collagen is composed of 14.3% hydroxyproline, the overall collagen amount

of the samples can be calculated using hydroxyproline assay (Anandacoomarasamy and March, 2010). There was more collagen production observed in CG EX as compared to CGC and CG, this may be due to CS supplied exogenously for regular time period. As already it is known that CS enhances TIMP-1 production which inhibits MMPS (matrix metalloproteinase) which further degrade type 2 collagen thus helping in more collagen production.

Also it was observed that there was gradual increase in collagen content over time in CGC 5 % but when compared to control (CG) production was comparable this can be again due to non availability of free CS in CGC 5 % as shown in Figure 2.12. The above observation guided us to think further improving CGC scaffold and to see an alternate method which delivers the CS in continuous rate for long time without requirement of booster dosage.

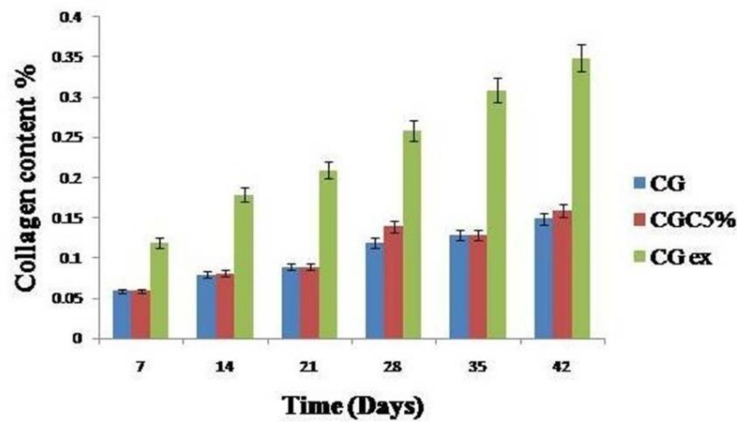


Figure 2.12) Collagen estimation by hydroxyproline assay for 42 days, samples done in triplicates and $p<0.05$

2.4 Conclusion

In conclusion, CGC scaffold was synthesized by cryogelation method. The synthesized cryogel has large and interconnected pores as observed by SEM and flow rate, respectively. The most important parameter which is vital in cartilage tissue engineering is mechanical strength, and the results confirmed that synthesized scaffold showed optimum mechanical strength, thus could be used for implantation and load bearing studies. Rheology supported the elastic nature of the scaffold. *In vitro* studies did not show any cytotoxic effects on the seeded chondrocytes. However, there was not much proliferation observed in CGC as compared to the samples where CS was supplied exogenously. This may be attributed to non-availability of chondroitin sulfate to support proliferation. Similar results were also obtained in GAG and collagen assays. When observed in CG EX there was a high increase in biochemical activities (GAG and Collagen), which can be attributed to the regular exogenous supply of CS at the regular time. However, for actual *in vivo* application in future, recurrent CS supply exogenously in the form of booster dosage will be costly and thus disadvantageous. However this system gave us insight for our next step.

Chapter 3

Comparative Study of Various Delivery Methods for Supply of Chondroitin Sulfate for Application in Cartilage Tissue Engineering

Chapter 3

Comparative study of Various Delivery Methods for Supply of Chondroitin Sulfate for Application in Cartilage Tissue Engineering

3.1 Introduction

The major cause of chronic disability among older people is osteoarthritis which triggers when there is loss of homeostasis in protection of articular cartilage (Anandacoomarasam and March, 2010). There are surgical treatments including arthroscopy, osteotomy and knee arthroplasty available. For patients with severe OA, total knee arthroplasty can be safe and cost effective treatment (Lützner *et al.,* 2009). There are some initial treatments like use of non-steroidal anti-inflammatory drugs (NSAIDS) analgesics and intra-articular corticosteroids and other drugs for short term pain relief (Richmond *et al.*, 2009). Current strategies of regenerative medicine are focused on injection of cells in combination with scaffolds and biomolecules. In recent years, considerable interest has been given to bioactive molecules that can be key products in reducing pain, inflammation and in some way repairing cartilage (Ingber *et al.,* 2006). Thus, tissue engineering field not only requires the scaffold matrices to fill the tissue void or provide structural support to cells that have the ability to form tissues within the body but also provide one of the other major component which is bioactive molecules (Gothard *et al.,* 2009). Research in the area of scaffold fabrication has lead to fabrication of newer types of scaffolds that have consequently revolutionized the field of tissue engineering and scaffolds. Now scaffolds have advanced from first generation in 1960’s and 1970’s which dealt with mimicking physical properties of replaced tissue to second generation in 1980’s and 1990’s that were biodegradable, to the

current third generation biomaterial focused with incorporation of bioactive molecule into it by different modes, so that scaffold could mimic ECM to its closest (Kuo *et al.,* 2006). So here scaffolds may not just interact with cellular components but will also include biological cues or bioactive factors which overall will help in cell growth and matrix enhancement thus speeding up the process of cartilage repair (Chan and Leong, 2008). In the past several decades the field of drug delivery has made tremendous growth. Traditional pharmaceutical formulations such as tablets, capsules, injections have become incorporated in our daily health care system. In the treatment of health related dysfunctions, it is desirable that drug reaches the site of action with desired concentration and dosage required for treatment of disease. Another concern is that it should remain constant over a sufficiently long time period but the process of pharmaceutical drugs is restrained because of factors such as degradation, interaction with other cells and incapacity of them to penetrate tissue (Vilar *et al.,* 2012). Drug delivery is a process or method of administering a pharmaceutical compound to attain a therapeutic effect in humans or animals. The routes like nasal, pulmonary are gaining significance (Janssen *et al.*, 2014). But there are many hurdles in drug delivery system design like, for OA the most common mode to deliver the drug is via intra-articular drug delivery, but the field of polymers for intra-articular delivery is very fragmented, also many factors have to be considered for delivering drug with use of these vehicles, like particle size which may affect different drug delivery system kinetics, biocompatibility of drug and drug delivery system (DDS) is another factor in joint. The polymer to be selected for DDS should be biocompatible and biodegradable so that residues don't cause damage. There are many drug delivery vehicles present which include liposomes, proliposomes, microspheres, gels, pro drugs, cyclodextrins and others (Chan and Leong

2008). Microparticles are one of the types of DDS where the particle size ranges from one micron to few mm (Pavan Kumar *et al.,* 2011). The main advantage of the DDS is that microencapsulation technology allows protection of drug from the environment, increases bioavailability of the drug enables targeting the drug to a specific site, reducing dosage frequency and toxicity of various drugs, reduces local side effects, can be stored in dry state or suspension, as shown in Figure 3.1. Hence, they play an important role as drug delivery systems, aimed to improve bioavailability of conventional drugs (Madhav and Kala, 2011). Microparticles have already been used as drug delivery vehicle for many diseases like cancer where the delivery of drug at site of action is requisite (Davoodi *et al.,* 2015), ocular treatment (Choy *et al.,* 2008), heart disease (Sy and Davis, 2010), Parkinson's treatment (Di Stefano *et al.,* 2009). Many modes have been already used for OA such as previous reports of targeting diclofenac sodium in joint using gelatin magnetic microspheres to reduce inflammation (Saravanan *et al.,* 2011), methotrexate entrapped in liposome when injected via intra articular could suppress the development of arthritis (Canto *et al.,* 1999; Peppas *et al.,* 2000).

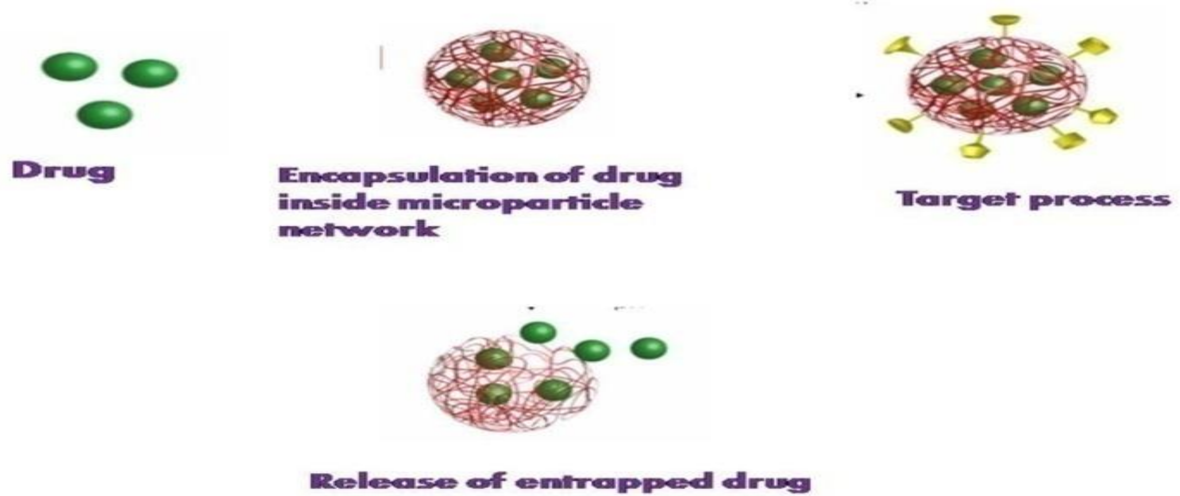


Figure 3.1) Microencapsulation technology allows active products to be protected, delivered and chemically programmed to release where and when required

Not much of work has been done using microparticle as drug delivery vehicle for osteoarthritis, but it can be used as a tool for OA as microparticles have already shown promising effect in case of other diseases so using them as a vehicle for this disease can open new insight. The limitation for intra-articular drug delivery is that it often requires frequent injections that can be costly, impact on patients, rapid degradation and clearance of injected agent and risk of other complication and infections. So the use of microparticle as sustained drug delivery could be a possible promising treatment for the disease. So the motivation behind this research work was to use the approach of microparticle for OA (Zhang and Huang, 2011). Different type of materials have been used for microparticle such as synthetic polymer like acrolein, epoxy polymers or natural polymers like protein, gelatin, carbohydrates, agarose, chitosan, starch, gelatin (Ramteke *et al.,* 2012). For DDS it is better to use the biodegradable polymer as a material for synthesis so that microparticles are biocompatible to the system and no residues are left which can cause some immune response (Kong *et al.,* 2011). There are different method for its synthesis like spray drying, solvent evaporation, complex coacervation, single emulsion technique, double emulsion technique (Sahil *et al.,* 2011). The mechanism of drug release occurs as a consequence of degradation

and erosion of polymeric device. So the understanding of polymer nature is essential to understand the mechanism of release. When polymer degrades the drug diffuses through channels generated by breaking of polymer chains without loss of volume in the particle (Vilos and Velasquez, 2012). Some properties need to be considerate of size, surface chemistry, and drug-polymer ratio, charge when trying to encapsulate drug and polymer together. So the use of microparticles to provide for localized and sustained delivery of a drug which has shown promising results in treating OA, following intra-articular injection may be an attractive strategy for treatment of arthritis (Mwangi *et al.,* 2015).

But in recent years one of the other field that is microsphere based tissue engineering scaffolds have attained significant attraction, where microspheres are embedded inside scaffold. So microspheres scaffolds will not only serve us stiffness gradients but the supply of drug loaded microparticles that can serve as trigger factor for drug sustained release. Already microsphere scaffolds are used in advanced tissue engineering application such as gene therapy (Stephens *et al.,* 2000). Microspheres scaffolds are generally a polymer matrix used for drug encapsulation for drug release at a relatively slower rate and for sustained long time period. This system would be definitely advantageous in case of OA as cartilage repair also takes place at slow rate (Dhandayuthapani *et al.,* 2011).

Many of the drugs used for treatment are not available for longer duration and causes toxicity in high doses. To overcome this microparticles enacapsulated with drugs and also with scaffolds can be used for protected and controlled delivery of drug for longer duration using drug incorporated microparticle scaffolds which would be able to mimic ECM to closet. The main focus of this work was the synthesis of efficient delivery modes for cartilage tissue engineering. To accomplish this objective chondroitin sulfate was delivered to chondrocytes

by different modes like incorporation in scaffold during synthesis, incorporated in microparticles for sustained release and further the drug loaded spheres were incorporated in a scaffold for a slower release of the drug so that it can be accessible to chondrocytes for longer period. Thus this study will give newer insights into the efficient and novel delivery modes for CS which has already shown many benefits in treating OA. So here different modes were synthesized and analyzed by SEM and *in vitro* release study was done and further primary goat chondrocytes were used for *in vitro* testing of all modes by MTT, SEM, GAG and Collagen assays.

3.2 Materials and methods

Materials

Gelatin (cold water fish skin), Chondroitin sulfate, Dulbecco modified Eagles medium (DMEM), Collagenase type2, penicillin-streptomycin, nystatin, papain, trypsin-EDTA solution, 1, 9 dimethyl methylene blue, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma Chemical Co (St. louis, MO, USA). Glutaraldehyde was obtained from S.D. Fine Chemicals Ltd (Mumbai, India). Fetal bovine serum was purchased from Hyclone (UT, USA). Light liquid paraffin and TritionX-100 were obtained from Merck (Mumbai, India). All the chemicals used were of analytical grade and were used without any further purification.

3.2.1 Synthesis of gelatin spheres with incorporation of chondroitin sulfate into it during synthesis

In order to encapsulate the chondroitin sulfate gelatin microspheres were synthesized. For the synthesis of gelatin microspheres emulsion-polymerization technique was used with some modification (Wissemann and Jacobson, 1985). The size of microspheres is important

parameter as it affects the release of the drug. There are many factors that influence particle size of microspheres like gelatin concentrations, stirring rate of the system, the volume ratio of aqueous and oil phases, crosslinker concentration and cross linking time (Dinarvand *et al.,* 2005). Release pattern of the drug can be modified via changes in drug particle size, drug load or chemical modifications that affect the solubility, as altering the size of the drug may alter the diffusion and erosion phase (De Young *et al.,* 2011).

Different parameters were standardized in order to form the gelatin spheres or to encapsulate chondroitin sulfate, like gelatin concentration (5gm,10gm,15gm) for the formation of spheres, glutaraldehyde crosslinking concentration was also optimized 2%, 4%, 6% , 8 % , Triton X-100 was optimized with 0.2, 0.4. 0.5 , 1 (%) and at last stirring speed from 50, 100, 200, 400(rpm). All these were optimized to obtain spheres which don't stick to each other and maintain proper shape.

Finally, the standardized protocol was used for encapsulation of the drug inside gelatin spheres, as mentioned below.

Spheres were prepared by dissolving 15 gm gelatin (15%) in 100ml of 20 mM sodium phosphate buffer, pH (5.5). Emulsion system was prepared by dissolving 1.8ml of 1% of triton X-100 into 100 ml paraffin at 40 ºC and solution were stirred at 200 rpm for 10-15 min. As a crosslinking agent 8% glutaraldehyde was mixed in previously made gelatin solution containing freshly prepared chondroitin sulfate. The above solution was mixed immediately to paraffin emulsion and stirred for half hour, 200ml of 200 mM of sodium phosphate buffer was added. To let the beads settle down the whole solution was kept at room temperature. Washing of beads was done by sodium phosphate buffer and finally beads were transferred to beaker containing a fresh buffer. Again 2.4 ml of 8% glutaraldehyde was

added to maintain the stability of beads and pH was adjusted to 7 using saturated solution of sodium tertraborate, and the beaker was kept at room temperature for 1 h. Further to increase stability of beads the pH was increased to 9 and sodium borohydride solution was added followed by incubation on a shaker for few hours. Finally the beads were extracted by washing with sodium phosphate buffer and 60% ethanol and were kept for drying for further analysis.

3.2.2 *In vitro* release profile

In order to measure the amount of chondroitin sulfate that has been incorporated into the synthesized spheres *in vitro* release profile was studied. Microspheres (25mg) were taken in 1 ml PBS followed by incubation on incubator shaker at 37 ºC for 42 days. DMMB method was used for estimation of the release of chondroitin sulfate from gelatin spheres. The amount of CS released was measured spectrophotometrically at 540 nm with PBS as a control. This analytical test is necessary as it will give us insight as to how long CS can be made available to chondrocytes (Jiang *et al.,* 2011).

3.2.3 *In vitro* effect of chondroitin sulfate (CS) on primary goat chondrocytes by various delivery modes

The effect of chondroitin sulfate on growth and proliferation of chondrocytes were analyzed by making the drug molecules available to cells by following different modes.

a) CS blended in the chitosan-gelatin scaffold forming an interpenetrating network (CGC)[already discussed in chapter 1]

b) CS incorporated into gelatin spheres (CGS): In order to avoid the booster dosage drug was encapsulated in spheres to obtain control release at the defect site. Additionally microspheres

loaded with the drug were supplied onto chitosan-gelatin matrix mixed in the media. Scaffolds were further studied by MTT, SEM, GAG and collagen estimation.

c) CS incorporated gelatin spheres embedded into chitosan-gelatin scaffold (CGM)

Here CS loaded microspheres were incorporated in the chitosan-gelatin scaffold (CGM). Then CGM was studied by SEM, MTT, GAG and collagen estimation. The main aim of incorporating spheres inside scaffold is to slow down the release of the drug, as it is required in cartilage repair process which is slow, as shown in Figure 3.2.

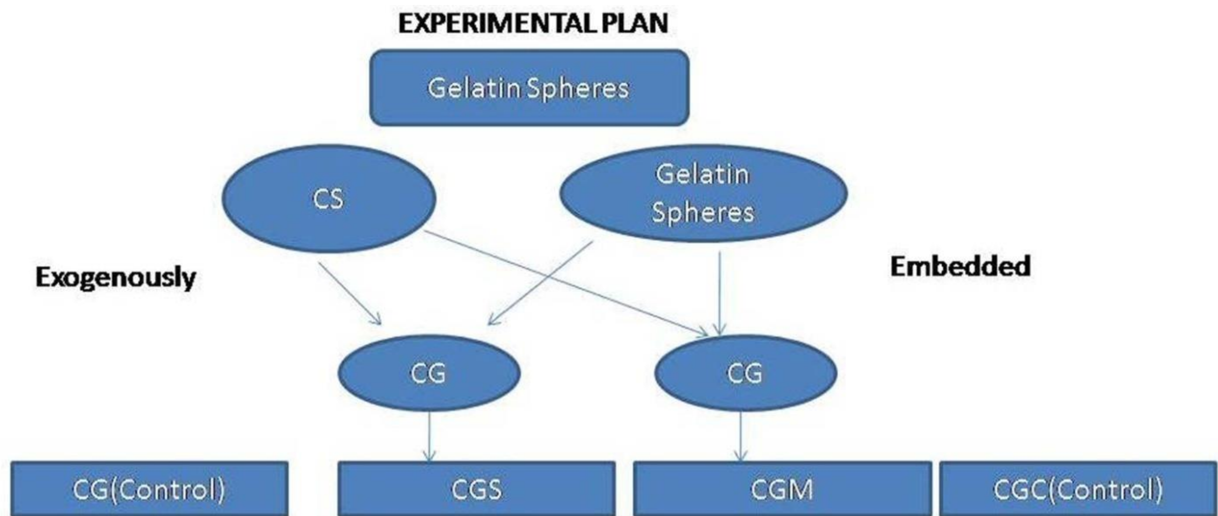


Figure 3.2) Schematic representation of the experimental plan for different modes of delivery for CS. CS (chondroitin sulfate), CG (chitosan-gelatin scaffold), CGS (CS incorporated into gelatin spheres supplied on CG scaffold), CGM (CS incorporated into gelatin spheres embedded into CG scaffold)

3.2. 4 Determination of *in vitro* cell viability of delivery mode synthesized

In order to check the effect of CS on the proliferation rate of chondrocytes delivered by all modes (a, b, c), MTT was done. MTT is 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of dye into formazan crystals by living

cells, which determines mitochondrial activity. Number of viable cells is related to mitochondrial activity. Scaffolds with all modes and control CG were saturated with DMEM overnight then seeded with chondrocytes isolated at density $1x10^5$ cells/ml in 24 well plates. Two dimensional (2D) cultures were also taken as one of the other control where cells seeded at a same density on tissue culture treated plate. To perform the MTT the previous media was discarded and 500 µl of 0.5mg/ml MTT solution was added in to each well. Plates were then incubated for 4-5 h in an incubator at 37 °C. After the time period of incubation is over, 1.5 ml of DMSO was added for 15 min. The amount of purple formazan formed and was measured by spectrophotometer at 570 nm by using DMSO as blank. The assay was done on first day followed by every 7th day for a period of 6 weeks (Van *et al.*, 2011).

3.2.5 Microscopic analysis of scaffold seeded with chondrocytes

Chondrocytes cultivated scaffolds were analyzed by scanning electron microscopy (SEM) on every 7th, 14th, 21st, and 28th day. The cells on the scaffold for all the modes were fixed by using 2.5% glutaraldehyde followed by incubation in ethanol gradient from 20% to 100% for about 15 min in each gradient. In order to check whether the microspheres were embedded in the scaffold, the sample was also kept for SEM analysis. Then scaffolds were dried in desiccators and UV sterilized for some time and then mounted on aluminum stubs and vacuum sputter-coated with gold. Samples were then analyzed by SEM with constant voltage 20 kV and high pressure. The images were taken for analysis.

3.2.6 *Glycosaminoglycan (GAG) estimation*

In order to determine the amount of GAG secreted by chondrocytes on scaffolds on all the modes, GAG was estimated by dimethyl methylene blue (DMMB) method. The scaffold

seeded with chondrocytes were washed with PBS and stored at -20 °C. Then samples were lyophilized and dry weight of samples was taken. Samples were subjected to papain digestion overnight. The supernatant of the digest was collected in a tube and mixed with DMMB dye and absorbance was measured at 540nm with scaffold without cells as blank. The assay was done for 6 weeks for all delivery modes along with control (Bhat *et al.,* 2013).

The GAG content was calculated by the formula

GAG Content (%) = [(Chondroitin- 4- sulphate (µg ml $^{-1}$) x dilution factor

x papain Volume) / (dry weight of sample (g) x 10^6)] x 100

3.2.7 Collagen estimation

The extracellular framework and 2/3rd of the dry mass of adult articular cartilage are polymeric collagen. The majority of collagen present is type II along with III, VI, IX, XI, XII, XIV all contribute to mature matrix (Poole, 1997). Type II collagen represents 90 to 95% of collagen in ECM and forms network with proteoglycans aggregate. The basic structure of collagen is that it contains a region consisting of 3 polypeptide chains (alpha chains) wound into a triple helix. Glycine and proline is amino acid composition. The basic triple helix structure of polypeptide chains provides articular cartilage with essential shear and tensile properties to stabilize matrix (Fox *et al*., 2009).

Collagen was estimated by hydroxyproline assay. Scaffolds were kept at -20 °C, lyophilized and dry weight was measured and digested by papain. The supernatant (250µl) was collected in vial and incubated at 120 ºC in the oven overnight. The contents were transferred into glass scintillation vial and incubated at 90° C till brown residue was formed at the bottom. The tubes were kept at room temperature and 1ml of sodium phosphate buffer was added. The resultant mixture was allowed to react with chloramine T solution at room temperature

and mixed with 250 μl of pDAB and incubated at 60 °C for 24 h. The colored mixture was measured spectrophotometrically at 540 nm. Scaffold without cells treated in the same way was used as blank (Bhat *et al.,* 2013).

The total collagen content was determined by the equation.

$$\text{Collagen content (\%)} = [(\text{hydroxyproline } (\mu g\ ml^{-1}) \times \text{dilution factor} \times \text{proportion hydrolysed}) / (\text{dry weight of sample} \times 0.143 \times 10^6)] \times 100$$

3.3 Results and Discussion

3.3.1 Synthesis of gelatin microspheres and incorporation of chondroitin sulfate

Due to many disadvantages like poor stability, low solubility there is an increasing need to deliver the drug molecules in a controlled manner. In this context we have used microspheres as controlled long term DDS. This microsphere as DDS has numerous advantages over traditional method of administration. These advantages include constant rate of delivery, protection of drug, dosage reduction, and efficient delivery (Varde and Pack, 2004). Microspheres fabricated from biodegradable polymers are being used as controlled drug delivery vehicles (Jayan *et al.,* 2009). Here we have synthesized microspheres by emulsion polymerization technique; this process has some advantages like high molecular weight polymers can be made at fast polymerization rates, final product need not to be altered (Ramteke *et al.,* 2012). Gelatin was used as polymer for microspheres synthesis, it is an interesting biomaterial as it is biodegradable, it can be broken down by cellular action through secretion of specific matrix metalloproteinase, and no toxic byproducts are created by its degradation as it is non toxic. Mainly its degradability is prerequisite property as degradation is required to release drug. Other additional property which is advantageous that

gelatin can be dissolved in aqueous solutions, so it does not involve the use of toxic solvents (Solorio *et al.*, 2012).

As our focus was to construct a delivery vehicle for cartilage repair, we encapsulated CS which has already shown positive potential in treating OA. But the limitation is that this drug would be more effective if it is absorbed properly, so for it to act with its proper biological activity its entrapment is necessary, so by using microspheres as vehicle it can be entrapped or microspheres with CS can be embedded into scaffold so that it can be delivered in proper manner with controlled release (Cai *et al.,* 2007). Gelatin spheres loaded with CS has already shown potential treatment for OA (Brown *et al.,* 1998). However in our work, we have further tried to study the effect of synthesized gelatin spheres loaded with CS and supplied exogenously on CG matrix so that this system can mimic ECM to closest. We further analyzed its effect on chondrocyte proliferation and ECM production. The purpose of studying the effect of delivery modes was because cartilage repair is a slow process and drug should be delivered in a sustained manner for a long time. Another novelty of this work is an encapsulation of these drug loaded spheres inside scaffold and its effect on chondrocyte proliferation and ECM production was studied.

3.3.2 In vitro release profile

The drug release kinetics from microspheres is effected by many factors like composition, structure, swelling, degradation rate of microspheres and solubility, stability, charges of drug, speed, method of drug incorporation, the interaction of microspheres and drug, release environment such as presence of enzymes, cross linker concentration (Fu and & Kao, 2010).

The release pattern of the drug from microspheres can be divided into those that release drug at a slow zero or first order rate and other with speedy dose, followed by slow zero or first

order release of the sustained compound. The aim is to release the compound and to maintain in target tissue for desired time (Dash *et al.,* 2010).

Several events contribute to the overall kinetics of drug release from microspheres including chemical degradation of the polymer by hydrolysis, polymer erosion, diffusive transport of drug from polymer (Versypt *et al.,* 2003).

The release of CS from gelatin microspheres was estimated by DMMB (Dimethyl methylene blue) assay at regular intervals for 42 days. Percentage release was also calculated and it was observed that more than 90% of drug released till the end of 42 days as shown in Figure 3.3.

Here it can be seen that CS was released at constant rate, where in the CS released increased an increase in number of days, while maintain a sustained rate, which is an added advantage.

The release of CS at a constant rate also shows the degradability of microspheres as one of the factors responsible for the release of drug from microspheres is polymer degradation. So overall from the release profile it can be concluded that these microspheres are suitable for cartilage tissue engineering application as our focus was to study the effect for 42 days, as first cartilage repair is slow and chondrocytes are slow growing cells that take time to proliferate and grow. Optimization with respect to number of days was done based on the previous work done on CAG cryogel scaffolds in our lab (Bhat *et al.,* 2011).

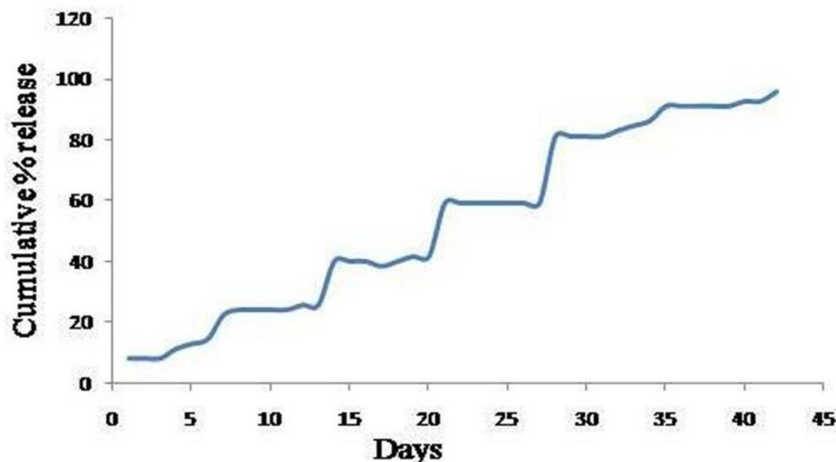


Figure 3.3) Cumulative percent release of CS (Chondroitin sulfate) from microspheres till 42 days (the release of CS from gelatin spheres was determined by DMMB, dimethyl methylene method)

3.3.3 In vitro **effect of chondroitin sulfate released on primary goat chondrocytes by various delivery modes.**

a) CS incorporated into gelatin spheres (CGS)

The major limitation of drug supply or bioactive molecule in free form is that it is exposed to enzymatic degradation, instability, so it requires booster dosage which increases the cost of treatment (Joseph *et al.,* 2009). Therefore for the protection of drug and release of drug in a sustained manner we incorporated CS into gelatin microspheres. Further these microspheres were delivered to cell seeded on chitosan-gelatin scaffold. So that apart from the bioactive system this approach also provided 3D matrix. Relying on micro-encapsulation technologies can open a new base for treatment of OA.

a) CS incorporated gelatin spheres embedded into chitosan-gelatin scaffold (CGM)

In the case of cartilage repair as the process is very slow so we require the slow release of drug or bioactive molecule (CS in this case). So we incorporated these spheres (CGS) inside chitosan- gelatin scaffold. This system has many advantages over present delivery modes of the oral route or intravenous route. As CS has already been administered orally but it has some limitation like the destruction of drugs by gastric juices, unpleasant taste of the drug, not useful in terms of emergency (Reddy *et al.,* 2011). Also when the drug is delivered intravenously then there are chances for blood borne infections, many infections due to non maintenance of aseptic conditions, arterial damage and chances of over dose. So delivery of drug encapsulated in microspheres and loaded inside scaffold could be one of the best, non

harmful modes of delivery. In this approach the scaffold along with bioactive molecule have to be implanted into the system once. This mode would mimic ECM of cartilage. Already a variety of microspheres have been added to different scaffolds like PLGA microspheres incorporated into gelatin scaffold (Pramanik *et al.,* 2009). In another chitosan loaded with TGF-beta has been incorporated into the scaffold (Lee *et al.,* 2004). Thus, we have designed novel mode CGM that can take the generation of tissue engineering from one level (scaffold with cells) to next level (Scaffold, cells, bioactive component) so to mimic native cartilage ECM to the closest.

3.3.4 SEM analysis of microspheres and microspheres incorporated into scaffold

SEM of spheres with the drug was done to analyze their morphology and size. They showed smooth rounded surface as shown in Figure 3.4. The microspheres were found to be in the range of 50-100 µm diameters. It is also known that smaller microspheres release drug at faster rate. Also the rounded morphology ensures complete protection of drug and smooth surface with which the drug can be uniformly released (Neelesh *et al.,* 2004).

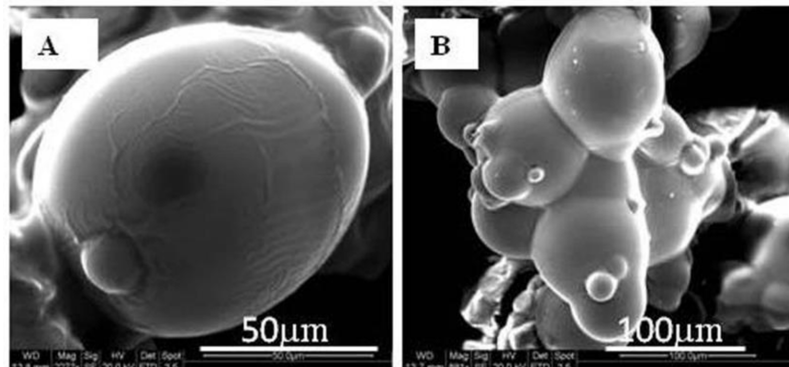


Figure 3.4) Scanning electron microscopy images of gelatin microspheres loaded with CS (chondroitin sulfate) at (a) 2276X magnification b) 881X magnification

3.3.5 SEM analysis of microspheres embedded in scaffold

Microspheres embedded scaffolds were scanned by electron microscopy to observe the presence of spheres in the scaffold as shown in Figure 3.5. We observed the presence of microspheres in the pores of the scaffold. The microspheres were distributed on scaffold, leaving proper space so as not to block the pores of scaffolds, as this could lead to an inhibition of exchange of nutrients and gaseous. Moreover, cells will produce ECM which will also block the pores so hindering the availability of oxygen to lower parts of the scaffold, as availability of oxygen can lead to chondrocytes cell death. Hence, optimization with respect to the concentration of microspheres is important to avoid cell death. In the present study the size and microspheres distribution was sufficient which allowed chondrocytes to migrate without hindrance.

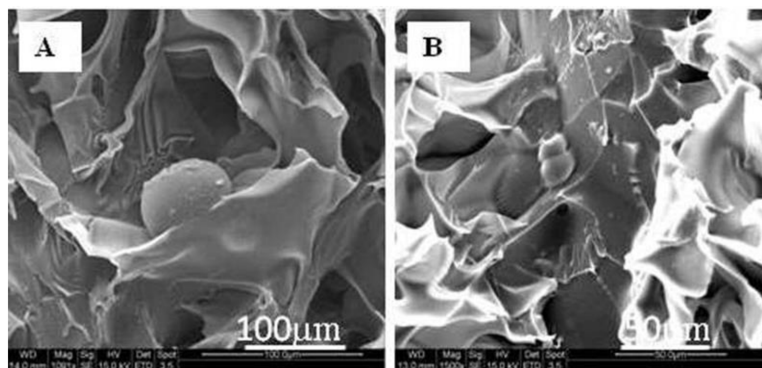


Figure 3.5) SEM (Scanning electron microscopy) showing gelatin microspheres incorporated into chitosan-gelatin scaffold (A&B)

***In vitro* studies**

3.3.6 To determine the chondrocytes viability (MTT assay)

In order to assess the biocompatibility of synthesized cryogel scaffold for all the modes towards the seeded chondrocytes 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay was performed. The NADPH present in viable cells is capable of reducing MTT to formazan which can be measured spectrophotometrically. The absorbance measured determines the rate of cell proliferation. Here in order to observe the efficacy of delivery modes of CGS and CGM, 2D and 3D controls were taken. For 2D control, cells were seeded on treated tissue culture plate and for 3D controls CG and CGC 5% were taken. As already discussed compared to 3D, 2D system showed initial proliferation for 21 days and then cell death occurred due to lack of space. Our focus was primarily on different mode CGS and CGM. There was a considerable increase in proliferation in CGS and CGM as compared to control CG, CGC 5% this may be attributed due to the presence of free CS, as shown in Figure 3.6. Also it holds validation as CS role in chondrogenesis has been known. CS is major GAG molecule which helps in cartilage formation and thus in this case presence of free CS helped in increasing chondrocytes viability (Varghese *et al.,* 2008). Literature has also shown that CS helps in cell-cell interaction, mediating cell signaling recognition through cell surface receptors so also it is known that cell signaling through integrins regulates several chondrocytes functions, including differentiation, response to mechanical stimulation and cell survival (Gao *et al.*, 2014). Hence, CS helps in this mechanism and there are chances of more proliferation of chondrocytes. The presence of free CS in CGS and CGM modes provides signals for cell-cell interaction which further increase the proliferation rate. Also, when we compare CGS and CGM there was a slight increase in proliferation activity of CGS

as compared to CGM. This might be due to CGM the microspheres loaded with CS is incorporated inside scaffold so clogging of pores and thus less surface area for cell attachment. Also when we see the CGM system here it mimics ECM to closet with scaffold along with bioactive component and also CS does not provide as booster dosage. This study holds validation as it has been already known that chondroprotective such as chondroitin sulfate can be a promising approach for therapeutic approach. Their action can be explained by the following mechanism 1) being basic components of cartilage and synovial fluid; they stimulate the anabolic process of the cartilage metabolism 2) they can delay inflammations induced catabolic process. The process can help to stimulate cartilage regeneration by chondrocytes stimulation. So because of known role of CS we tried encapsulating CS in different delivery modes so that it can help in the stimulation of chondrocytes which in turn can induce the matrix synthesis (Jerosch, 2011).

Also it is known that CS is an important component as it plays an important role in controlling chondrocytes metabolism and functions such as organized cytoskeleton through integrin-mediated signaling via-cell matrix interaction. Several chondrocyte functions such as differentiation, metabolism, matrix remodeling, response to mechanical stimulation and cell survival are regulated by cell signaling through integrins. The pathways that control chondrogenesis have been known as wnt signal, protein kinase C, retinoic acid (RA) signal (Gao, et al., 2014). So because of all the above mechanisms holds validation for above results for the role of CS in chondrocytes proliferation. The limitation in CGC scaffold synthesizes was overcome by the development of CS encapsulation in microparticle with different modes. We were successful in synthesizing mode which can protect CS and release in a controlled manner. The development of novel delivery modes such as CGS and CGM

were able to achieve our goal of protecting CS and releasing in a controlled manner for a long time duration, which is required in cartilage repair as cartilage healing is slow.

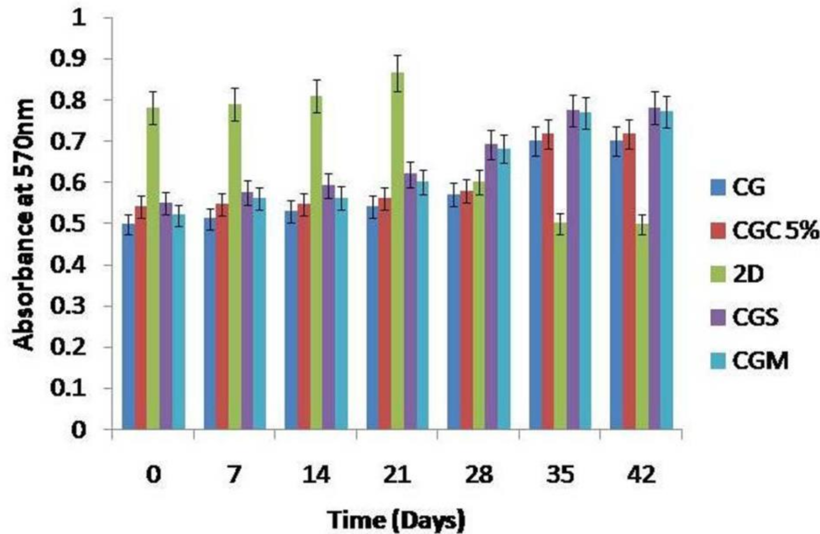


Figure 3.6) In vitro cell viability of CG, CGC5%, 2D, CGS, CGM by MTT assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide. Cell culture experiment was run for 42 days. CG (chitosan-gelatin scaffold), CGC5%(chitosan-gelatin-chondroitin sulfate5% scaffold), CGS (CS incorporated into gelatin spheres supplied on CG scaffold), CGM (CS incorporated into gelatin spheres embedded into CG scaffold). The experiment was done in triplicates and p<0.05.

3.3.7 Scanning electron microscopy of chondrocytes cultured on different modes

SEM was done of chondrocyte cultured on all scaffolds synthesized; we could clearly observe the attachment of chondrocytes on all delivery modes CGS, CGM with its maintained rounded morphology.

The attachment in all modes may be due to the presence of gelatin as it has signals which help chondrocytes to attach. But when we observed the SEM image of CG and CGM in CG there was not much cell attachment but in the case of CGM where free CS is present the

attachment was more and chondrocytes morphology was also maintained as shown in Figure 3.7. This might be due the availability of CS which helps cells to attach, proliferate and maintain its morphology (Ingavle *et al.,* 2012). This also confirms our MTT result that these scaffolds are supporting chondrocytes attachment and proliferation.

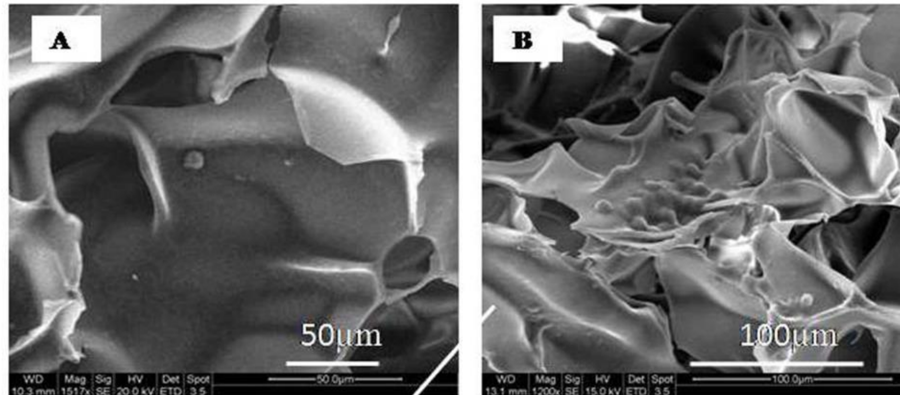


Figure 3.7) SEM (Scanning electron microscopy) images of Chondrocytes after 1st week in culture A) CG B) CGM

3.3.8 Glycosaminoglycan analysis on chondrocytes seeded on the scaffold

The GAG content of engineered cartilage is a measure of biochemical and mechanical quality. For successful cartilage repair it is important to measure the GAG content, how it is maintained or can be increased (Ramaswamy *et al.,* 2008). GAG is one of the important components of ECM cartilage. So, GAG is an important factor to maintain long term stability of repair tissue (Sharma *et al.,* 2007). As already the effect of CS on osteoarthritis has been shown to improve the efficacy of CS as a therapeutic agent in knee OA treatment would be great interest so GAG activity for different delivery modes was estimated by DMMD dye method (David *et al.,* 2009). As shown in Figure 3.8, microspheres with chondroitin sulfate on chitosan-gelatin scaffold (CGS) and microspheres with drug embedded in the scaffold

(CGM) there is an increase in GAG production as compared to CG control where there was absence of any spheres. This results holds validation as GAG like chondroitin sulfate helps in increasing matrix synthesis (Henrotin *et al*., 2010; Bassleer and Franchimont 1992; Schwartz and Dorfman, 1975). So the systems like CGM and CGS due to the presence of free CS encapsulated microspheres could provide long term increase in GAG production which is a very important strategy in cartilage repair to increase the matrix synthesis. So this system would also overcome the limitation of booster dosage. Here the role of CS is clear but the mechanism by which this helps in increasing GAG production is still unclear. When CGS and CGM system was compared with CGC5% scaffold we observed that the GAG content was less in CGC5% as compared to CGS and CGM this might be due to unavailability of free CS .

So here we conclude that the system with microspheres encapsulating CS can be an effective long term mode to increase the CS production that in turn can help in cartilage repair.

CS has already been recognized as a therapeutic approach in OA. Also known that CS supply could give building blocks for the production of matrix component, so CS can help in favor of matrix regeneration and account for its beneficial effects. Since the mechanism of CS as a therapeutic agent is still be elucidated and is under debate.

But some its known that CS help in increase in type 2 collagen and GAG in chondrocytes, the effect may be due to MMP(matrix metalloproteinase) down regulation, CS shown to inhibit MMP-1, ADAMTS-4 and 5 in human, bovine, porcine chondrocytes. MMP-13 inhibition can happen through inhibition of p38 and ErK1/2 activation (Henrotin *et al.*, 2010).

So increase in GAG production may be validated by the above mentioned mechanism for the role of free CS towards increasing GAG production. Our aim was to provide the positive effect of CS which has already shown an effect in the treatment of OA. CGS and CGM system both provided the delivery of CS at constant rate and in sustained manner, by encapsulating in microspheres. The novel delivery mode synthesized CGS and CGM which are known as the third generation of biomaterial (cells with scaffold with bioactive components), these modes with increase in GAG content serve as better delivery mode than CGC 5%. Showing the potential of CGS and CGM as therapeutic delivery mode for OA.

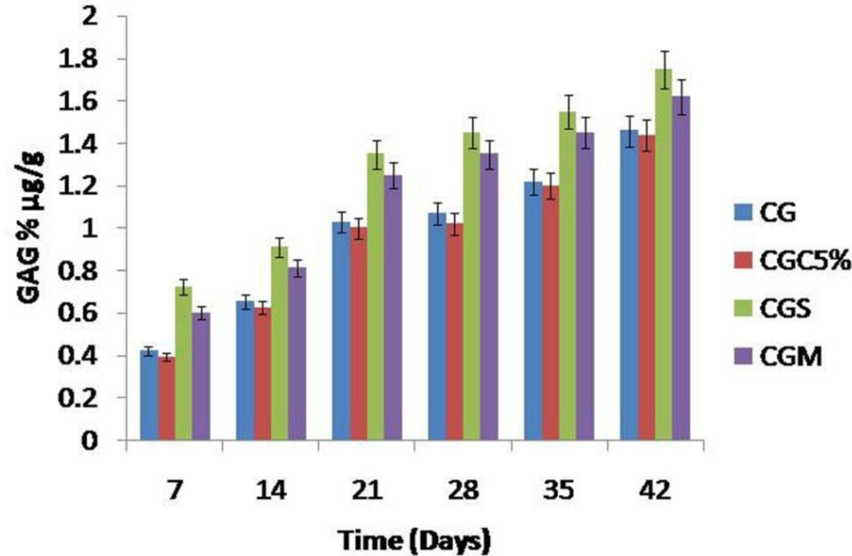


Figure 3.8) GAG (glycosaminoglycan) analysis using DMMB, dimethyl methylene method for 42 days. CG (Chitosan-gelatin scaffold), CGC5%(chitosan-gelatin-chondroitin sulfate5% scaffold), CGS (CS incorporated into gelatin spheres supplied on CG scaffold), CGM (CS incorporated into gelatin spheres embedded into CG scaffold). The experiment was done in triplicates and p<0.05.

3.3.9 Collagen estimation

Collagen was estimated by hydroxyproline assay. The estimation utilizes the ability of chloramine T to oxidize hydroxyproline to pyrole compound and then to pyrole 2 – carboxylic acid that reacts with p-dimethylaminobenzaldehyde (DAB) and its formation gives colored complex which can be measured spectrophotometrically. It was observed that there is an increase in collagen production in CGS and CGM as compared to plain scaffold CG, as shown in Figure 3.9. This can be due to the presence of free CS in both systems which reduces the degradation of cartilage ECM component as CS enhances TIMP-1 production which are inhibitors of MMPS(matrix metalloproteinase) which in turn degrade type 2 collagen (Imada *et al.,* 2010). Also CGC 5% collagen production was less as compared to another mode this might be due to unavailability of free CS. So from this study it can be concluded that in this system free CS also helps in collagen production for a longer time duration which would be advantageous. It is already known that collagen is an important component of cartilage matrix so here these delivery modes CGM and CGS help in increasing collagen production which has potential for treatment of OA. The results also show the role of CS towards the collagen production by enhancing collagen fibril formation. So above results showing increase in collagen production in CGS and CGM are validated by role of CS towards extracellular matrix component like collagen (Kvist *et al.,* 2006). The enhanced collagen production in CGS and CGM shows the significance of these modes. These modes worked much better as compared to scaffold showing the role of third generation biomaterials in the current time period, providing more efficacies in the treatment of OA. With use of CGS and CGM other goal i.e. to provide sustained release of CS could also be achieved.

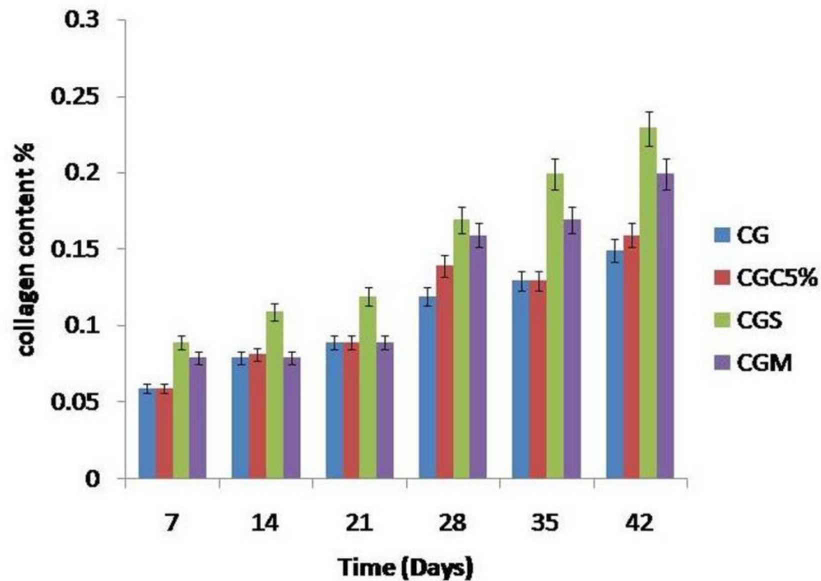


Figure 3.9) Collagen estimation by hydroxyproline assay.CG (chitosan-gelatin scaffold), CGC5%(chitosan-gelatin-chondroitin sulfate5% scaffold), CGS (CS incorporated into gelatin spheres supplied on CG scaffold), CGM (CS incorporated into gelatin spheres embedded into CG scaffold). The experiment was done in triplicates and p<0.05

3.4 Conclusion

Different delivery modes were synthesized and their effects were studied by MTT, SEM, GAG and collagen studies. From the above studies it can be concluded that CGS and CGM delivery modes showed an increase in cell proliferation and matrix production. Also when compared with CGC 5% the proliferation rate and matrix production was lower in CGC 5% which may be attributed towards the non availability of free CS in CGC 5% . Whereas systems like CGS and CGM showed enhanced matrix production and cell proliferation. This system would be advantageous for OA and this system is overcoming limitation of the traditional method of delivery. As CS potential for OA has already been shown but limitation exist like booster dosage requirement, chances of infection, cost, using CGS and CGM as delivery modes might be able to overcome limitation of traditional methods as CS can be sustained and released for longer time duration, which would be advantage in cartilage repair

as cartilage healing takes time. Therefore availability of CS for longer term duration would be certainly advantage and also would overcome the limitation of traditional drug delivery. Showing huge potential of CGS and CGM for treatment of OA.

Chapter 4

Encapsulation of bioactive molecule for sustained release and study effect of Nutritional supplements as a novel strategy for repair of cartilage injuries

Chapter 4

Encapsulation of bioactive molecule for sustained release and study effect of nutritional supplements as a novel strategy for repair of cartilage injuries

4.1 Introduction

Microencapsulation is a process in which small discrete solid particles are surrounded by intact shell. Microspheres size varies from 0.1-1000 μm and act as drug carriers for controlled release (Shi *et al.,* 2003). The main advantages of microencapsulation are: active drug/bioactive molecules are released at controlled rates over prolonged periods of time; easy administration; protection of drug by enzymatic degradation; the reactive or incompatible component can be separated. The material encapsulated inside the microparticles is known as core or internal phase (Desai *et al.*, 2006). There are different types of materials (bioactive compounds) that can be encapsulated. Bioactive molecule is nonessential and exhibits the potential which stimulate metabolic process which results in promotion of health improvement. The focus towards bioactive is increasing because of their role as antioxidants, enzyme inhibitors or inducers. Plant products, vitamins, whole grain have been the source of large number of bioactive compounds with therapeutic potential, of which large number have been developed into drugs that are consumed worldwide for treatment of osteoarthritis (Venkatesha *et al.,* 2016). Role of different type of vitamins have been studied for example the potential of vitamin D for specific benefits in OA. It has been reported that vitamin C may slow progression of OA (Nawabi *et al.*, 2010). Role of vitamin C have been studied in skin

diseases, especially in the case of ageing, but there is limited information on the role of vitamin C in OA (Crisan *et al*., 2015).

Ascorbic acid (AA) is a naturally occurring organic compound with most abundant antioxidant properties. D-Glucose is converted into D-mannose-1-P via glucose 6-P-isomerase, further conversion of D-mannose into GDP-D mannose and GDP-L-galactose. Galactose dehydrogenase converts GDP-L-galactose into L-galactono-1, 4 lactone which is converted into AA via L-galactono-1-4 lactone dehydrogenase (Smirnoff, 2000). Ascorbic acid also plays the role that is linked to inflammatory processes and involves immunity. Hence ascorbic acid has a defined role to stimulate the immune system, which could be advantageous as it is known that the onset of many diseases occurs due to weak immune system. Also AA usually helps in collagen synthesis by acting as co factor for prolyl, lysly hydroxylases and increasing proteoglycan synthesis (Schwartz and Adamy 1977). Therefore it acts as an immunostimulant, anti-inflammatory and antibacterial agent and helps in cartilage matrix production. Therefore these properties can be used as a potential tool to treat many diseases such as osteoarthritis, skin diseases etc. Literature survey has shown that AA could slow down the progression of OA in humans and guinea pigs. The role of ascorbic acid in collagen synthesis is already known, however AA may also exert a chondroprotective effect by enhancing gene expression and protein synthesis of cartilage matrix component. Despite several formulations containing AA in the market, few are topically effective. The major limitation of these formulations are 1) low concentration of AA 2) incomplete absorption and metabolism of different forms 3) AA oxidized to dehydroascorbic acid due to exposure to air, light and high temperature which results in short shelf time of formulations containing AA. In basic conditions the oxidation of AA occurs rapidly and results in

irreversible hydrolyzation of AA into 2,3 –L diketogulonic acid which is inactive form. So there is high need to protect AA by encapsulating it in a delivery vehicle so that it can be available for longer time duration. Therefore we tried encapsulating AA in gelatin spheres (Sorice *et al.,* 2015; Naskar *et al.,* 2013*)*

Vitamins, minerals and nutritional supplements are micronutrients that benefit the body. Such as, vitamin A helps in vision and bone growth, also vitamin E boost up the immune system and repair DNA. Due to lack of mineral and vitamin, body might lose ability to heal and protect itself. Micronutrients can also have an effect on mental fitness (Belcaro *et al.,* 2010). But nutritional supplements are relatively new line of treatment; very little information is known about them and has fewer side effects (Gregory *et al.,* 2008). Nutritional supplements derived from foods and herbs have shown biologic effects, with some potential in clinical trials in OA patients. The major advantage is that these approaches provides safe symptom relief, and mediate beneficial joint remodeling in early OA that opens its channels for future scope of investigation. Forty seven percent of adults utilize non prescribed medications for OA including nutritional supplements, such as phytoflavonoids, polyphenols, and bioflavonoids which are natural compound found in trees, spices, wines and vegetables based on their anti-inflammatory and anti-catabolic actions, and protective effects against oxidative stress (Leong *et al.* 2013).

We have used collaflex and retinoic acid as drug supplements. Collaflex contains collagen peptides that are formed by enzymatic process that converts large native collagen molecule into small bioactive peptides. Retinoic acid is a metabolite of retinol that mediated basic function of growth and development. It is well known to have diverse immunomodulatory actions. Role of all trans retinoic acid has been investigated on mice model where retinoic

acid has shown anti-arthritic effect suppressing the clinical signs of arthritis (Kwok *et al.*, 2012). Moreover the anti-inflammatory effect of retinoic acid has also been demonstrated (Nozaki *et al.,* 2006). In another study it has been shown that retinoid signaling might have a potential central role in OA and that components of the pathway may provide potential disease biomarkers or targets for therapeutic intervention (Davies *et al.,* 2009). Availability of growth factors and micronutrients are known to improve the functionality of the cartilage. To achieve this for longer time duration bioactive molecules using AA, collaflex and retionoic acid are used for controlled drug delivery.

In our work we have tried to encapsulate the ascorbic acid molecule inside the gelatin spheres and further embedded this in the scaffold, thereby trying to achieve the tissue engineering construct that covers the basic components of tissue engineering i.e. scaffold, chondrocytes and bioactive factors. After synthesis the *in vitro* release profile was studied and then further all delivery modes synthesized were studied by MTT, SEM, GAG, Collagen assay. Moreover, two nutritional supplements such as collaflex and retinoic acid were also studied in 2D and 3D by MTT, Collagen, GAG assay.

4.2 Materials and methods

Materials

Gelatin (cold water fish skin), ascorbic acid (S. d. Fine chem. Limited), collaflex (Sanofoi), retinoic acid (sigma), Dulbecco modified Eagles medium (DMEM), collagenase type2, penicillin-streptomycin, nystatin, papain, trypsin-EDTA solution, 1, 9 dimethyl methylene blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma Chemical Co (St.louis, MO, USA). Glutaraldehyde was obtained

from S.D. Fine Chemicals Ltd (Mumbai, India). Fetal bovine serum was purchased from Hyclone (UT, USA). Light liquid paraffin and TritionX-100 were obtained from Merck (Mumbai, India). All other chemicals used were of analytical grade and were used without any further purification.

Methods

4.2.1 Synthesis of gelatin spheres and incorporation of ascorbic acid into it during synthesis

In order to encapsulate the ascorbic acid molecule inside gelatin spheres, emulsion polymerization technique was used with some modification. Gelatin (15%) was dissolved in 100 ml of 20 mM sodium phosphate buffer, pH (5.5). AA (2mg/ml) was added to the above solution. Further, 1.8 ml of 1 % triton X-100 was added into 100 ml paraffin at 40 ºC and solution was stirred at 200 rpm for 10-15 min. In a solution containing gelatin and ascorbic acid, 8% glutaraldehyde was mixed. The above solution was quickly added to paraffin emulsion and stirred for half an hour. Subsequently 200ml of 200 mM of sodium phosphate buffer was added into the whole solution and kept at room temperature for the beads to settle down. Beads were washed to remove any other chemicals using sodium phosphate buffer. To increase the stability of the crosslinking, 8% glutaraldehyde was added and pH was adjusted to 7 by using saturated sodium tetraborate.

Further, to increase the stability of beads, the pH was increased to 9 and sodium borohydride solution was added followed by incubation on a shaker for few hours. Finally the beads were extracted by washing with sodium phosphate buffer, 60% ethanol and kept for drying for further analysis (Wissemann and Jacobson, 1985).

***4.2.2 In vitro* release profile**

In order to measure the amount of ascorbic acid that has been incorporated into the synthesized spheres, *in vitro* release profile was studied. Microspheres (25mg) were taken in 1 ml PBS followed by incubation on incubator shaker at 37 ºC for 35 days. A simple and highly sensitive direct spectrophotometric method was developed for the determination of L-ascorbic acid using sodium oxalate for estimation of the release of ascorbic acid from gelatin spheres. This analytical test is necessary as it will give us insight about the duration for which ascorbic can be made available to chondrocytes (Selimović *et al.,* 2011).

4.2.3 SEM analysis of microspheres

In order to analyze the morphology and size, SEM analyses were carried out. Microparticles were synthesized, dried and mounted on aluminum stubs and vacuum sputter-coated with gold. Samples were analyzed by SEM with constant voltage at 20 kV and high pressure. Random images were taken and analyzed for results.

4.2.4 Different delivery modes synthesized to study *in vitro* effect of ascorbic acid on primary goat chondrocytes

In this study in order to study ascorbic acid encapsulation in different modes and its effect on chondrocytes proliferation and matrix production, the following modes were analyzed

a) The cells were seeded on tissue culture plate (2D)
b) 2D ex (exogenous): The cells were seeded on tissue culture plate and microspheres were added, to observe the effect of ascorbic encapsulated into gelatin spheres. Here there is no support provided in the form of a scaffold. This mode has advantage as it can be directly injected into the damaged area.
c) 2D asc o: The drug (AA) in free form was added just once; this is external control to show how the drug in free form acts.

d) CG: chitosan-gelatin scaffold was used as control

e) CG ex: The scaffold was provided with ascorbic acid encapsulated in gelatin spheres. AA encapsulated microspheres were added exogenously so that both matrix and sustained release of AA could be provided to the culture.

f) CGM: This mode was synthesized by adding AA encapsulated gelatin spheres embedded in to chitosan-gelatin scaffold. Here not only the scaffold was used as support to provide attachment surface to chondrocytes but also the continuous delivery of AA was also provided in a sustained manner. Also AA spheres were embedded inside scaffold so that the release would be slow which is an additional advantage in the case of cartilage repair which is a slow repair process.

g) CG asc o: Control where AA was supplied as free form only once on chitosan-gelatin scaffold

Modes for collaflex and retinoic acid

A) 2D(chondrocytes seeded on monolayer 24 well plate)

B) 2DX (collaflex and retinoic acid added exogenously on culture)

C) CG (chitosan-gelatin scaffold as control)

D) CGX(chitosan –gelatin with collaflex added exogenously)

4.2.5 Seeding of chondrocytes on different delivery modes

After the chondrocytes were isolated and expanded on tissue culture flask. Once they reach confluency they were seeded on tissue culture treated plate for 2D culture and tissue culture non treated plate in 3D culture for modes a, b, c, d, e, f, g and A, B, C,D at a cell density of $1X10^{5}$ cells/ml. Scaffolds were first sterilized by ethanol treatment and UV treatment and seeded with chondrocytes. The modes were studied for *invitro* studies like MTT which is to

observe the behavior of chondrocytes with different delivery modes. And then GAG and collagen assay which is to study the matrix behavior with modes

4.2.6 Determination of *in vitro* cell viability of chondrocytes (MTT).

The effect of AA on proliferation rate of chondrocytes delivered by all modes(a,b,c,d,e,f,g) was analyzed by MTT 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay based on conversion of MTT dye into formazan crystals by living cells, which determines mitochondrial activity. Cells were seeded at a density of $1x10^5$ cells/ml in 2D mode and scaffold modes. Then in order to carry out the assay the previous media was replaced by 500 µl of 0.5mg/ml MTT solution in to each well. Plates were then incubated for 4-5 h in an incubator at 37 °C. After the time period of incubation is over 1.5 ml of DMSO was added for 15 min. The amount of purple formazan formed was measured by spectrophotometer at 570nm by using DMSO as blank. The assay was done on the first day followed by every 7th day for a period of 5 weeks. Similar procedure was followed for collaflex and retinoic acid for different modes (Jabbar *et al.,* 1989).

4.2.7 Glycosaminoglycan (GAG) estimation

The estimation of GAG secreted by chondrocytes on scaffolds in all modes was analyzed by dimethyl methylene blue (DMMB method). The scaffold seeded with chondrocytes were washed with PBS and stored at -20 °C. Then samples were lyophilized and dry weight of samples was taken. Overnight digestion of samples was carried out. The digest supernatant was collected in a tube and mixed with DMMB dye and absorbance was measured at 540nm. The assay was done for 5 weeks for all delivery modes along with control. The 2D modes

were processed in the same manner. Similar procedure was followed for collaflex and retinoic acid modes.

The GAG content was calculated by the formula

GAG Content (%) = [(Chondroitin- 4- sulfate (µg ml^{-1}) x dilution factor x papain Volume) / (dry weight of sample (g) x 10^6)] x 100

4.2.8 Collagen estimation

Hydroxyproline assay was used to estimate collagen synthesized by cells in all the different modes. Scaffolds were kept at -20 °C, lyophilized, the dry weight measured and digested by papain. The digest was collected in a vial and incubated at 120ºC in the oven overnight. The contents were poured into glass scintillation vial and incubated at 90°C till brown residue was formed at the bottom. Tubes were kept at room temperature and 1ml of sodium phosphate buffer was added. The resultant mixture was allowed to react with chloramine T solution at room temperature and mixed with 250 µl of pDAB and incubated at 60°C. The colored mixture was measured spectrophotometrically at 540 nm. The 2D modes were processed in similar manner (Bhat *et al.*, 2013). Similar procedure was followed for retinoic and collaflex. The total collagen content was determined by the equation.

Collagen content (%) = [(hydroxyproline (µg ml^{-1}) x dilution factor x proportion hydrolysed)

/ (dry weight of sample x 0.143x 10^6)] x 100

4.3 Results and discussion

4.3.1 Incorporation of ascorbic acid into gelatin spheres and SEM analysis

In this study we tried encapsulating AA into gelatin spheres. Not only we have tried to encapsulate AA inside spheres but we have synthesized different modes of its delivery so that their overall effect on chondrocytes proliferation and cartilage matrix synthesis can be studied. The novelty of this work is that different modes were tried for cartilage matrix.

SEM analysis showed that microspheres were smooth with rounded surface. Microspheres were in the range of 50-200µm, as shown in Figure 4.1. The size is important as it determines the release of the drug.

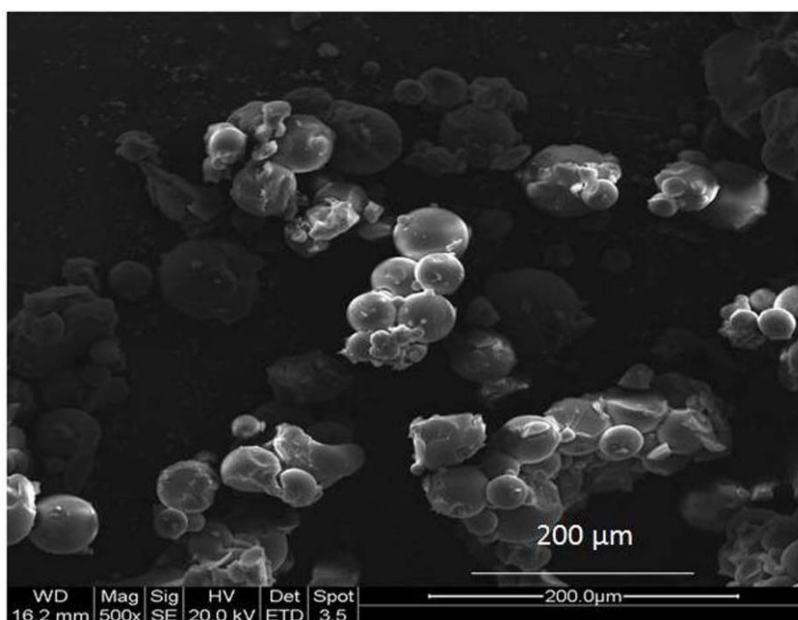


Figure 4.1) Scanning electron microscopy analysis of ascorbic acid encapsulated in microspheres

4.3.2 *In vitro* release profile

Release profile depends on many factors, like polymer hydrophobicity, particle size, stirring speed, drug concentration, the concentration of crosslinker and particle coating. Hence, *in vitro* release profile can be controlled by standardizing these parameters (Yang *et al.,* 2001).

We tried studying encapsulation pattern of AA encapsulated in gelatin spheres. The release of AA from microspheres was estimated by sodium oxalate as stabilizer**,** as shown in Figure 4.2. The pattern was studied and it was observed that drug was released for 35 days, which is a good parameter obtained as in our study it would be beneficial because cartilage repair is a slow process, so the overall slow release of drug and for longer time period is a added advantage. A good observation was that more than 90 % of the drug was released by end of 35 days, so the drug was utilized properly and didn't added to the cost of production. Therefore, by this method we were successful in encapsulating AA and achieve its sustained release for specific time period. Initially there is a sudden burst release, then release is maintained for a time period of 35 days. 95 % of drug was released till time period of 35 days.

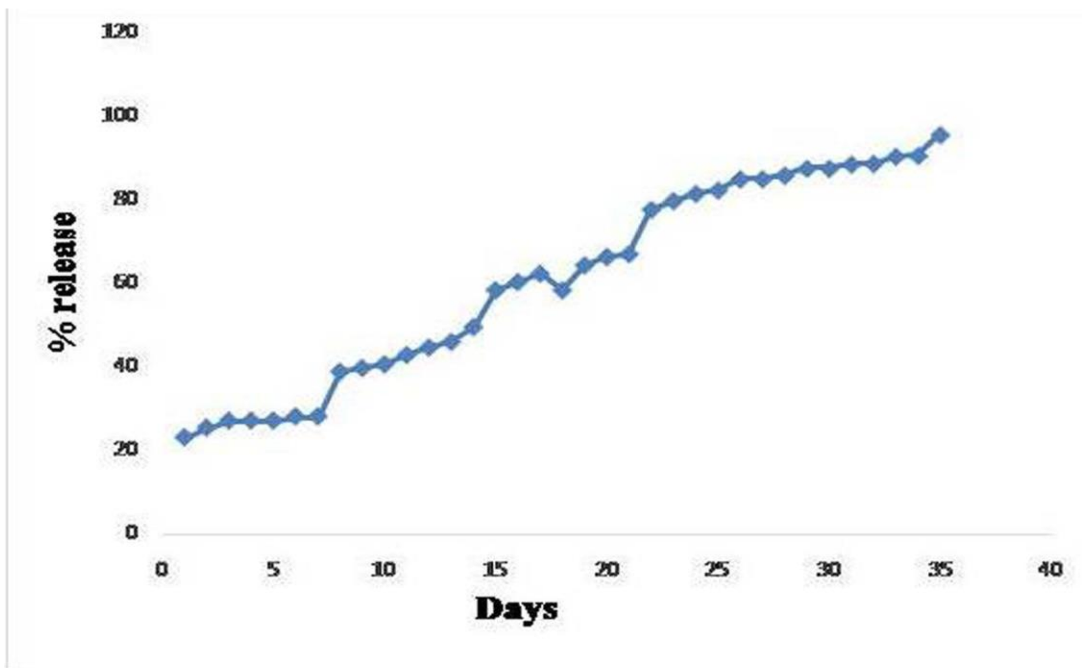


Figure 4.2) Percentage release of ascorbic acid from gelatin spheres

4.3.3 MTT assay

Different delivery modes with 2D (2D, 2D ex, 2D asc o) and with 3D (CG, CG ex, CGM, CG asc o) were studied for cell viability and biocompatibility assay. As shown in Figure 4.3, it was observed that when 2D and 2D ex were compared the proliferation was more in case of 2D ex this may be due to continuous supply of AA in a sustained manner and in 2D after 21 days the cell proliferation goes down this is due to confluency attained as in 2D there is no much space for cell to proliferate well and may lead to accumulation of toxic waste and cell death. In case of 2D ex also there was low cell proliferation level after 21 days but it was more than 2D this can be due to AA stimulating cell proliferation level (Ronzière *et al.,* 2003). As compared to other control 2D asc o where the same amount of drug which was encapsulated in microspheres was supplied in free form the proliferation is high in the first week, after which the proliferation rate decreased, which may be due to AA degradation, or instability. This shows that synthesized 2D ex proved to be more advantageous where AA could be protected and made available to chondrocytes for longer time duration. We also studied the 3D mode, Figure 4.3 we designed this mode so that in this case both support in form of scaffold could be provided along with supplementation of bioactive molecule (AA). when we compared CG (control) with CG ex, there was more proliferation in case of CG ex than in case of CGM where AA encapsulated in gelatin spheres was embedded in scaffold here also increase in chondrocyte proliferation as compared to CG, this may also be due to free AA released in sustained manner (Kim *et al*., 2003). When compared with other control CG asc o in that proliferation was there but it decreased after few weeks, because the drug was not available. But the novel drug delivery system we designed CG ex and CGM supported increased chondrocytes proliferation and could be maintained for longer time

duration, thereby achieving sustained. When CGM and CG ex were compared the proliferation was slightly lower in case of CGM, which might be because the microspheres were embedded so release slow which is beneficial in our case because cartilage repair is slow process. Thus among the different modes we synthesized in 2D and 3D exogenous mode (2D ex and 3D ex) and where microspheres was embedded in scaffold (CGM) in all the cases there was sustained increase in proliferation so both of these modes worked better. But there would be an added advantage of 3D mode which will also provide support for attachment of cells.

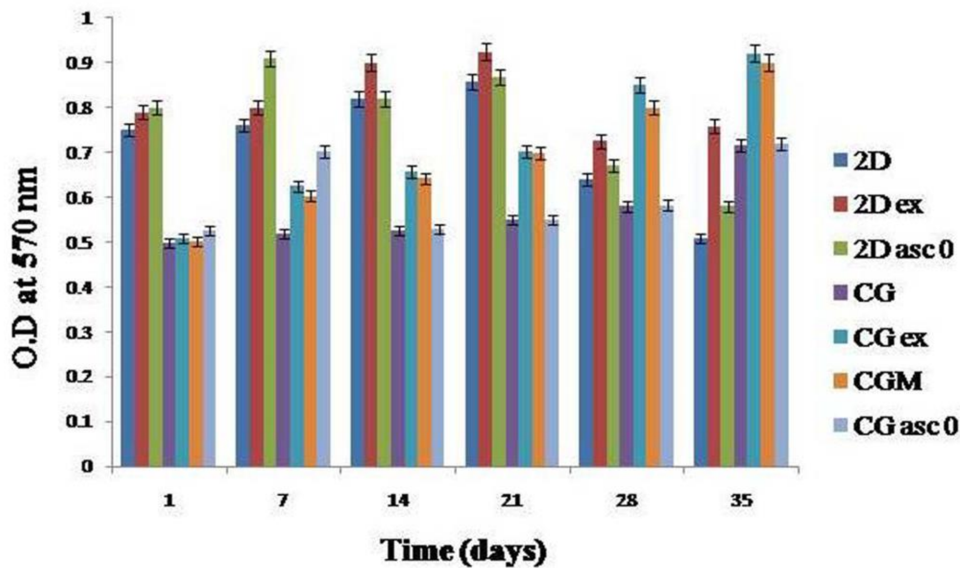


Figure 4.3) MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, assay for different modes 2D (Chondrocytes seeded on monolayer 24 well plate), 2DX (collaflex added exogenously on 2D, CG (chitosan-gelatin Scaffold),CGX(chitosan –gelatin with collaflex exogenously. All the experiments were performed in triplicates and p-value ($p < 0.05$)

For collaflex when we compared 2D and 3D there was more proliferation in 3D with exogenous added drug (CGX). In 2D there was proliferation in the beginning but after some time period it decreased which may be due to build up of toxins, as shown in Figure 4.3 (a).

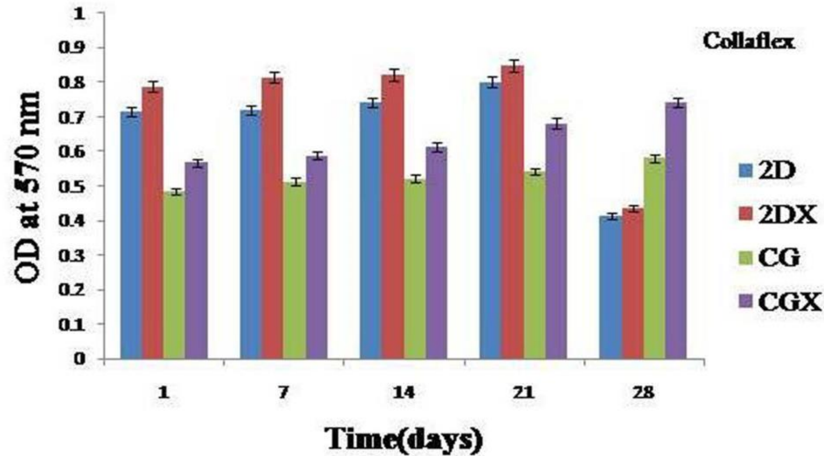


Figure 4.3 a) MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, for collaflex in 2D and 3D. 2D (Chondrocytes seeded on monolayer 24 well plate),2DX (collaflex added exogenously on 2D, CG (chitosan-gelatin scaffold),CGX(chitosan –gelatin with collaflex exogenously. All the experiments were performed in triplicates and p-value ($p< 0.05$)

For retinoic acid the concentration range .01, 0.1, 0.5,1, 1.5, 2, 2.5,3 (µM) was tested on 2D. Stimulation was observed in 0.5 (µM), so this concentration was used for further testing. There was cell death observed at concentration above 0.5 (µM), this may be due to excess retinoic acid which may impair proliferation and differentiation of chondrocytes (Li *et al.,* 2014)

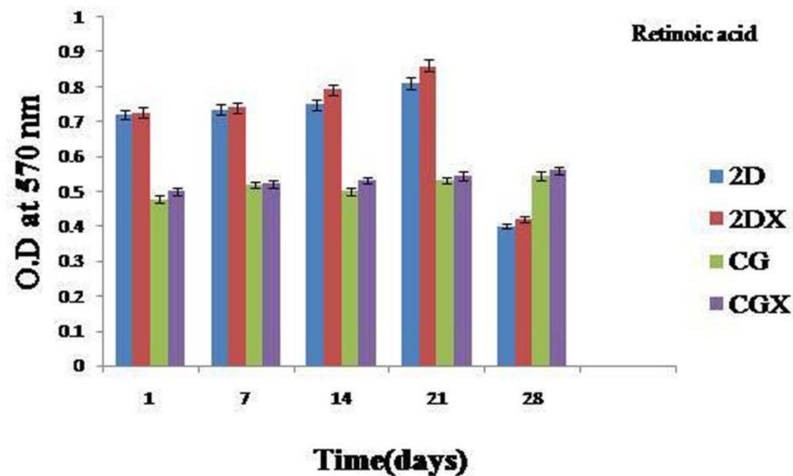


Figure 4.3 b) MTT*, *3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, assay for retinoic acid in 2D and 3D. 2D (chondrocytes seeded on monolayer 24 well plate), 2DX (retinoic acid added exogenously on 2D, CG (chitosan-gelatin scaffold), CGX (chitosan – gelatin with retinoic acid exogenously. All the experiments were performed in triplicates and p-value (p< 0.05)

Further concentration at which stimulation was observed was used for 3D and 2D for about 1 month. As shown in Figure 4.3 (b) it was observed, that there was a slight increase in proliferation in 2DX and CGX as compared to 2D and 3D. No significant increase in proliferation shows the limitation of retinoic acid to stimulate chondrocytes growth so showing the limitation of this system with exogenous retinoic acid.

4.3.4 GAG estimation

In 2D mode the GAG production was studied, 2D asc o was used as a control. When compared 2D ex with 2D there was increased rate of GAG production where microspheres with AA were added, as shown in Figure 4.4. This might be due to AA role in increasing GAG production (Sommer *et al.,* 2007).

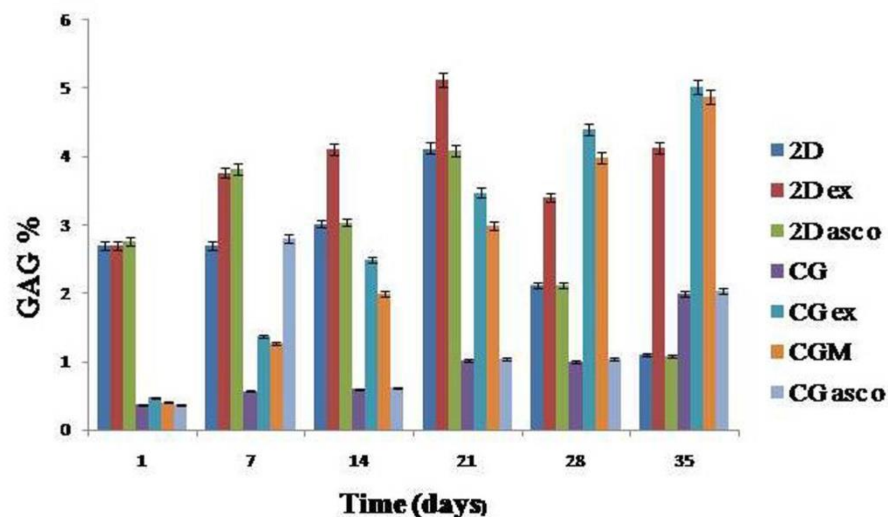


***Figure 4.4*) GAG(glycosaminoglycan) production of all different modes, 2D (Chondrocytes seeded on monolayer 24 well plate), 2DX (collaflex added exogenously on 2D, CG (chitosan-gelatin scaffold),CGX(chitosan –gelatin with collaflex exogenously. All the experiments were performed in triplicates and p-value ($p < 0.05$)**

The mechanism might be that ascorbic acid may serve as sulfate career in GAG metabolism or may inhibit arylsulfatase B, arylsulfatase is an enzyme that helps in removal of sulfates group from GAGs (Kao *et al*., 1990). But in case of the control, 2D asc o there was an increase in GAG production for a few weeks and then the production decreased. This might be due to instability of AA instability or its degradation. Thus the approach to encapsulate the AA will make it available for longer time duration has advantage over the free supply of the drug. Here the drug is utilized more properly and available for longer time duration. So these results in which AA is available in microencapsulation for a longer time might be due to AA protection, retard its core release, mask its acid taste, color change or prevent AA instability. So because of using microencapsulation technology many of the useful properties

of AA such as help in stimulating cartilage matrix production can be protected and utilized in an efficient manner (Uddin *et al.,* 2001).

We also synthesized 3D delivery mode so as to provide scaffold support to the cells so that they can attach and proliferate in a better manner, moreover the scaffold could be alternate support for damaged ECM until the time the native cartilage formation takes place. Here the scaffold would not only serve to provide support to chondrocytes but also act as a reservoir of bioactive molecule that can be supplied slowly (Hofmann and Garcia 2011). When CG was compared with CG ex, there was increase in GAG production with microspheres containing AA, which may be attributed to the role of AA in increasing cartilage matrix components like GAG (Ibold *et al.,* 2009). Moreover, synthesis of CGM was another approach where microspheres with AA were embedded in scaffold and here the GAG production was more than control CG but lower as compared to CG ex, there was 0.97 fold decrease in CGM as compared to CG ex, which might be attributed to microspheres being embedded inside scaffold resulting in slow release, Figure 4.4**.** The approach of encapsulating AA in microspheres and further embedding them inside scaffold provides two advantages first is the scaffold and second sustained release. Moreover from the above results it can be inferred that this approach utilizes the AA drug more efficiently and helps in extracellular GAG production.

When GAG production for collaflex drug was estimated in 2D and 3D at stimulating concentration. GAG production was higher in 3D, as shown in Figure 4.4(a). This may be because the scaffold (3D system) provides support to cells so that they can attach and secrete matrix. Rousselot 2011 showed the role of collaflex in enhancement of cartilage matrix markers, using 3D system. There was 1.4 fold increase in GAG production in CGX (CG

scaffold with collaflex drug) when compared to CG (scaffold). This shows the combination of collaflex with scaffold (CGX) works better than scaffold (CG).

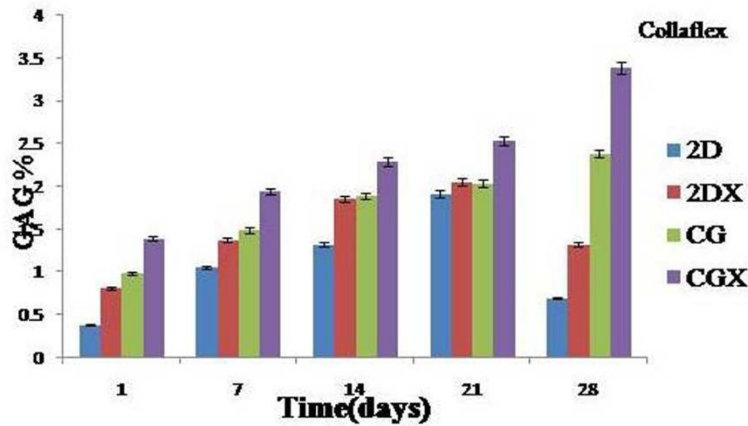


Figure 4.4a) Estimation of GAG(glycosaminoglycan) by DMMB(dimethyl methylene blue) for various modes 2D (chondrocytes seeded on monolayer 24 well plate), 2DX (collaflex added exogenously on 2D, CG (chitosan-gelatin scaffold),CGX(chitosan –gelatin with collaflex exogenously. All the experiments were done in triplicate and p-value ($p < 0.05$)

For retinoic acid, as shown in Figure 4.4(b), GAG production was analyzed with stimulated concentration (X), in 2D and 3D. There was slight increase in GAG production at stimulated concentration in both cases GAG.

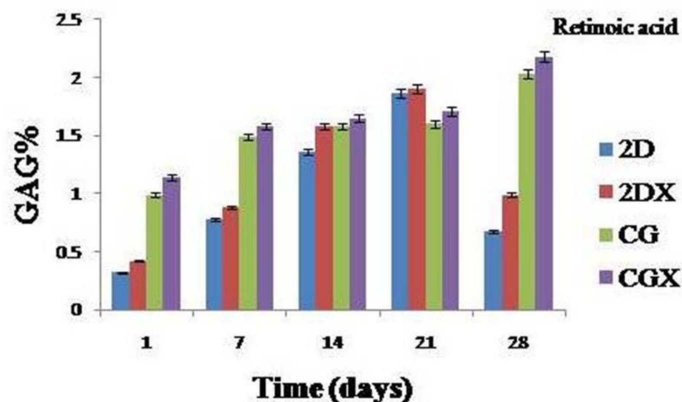


Figure 4.4 b) GAG(glycosaminoglycan) productions in retinoic acid. 2D (chondrocytes seeded on monolayer 24 well plate), 2DX (retinoic acid added exogenously on 2D,CG(chitosan-gelatin scaffold),CGX(chitosan-gelatin with retinoic acid exogenously. All the experiments were done in triplicate and p-value (p< 0.05)

4.3.5 Collagen production

In 2D mode the collagen production was studied for different modes of ascorbic acid such as 2D, 2D asc o and 2D ex. When compared with 2D, 2D ex exhibited more collagen production. There was 1.48 fold increases in collagen production in 2D ex as compared to 2D. This may be attributed to the role of AA in collagen production. AA plays a very critical role in maintenance of normal mature collagen network (Boyera *et al.,* 1998). The probable mechanism is that collagen peptide synthesis, post translational hydroxylations and activities of two hydroxylases are regulated by ascorbic acid (Murad *et al.,* 1991). We also synthesized CG, CG asc o, CG ex , CGM . Collagen production in CG ex was more than CG which may be due to presence of AA as shown in, Figure 4.5. There was 1.27 fold and 1.4 fold increase in collagen production in CG ex and CGM as compared to CG. This shows higher efficiency of these two delivery modes in 3D system. The presence of AA is helps in increasing the collagen production, whereas the CG asc o where the drug is supplied once there was an initial increase in collagen production for few days with a subsequent decrease which might be due to instability of AA.

In case of CGM, the collagen production was more as compared to CG which shows that AA in form of microencapsulation could provide the drug in a sustained manner and for longer time duration which could be an added advantage (Robertson and Schwartz 1953).

The present approach of encapsulating AA could provide better and increased collagen production for long time duration.

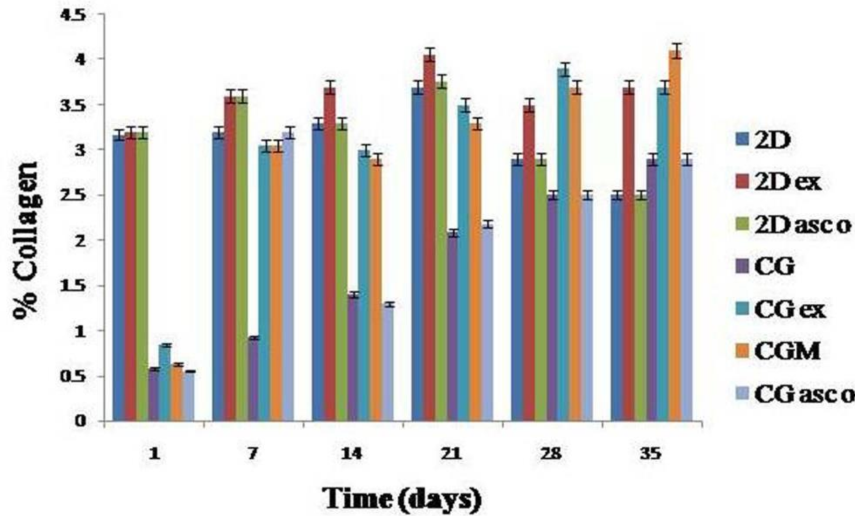


Figure 4.5) Collagen production estimation by hydroxyproline method for various modes. All the experiments were performed in triplicates and p-value ($p < 0.05$)

Collaflex is unique blend of hydrolyzed collagen which may improve joint flexibility. In collaflex and 3D (scaffold) combination there was higher production of collagen in 3D this may be due to collaflex because they themselves are collagen peptide formed by enzymatic degradation of collagen molecules, moreover collaflex improves the health conditions by stimulating collagen synthesis, blocking the damage caused by free radicals thus heals articular cartilage wounds.

There was 1.6 fold increases in collagen production observed when drug was combined with scaffold (CGX) than CG, as shown in Figure 4.5(a). This shows the applicability of these scaffolds.

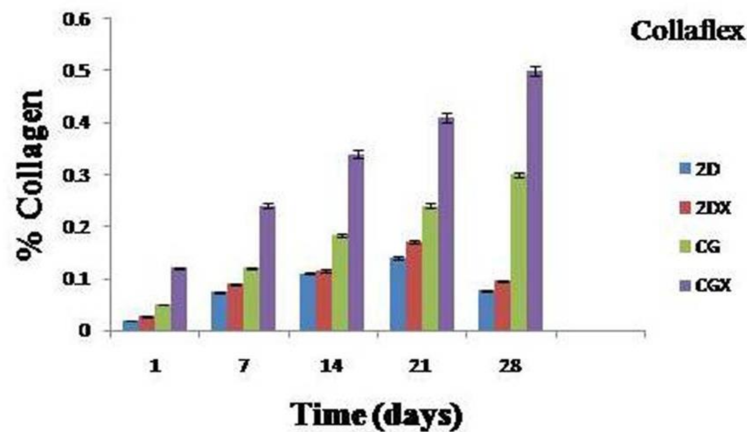


Figure 4.5 a)Collagen production with collaflex drug. 2D (chondrocytes seeded on monolayer 24 well plate), 2DX (collaflex added exogenously on 2D, CG (chitosan-gelatin scaffold),CGX (chitosan –gelatin with collaflex exogenously. All the experiments were performed in triplicate and p-value ($p < 0.05$)

Collagen content was estimated at stimulated concentration of retinoic acid in 2D and 3D. There was not much increase in Collagen content, as shown in Figure 4.5(b). So this might be due to retinoic acid not helping much in increase in extracellular matrix production.

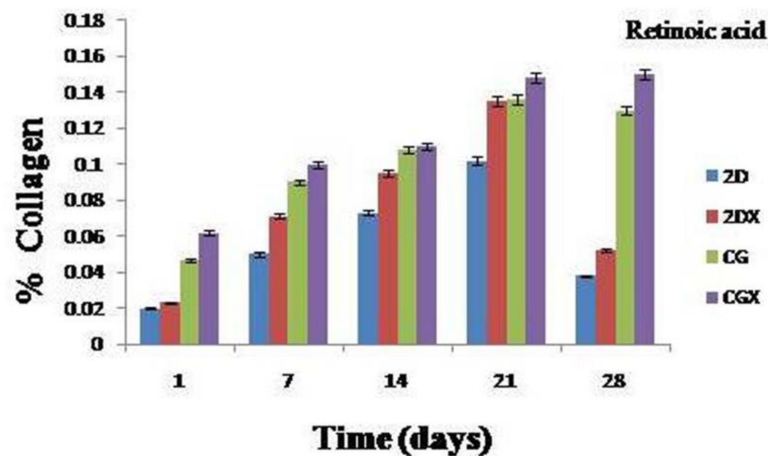


Figure 4.5 b) Collagen productions with retinoic acid. 2D (chondrocytes seeded on monolayer 24 well plate), 2DX (retinoic acid added exogenously on 2D, CG (chitosan-gelatin scaffold),CGX(chitosan–gelatin with retinoic acid exogenously. All the experiments were performed in triplicate and p-value ($p< 0.05$)

4.4 Conclusion

Because of instability and lack of protection of AA in free form we have encapsulated AA in the microspheres. AA was encapsulated in the microspheres and then its release was studied by *in vitro* release assay. We observed that drug could be made available for longer time duration which would be beneficial in cartilage damage as repair takes time.

Here different delivery modes were analyzed and studied which included both 2D and 3D scaffolds. They all were analyzed to study chondrocyte proliferation behavior and cartilage matrix behavior. What we observed that wherever the AA was encapsulated inside microspheres there was better cell proliferation and increase in matrix production compared to control when AA was supplied exogenously in free form. This shows that our approach to encapsulate AA inside microspheres could not only provide us with sustained release of AA and increase in GAG and collagen production, but it could work better than free form of the drug. The free form of the drug could not be made available for sustained time period and matrix production also decreased, but in the case of microencapsulation of AA there was better chondrocyte proliferation and also enhanced matrix production with sustained release. This approach with different AA delivery mode can provide new insight for treating OA. Also these modes can be further improvised and can be tried for different *invivo* models or osteoarthritic animal models.

Collaflex drug showed an increase in matrix production when combined with scaffold and for longer time duration. Retinoic acid effect was also studied but there was not much increase in extracellular matrix production.

Chapter 5

Role of Gold Nanoparticles as Drug Delivery Vehicles for Chondroitin Sulfate in the Treatment of Osteoarthritis

Chapter 5

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5.1 Introduction

There is a challenge to cure chondral injuries because of the complex structure and biochemical function of articular cartilage. Due to avascular nature articular cartilage has limited self repair capacity. The acute and repetitive impact and torsional joint loading that occurs due to obesity, age, sports injuries, and heredity can damage articular cartilage surfaces causing pain, joint dysfunction and effusions. Sometimes if damage continues it can lead to progressive joint degeneration known as OA (Safran and Seiber, 2010). Repair process occurs but never has been documented for stable regeneration. There is a huge challenge for an orthopedic surgeon for treatment options for cartilage lesions in the knee. A wide range of options are available such as simple arthroscopic interventions, marrow tapping techniques, osteochondral auto grafting, cell based techniques, growth factors and newly emerging gene therapy techniques (Falah *et al.,* 2010). The mechanism of osteoarthritis begins with the injury depiction and unstable joint. The instability increases the sliding between joint surfaces and lowers the efficiency of muscles. Eventually, there is an uneven distribution of load in cartilage and underlying bone, causing wear and shear, which leads to osteochondral degeneration. The trigger point which leads to mechanical process is the inflammatory reaction triggered by the injury and sustained during healing. The onset continues and inflammation could be responsible for start and progression continues which

are accelerated by mechanical factors like load due to obesity. The full mechanism leading to OA has not been fully established. Considering that OA is likely to occur despite available treatment options, the best solution may be prevention or control at the early stage of primarily treatment options like drugs (Fleming *et al.,* 2005).

Primarily, treatment options in the form of drugs are available for reducing pain and inflammation. Several classes are available for OA therapy including corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and aspirin are agents with potential disease modifying properties and nutraceuticals (Bhatia *et al.,* 2013). Paracetamol is the first line of pharmacologic mediator for the treatment of OA, it is effective for pain relief in OA. Non steroidal anti-inflammatory drugs are commonly associated with first line treatment for OA pain along with exercise and weight loss. They usually promote the healing of damage cartilage in the knee or to maintain the composition of the synovial fluid. Selective and nonselective cyclooxygenase (COX) inhibitors have antipyretic, anti inflammatory and analgesic effects and are generally used in curing many aching conditions. The availability of NSAIDs is easy and in prescription products which includes ibuprofen, naproxen, diclofenac, and celecoxib (Laar *et al.,* 2012). Acetaminophen is taken regularly every day, whereas NSAIDs should be given at the lowest effective dosage for the shortest period of time. On the basis of a meta-analysis of clinical trials, NSAIDS are superior to acetaminophen. Another cream like capsaicin derived from pepper plant is effective in managing pain and can exert further benefits and can be an alternative to oral/inflammatory agents (Heidari, 2011). Prolonged use of such drugs has been associated with side effects causing gastro-intestinal tract erosion, ulcers, deleterious renal effects, problems with liver and kidney, and sensory problems (Hochberg and Dougados, 2001).

A problem linked with presently available drug side effects along with increasing knowledge of cartilage biochemistry and OA pathogenesis, has focused research in reducing the progression of OA and promoting cartilage matrix synthesis. So the current focus has been identified with term known as chondroprotective agents. The major function of the agent is to stimulate chondrocyte, synthesis of collagen and proteoglycans, inhibit cartilage degradation and prevent fibrin formation. Such chondroprotective agent includes hylauronic acid, glucosamine and chondroitin sulfate (Van *et al.,* 2012). This new concept for helping chondrocytes is to heal cartilage, rather than depend on analgesics. Amongst the two major chondroprotective nutrients are glycosaminoglycan (GAG's) and antioxidants. GAGs are repeating sugar units with sulfate groups and one type sugar , most common of GAG is chondroitin sulfate it is both available as a nutrient and as a drug. The major advantage of GAGs are providing precursors for GAG synthesis, direct inhibition of degradative enzymes such as elastase, collagenase, chondroitinase, stimulation of anabolic metabolism of chondrocytes (Busci, 1990).

Despite the potential of CS in treating OA, very little is known about the bioavailability and anti-inflammatory activity and its uptake mechanism (Surapaneni *et al.,* 2014). CS inhibits synthesis of various inflammatory intermediates such as nitric oxide, cyclooxygenase and prostaglandin. CS also possess anabolic effect as it increases the hylauronate in synovial and increase type 2 collagen and proteoglycans in human chondrocytes (Omata *et al.,* 2000).

Besides, continuous administration of CS for a stipulated time period has been reported to provide a remnant effect for a prolonged duration, a distinct advantage over analgesics and NSAIDs. However, an important limitation associated with its use is its poor absorption

through the digestive system leading to non-uniform distribution to cartilage, poor stability, and low solubility (Monfort *et al.,* 2008). On intravenous administration, its half life in blood is 3-15 min due to which it is not systemically (or effectively) distributed to articular cartilage (Johnson *et al.,* 2001). The delivery of medical agents to the human body for healing purpose is not new ,while in olden times the drug was delivered from hand to mouth , perhaps also by rubbing into skin, or placing on a wound. But in recent times, different vehicles are being developed like microparticles, nanoparticles, liposomes and other (Eberhard, 2008). Drug delivery systems like PLGA and gelatin are being explored for the possibility of encapsulating CS for improved distribution and bioactivity (Jiang *et al.,* 2011). Currently, nanoparticles are widely used for delivery of various bioactive molecules for improved drug efficacy, targeted delivery, reduced toxicity, and increased uptake (Malam *et al.,* 2009). Various methods are available for synthesis of nanoparticles with controlled size and morphology which enables the entrapment of a wide variety of bioactive molecules and their controlled release (Lohcharoenkal *et al.,* 2014).

The most potential point is that nanoparticles with optimized physicochemical and biological properties are up taken by cells more easily than larger molecules, so they can be productively used as delivery tools. The most important strategies to be concerned are the way of conjugating the drug to nanocarrier. A drug or bioactive molecule may be adsorbed or covalently attached to nanocarriers surface or else it can be encapsulated into it. The cell–specific targeting with nanocarriers may be achieved by using active or passive mechanisms (Wilczewska *et al.,* 2012).

So by using the nanomaterial as drug delivery vehicle can potentially reduce the drug dose needed to achieve therapeutic benefit which will lower cost and side effects. Also there can

be high flexibility in changing the size of nanoparticles to achieve passive and active drug targeting. Further nanoparticles (NPs) can also control and sustain release of drug during transport, or at the site of localization, changing drug distribution and consequent clearance of drug in order to improve therapeutic efficacy and decrease side effects. So from all the above points we can infer that nanotechnology could be strategically implemented in developing new drug delivery systems. Such a strategy can be applied to a drug which has poor biopharmacological properties, poor solubility (Bamrungsap *et al.,* 2012).

Nanoparticles have already been used as tool for drug delivery vehicle for many diseases. For example in the case of cancer like targeting anticancer drug delivery to pancreatic cancer cells, iron oxide nanoparticles as drug delivery vehicle for magnetic targeting of brain tumors (Yoshida *et al.,* 2012; Chertok *et al.,* 2008). Nano based drug delivery in mucosal immune diseases (Cario, 2012). There are major classes of nanoparticles delivery systems including liposomes, polymeric nanoparticles, lipid-polymer hybrid nanoparticles, silver and gold nanoparticles which can encapsulate drugs with high loading efficiency and protect them from undesired effects of external factors (Swami *et al.,* 2012).

Gold nanoparticles (AuNps) are promising nano carriers for drug delivery due to their unique properties like facile synthesis, functionalization, biocompatibility, ease in cell permeability, and high drug loading capacity (Kim *et al.,* 2009). Moreover, AuNps are also known to exhibit antioxidant properties by quenching reactive oxygen species (Barath, *et al.,* 2010). These properties have contributed to the use of AuNps in the treatment of conditions such as Alzheimer's, diabetes and arthritis. They have also been used as a drug delivery vehicle for cancer, heart failure, and arthritis (Dreaden *et al.,* 2012; Cheng *et al.,* 2013; Aryal *et al.*, 2009, Spivak *et al.,* 2009, Prades *et al.*, 2012, Leonavičienė *et al.,* 2012, Lee *et al.,* 2014).

Currently nanodiagnostics concepts focus around utilization of nanoscale properties for ultrasensitive biomarker development. Unmet diagnostic and therapeutic need in arthritis is there. Effective treatment in osteoarthritis are based on early detection of diseases nanotechnology has the potential to meet these needs. Nanotechnology has found potential in every possible field of science and engineering; it works on the synthesis of tools at nanometer scale which can be expected to function more effectively than micro and macro system. The major limitation in OA disease is hampered by side effects, and lack of suitable drug delivery systems that target, retains and deliver drugs locally. So there are many chances and potential that these challenges can be overcome by nanotechnology (De and Borm, 2008). In brief the rationale for using nanoparticles as another part of our strategy was to use potential of these particles to help in providing drug in much more efficient manner and to reduce the toxicity which will also help to bring down the cost of osteoarthritis treatment.

In this study we have synthesized Gold nanoparticles and characterized by TEM , XRD, Uv-Vis. and was evaluated using goat chondrocytes, and studied using various cell culture assays like MTT, GAG, collagen and hoescht staining.

5.2 Materials and Methods

Materials

Gold (III) chloride trihydrate ($HAuCl_4$ $3H_2O$), chondroitin sulfate, Dulbecco modified eagles medium (DMEM), collagenase type 2, penicillin-streptomycin, nystatin, papain, trypsin-EDTA solution, 1, 9 dimethyl methylene blue, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO, USA).

Sodium citrate was obtained from Merck (Mumbai). Fetal bovine serum was purchased from Hyclone (UT, USA). All chemicals used were of analytical grade.

Methods

5.2.1 Synthesis of gold nanoparticles

AuNps were synthesized according to the method described by Ramanaviciene *et al.*, using sodium citrate as the reducing agent. An aqueous solution of tetrachloroauric acid $HAuCL_4$ (1%w/v) was dissolved in 19.75 ml of deionized water. Sodium citrate (1 ml of 1% w/v) was added to the above solution with constant stirring (1000rpm) and heating (100°C). The heating and stirring were continued for further 5 min till the gold salt was completely reduced and the solution turned wine red in color (Ramanaviciene *et al.,* 2009).

Characterization

5.2.2 XRD analysis

It is diffraction of X- rays by regularly spaced atoms of a crystal, valuable for determining the arrangement of atoms. X-rays diffraction (XRD) data of the AuNps and also AuNps-CS combination were recorded using Miniflex II desktop X-ray diffractometer at room temperature in order to determine the crystallographic structure of gold nanoparticles (Park et al., 2000).

5.2.3 Ultra violet-Visible spectra (UV –Vis spectra)

UV-Vis spectra are often used as a supplementary technique for characterization or monitoring nanoparticle base system in suspensions and some of processes occurring there in.

UV-Vis spectra of colloidal gold and there combination (AuNps-CS) were obtained on spectrophotometer with a wavelength range of 400-800nm (Permyakov, 2012).

5.2.4 Transmission electron microscopy (TEM)

TEM has been found to be an excellent tool for characterizing the size of nanoparticles. TEM becomes an attractive method for the characterization of the size distribution of colloidal nanoparticles since it can provide size parameter.

For the TEM analysis a drop of AuNps was placed on carbon coated copper grid and dried under infrared lamp. The size and morphology of gold nanoparticles were determined using Transmission electron microscope (TEM) (Philips CM200).

5.2.5 Infrared spectrum

An important tool for the organic chemist is infrared spectroscopy, or IR Spectra are calculated by a special instrument, called IR spectrometer. IR is used to compile information about the structure and purity of compound. IR spectra are quick and easy to run. The infrared absorption spectrum of AuNps and AuNps-CS were analyzed using FT-IR Shimadzu IR Affinity-1 spectrophotometer in the 500-4000cm-1 frequency range.

5.2.6 Zeta potential

Zeta potential analysis is a technique designed for knowing the surface charge of nanoparticles in solution (colloids). The electrical potential at the boundary of the double layer is defined as zeta potential and has a value that ranges from +100Mv to -100Mv (Tantra *et al.,* 2010). Zeta potential was measured using zeta potential analyzer (Delsa™ Nano).

5.2.7 Chondrocytes isolation and maintenance

Chondrocytes were isolated from 10-12 month old goat articular cartilage region. Goat knee was obtained from the local slaughter house. The joint obtained was further processed under aseptic conditions by spraying 70% ethanol to remove surface contamination. Using sterile scalpel blades the cartilage was exposed. The cartilage pieces were collected in a sterile tube

containing PBS and 1% antibiotic cocktail (penicillin, streptomycin and gentamicin). The cartilage pieces were diced in a petriplate, collected in a centrifuge tube, washed thrice with a PBS-antibiotic solution and incubated with 0.25% trypsin-EDTA solution for 30-45 min on rotor shaker at 37°C. Repeated washes with PBS were given to remove trypsin. Then the samples was incubated for 16 h in collagenase digestion media (containing 0.3% collagenase type 2, DMEM, FBS, antibiotic). The cell suspension obtained was inoculated into a culture flask containing DMEM with 10% FBS and 1% antibiotic and incubated at 37°C with 5% CO_2 till they reached confluency (Bhat *et al.,* 2013). The chondrocytes were sub-cultured using trypsin-EDTA and maintained for further seeding. The chondrocytes were trypsinized and used for further experiments at a cell density of $1x10^5$ cells/ml.

5.2.8 In vitro effect of AuNps, CS and AuNps-CS on chondrocytes by MTT assay

Defined concentrations of AuNps, CS, AuNps-CS were selected (Table 1) for studying the synergistic effect of AuNps on CS for the bioactivity of goat chondrocytes. Goat chondrocytes were seeded in DMEM medium at a cell density of $1x10^5$ cells/ml in a 24 well plate and incubated for cell growth and attachment. Appropriate concentrations of AuNps, CS, and AuNps-CS in DMEM were added to the attached chondrocytes and the cells were incubated for further *in vitro* studies.

5.2.9 Determination of cell viability in presence of AuNps, CS and AuNps-CS combinations

In order to check the effect of AuNps and AuNps-CS on proliferation rate of chondrocytes, MTT (3, 4, 5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium) assay was performed. This assay is based on the conversion of MTT into formazan crystals by living cells, which detects mitochondrial activity. Since for cell populations the overall mitochondrial activity is related

to number of viable cells, this assay is broadly used to measure the *in vitro* cytotoxic effects of drugs on cell lines. MTT assay was carried out, chondrocytes were seeded and treated with AuNps-CS, AuNps, CS, at various concentrations. The control contains cells with no addition of AuNps or CS. After incubation for 24 hrs, the media was discarded and 500µl of 0.5mg/ml MTT was added to each well and incubated at 37 °C for 4-5 hrs. Subsequently, the supernatant was discarded and 1.5 ml of DMSO was added to the cells and kept for 15 min for the formation of formazan. The purple formazan product was measured spectrophotometrically at 570 nm using DMSO as blank (Stockert *et al.*, 2012).

5.2.10 Hoechst staining

Hoechst(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a dye used to stain DNA in eukaryotic cells. The cells were seeded on petriplates and incubated for 24 hrs. The growth media was discarded and the cells were given two washes with PBS. Cells were fixed with 10% PFA (paraformaldehyde) at room temperature for 2 min.

The fixed cells were given three washes with PBS. Permeabilization solution (0.1%Triton X-100 in methanol) was added to cover the surface of the cells and the plate was incubated for 1-2 hrs at room temperature. Finally, 1µl of Hoechst stain was added and incubated for 5min. The morphology of nuclei was observed using a fluorescence microscope.

5.2.11 Processing of samples for glycosaminoglycan (GAG) and collagen estimation

In order to determine the matrix production, cells treated with AuNps-CS were washed with PBS (pH 7.4) and pooled by trypsinization. Further, they were lyophilized and dry weight of samples was taken. Dry samples were then incubated in papain solution (1mg/ml) overnight for digestion. On completion of incubation, samples were estimated for GAG and collagen.

5.2. 12 Glycosaminoglycan estimation (GAG assay)

GAG was estimated by DMMB (dimethyl methylene blue) assay. From each of the papain digested samples the supernatant was collected, mixed with DMMB, and the absorbance was recorded at 540nm using a UV-Vis spectrophotometer. GAG was estimated using the formula

GAG content (%) = [(chondroitin- 4- sulfate (µg ml -1) x dilution factor

x papain volume) / (dry weight of sample (g) x 10^6)] x 100

5.2.13 Collagen estimation

Another important component of cartilage ECM is collagen. Hydroxyproline assay was used to estimate collagen. The supernatant after papain digestion was mixed with 250 µl of conc. HCL in glass scintillation tubes and kept in the oven at 120°C for 24 hrs followed by 90°C till a brown residue was formed. The tubes were cooled and the brown residue dissolved in 1ml of sodium phosphate buffer. Resultant solution was allowed to react with chloramine T solution at room temp for 20 min. Subsequently, 250µl of pDAB (para dimethyl amino benzaldehyde) was added and the solution was incubated at 60°C for 30 min. The samples were cooled to room temperature and the absorbance of the colored complex was measured spectrophotometrically at 540 nm. Hydroxyproline standards were similarly treated to obtain the standard curve. Collagen content was estimated using the formula.

Collagen content (%) = [(hydroxyproline (µg ml $^{-1}$) x dilution factor x proportion hydrolysed) / (dry weight of sample x 0.143x 106)] x 100

0.143 is the factor used to represent the percentage of hydroxyproline in total collagen[28.]

Statistical analysis: The experiments were carried out in triplicates and data are presented as the average of all samples analyzed and p value was calculated (Bhat *et al.,* 2013).

5.3 Results and discussion

5.3.1 Preparation of AuNps

AuNps were synthesized using sodium citrate as the reducing agent which was added to boiling solution of $HAuCl_4$. Gradually, the color of the solution changed from transparent to purple and after some time it changed to a deep-red wine color, indicating colloidal gold nanoparticles formation.

Color change occurs with reducing the size of particles as it approaches the nano scale range. Due to increased surface area of small nanosized particles, the properties of all materials change by reducing particle size, leading to increase in the surface to volume ratio of atoms, so this changes the physical properties of a material and formation of gold nanoparticles can therefore be observed by a change in color, that is red wine color, a shown in Figure 5.1. The layer of absorbed citrate anions on nanoparticle surface keep the nanoparticles separated, and the presence of this colloidal suspension can be recognized by reflection of a laser beam from particles. Moving to a smaller anion allows particles to approach more strongly and another color change is observed (Chen *et al.*, 2014).

In the past, various strategies have been used to synthesize gold nanoparticles organic and aqueous systems. Among these, synthesis of AuNps by citrate reduction is popularly used as it has the advantages of being inexpensive, nontoxic, and is a water based system (Nguyen *et al.,* 2010).

In the starting process gold is in solution in the Au^{+3} form , when we add the reducing agent that is sodium citrate, gold atoms are formed in the solution and their concentration increases

and till solution exceeds saturation. The residual dissolved gold atoms bind to nucleation sites and growth occurs.

Figure 5.1) Showing a wine red colored solution

5.3.2 TEM analysis for gold nanoparticles

TEM analysis has been found to be an excellent tool for characterizing the size of nanoparticles. The small size of nanoparticles has its importance in order to maintain their unique optical, electronic, thermal and chemical properties. And it's required to measure their size to understand nano-scale materials. Size, surface charge and shape determine various actions of nanoparticles including cellular uptake by reticuloendothelial systems, targeting to target cells, *in vivo* distribution and corona formation (Jo *et al.,* 2015). Particle size is one of the important key factors in the distribution of long circulating nanoparticles and achieving therapeutic efficacy. In one of study showed where nanoparticles of the size of 50 and 500 nm showed a higher level of agglomeration of larger nanoparticles in liver. Further it is shown that small size nanoparticles have a long blood residence time and high rate of extravasations into permeable tissues, showing the role of small size particles for achieving effective and targeted delivery (Alexis *et al.,* 2008). TEM analysis of the AuNps

showed that they were spherical with an average particle size of ~ 13nm, as shown in Figure 5.2 and might be this range of size could be suitable for achieving cellular effective uptake.

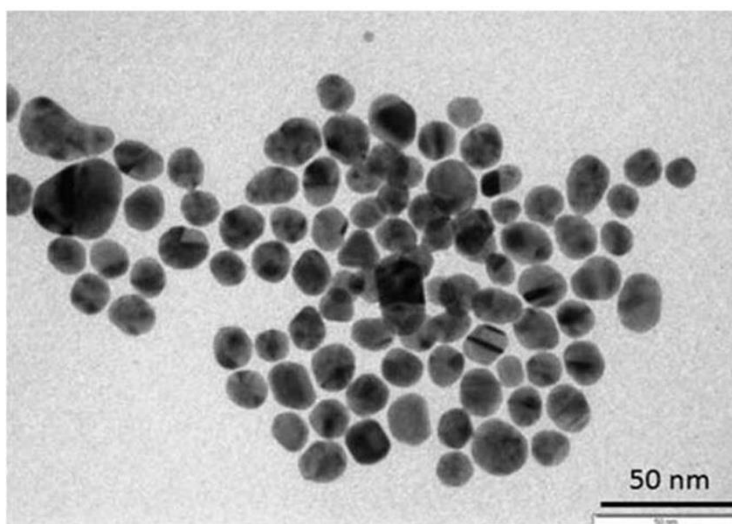

Figure 5.2) Transmission electron microscopy analysis of Gold nanoparticles

5.3.3 X ray diffraction of AuNps

XRD analysis is a unique method in determination of crystallinity of a compound. XRD is primarily used for determining the ID of crystalline material, ID of different polymorphic forms, distinguishing between amorphous and crystalline material, quantification of percent crystallinity of sample. Here monochromatic x-rays beams produce diffraction peaks scattered at specific angles from each set of lattice planes in sample. Peak intensities are determined by distribution of atoms within lattice. Overall the X ray pattern is a fingerprint of a periodic arrangement of nanoparticles or any other material. Bragg's law can be used to determine various distinctiveness of crystal or polycrystalline material. Measurements are made in angstroms; XRD avoids issues of representative samples and determining crystals as opposed to particles. XRD analysis was carried out for phase identification to elucidate the crystalline nature and structural information of the material based on unit cell dimensions. Diffraction peaks were obtained at 2θ values of 38.01, 44.4, and 64.5 corresponding to the

Bragg reflections of [111] [200] [220], confirming face centered cubic gold, as shown in Figure 3 (Jiang *et al.,* 2007).

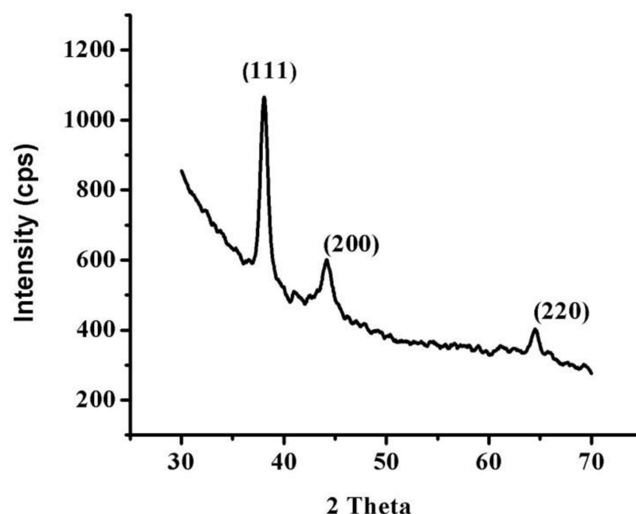

Figure 5.3) XRD (X-ray diffraction) analysis of gold nanoparticles

5.3.4 UV – Vis spectra of AuNps

Due to nanoparticles optical properties such as size, shape, concentration, agglomeration state and refractive index makes UV/Vis IR spectroscopy a valuable tool for identifying, characterizing them. Nanoparticles synthesized from gold and silver strongly interact with light of a specific wavelength and the unique optical properties of these materials are foundation for the field of plasmonics. The main advantage is that spectra technique is quick and cheap.

UV –Vis spectroscopy is a very valuable technique which determines gold nanoparticles size, concentration and aggregation level. The AuNps they do represent a distinct optical feature commonly referred to as localized surface Plasmon resonance (LSPR), that is the electrons oscillation in the conduction band of gold nanoparticles with a specific wavelength

of incident light. LSPR of AuNps does result in strong absorbance band in the visible region (500nm-600nm), which can be measured by UV-Vis spectroscopy. The spectrum is dependent on shape and size. With increasing diameter the peak absorbance wavelength also increases and in the case if nanoparticles are uneven or aggregated the absorbance spectrum shifts into far-red region of the spectrum (Abdelhalim *et al.,* 2012).

Optical properties of gold nanoparticles were studied using UV-Vis Spectra. The optical spectra of AuNps are shown in Figure 5.4. AuNps exhibited one intense peak with absorbance maxima at 530 nm, attributed to surface plasmon resonance of AuNps (Bac *et al.,* 2011).

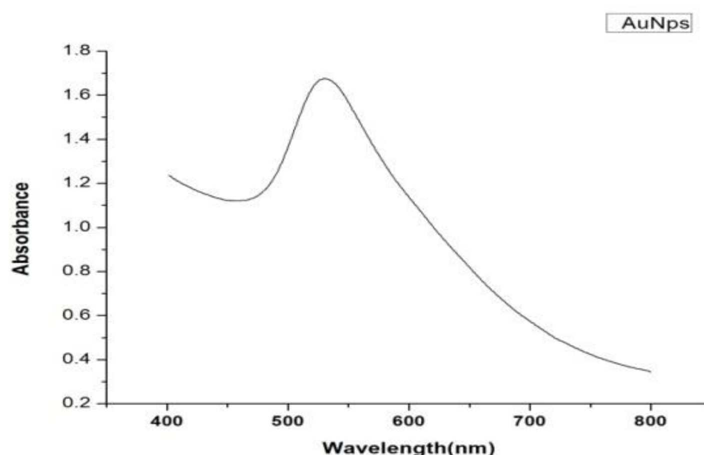

Figure 5.4) UV –Vis (Ultraviolet Visible spectroscopy) spectra of gold nanoparticles in the range of (500nm-600nm)

5.3.5 Gold nanoparticles and combination

AuNps display a mixture of physical, chemical, optical and electronic properties which are different from other biomedical nanotechnologies and provide platform from imaging and diagnosing disease, to selectively deliver therapeutic agents. These optical and electronic properties of AuNps are foremost important for use in multimodal drug delivery applications,

where these structures can manage increased drug pharmacokinetics/ biodistribution (Dreaden *et al.,* 2012).

One of the major advantages of AuNps that they are non-cytotoxic and also they have a large surface area where many molecules can bind to target area makes it useful in biomedical applications (Khan *et al.,* 2014).

Due to the small size nanoparticles they can easily travel into target cells and can bear high drug loading capacity. They have shown to bind with a wide range of organic molecules as they have reduced toxicity and tunable physical and chemical properties so can be used to deliver therapeutic agents (Lan *et al.*, 2013).

In addition, the nano-size facilitates easy entry into the cell interior without causing damage to cells (Cho, 2005). Encapsulation within gold nanoparticles also helps in prolonging the blood circulation time of drugs and may provide a synergistic effect due to their antioxidant properties (Park *et al.,* 2009). Already many therapeutic agents have been combined with gold nanoparticles and used to deliver to target site, like in the case of cancer disease. It is already known that treatment of cancer with chemotherapeutic agents has potential but dealing with many side effects. The drugs which are targeted to kill cancer often leads to damage to healthy cells in the nearby region. AuNps are versatile, biocompatible and they can be readily functionalized to serve as molecular vehicles. Many cancers treating drugs like doxorubicin, paclitaxel have been combined and used as therapeutic delivery tools (Cai, W *et al.,* 2008).

In this study, AuNps were combined with CS and tested for their efficacy to affect the bioavailability of CS to chondrocytes (AuNps-CS). Once the combinations were prepared

they were also analyzed by another technique like UV-Vis, FTIR and Zeta potential. It was done to estimate the change on AuNps and AuNps in combination with CS at all concentration tested the alteration in surface chemistry by FTIR, and changes in UV-Vis spectra upon interaction with CS and Measurement of Zeta potential.

5.3.6 UV-Vis spectra comparison of AuNps and AuNps-CS

In order to check that whether the combination of CS to AuNps is affecting the UV –Vis spectra because it is already known that if there are major shift spectra of AuNps it determines the aggregation level (Amendola and Meneghetti, 2009). If there is a major shift in spectra more is the instability in combination.

The UV-Vis absorbance spectrum of AuNps-CS exhibited a slight shift in the absorbance maxima to longer wavelength (537nm), as shown in Figure 5.5 (B) as compared to the absorbance maxima for AuNps, which may be due to the wrapping of CS around AuNps. Such a minimal shift is favorable as significant shifts to longer wavelengths indicate aggregation of AuNps. The absorbance profile of AuNps-CS was similar for all the combinations. This shows that combination didn't have much effect on their spectral properties. So this result is in favor of combination is not making much change in spectral properties so the spectral properties which determine the stability of AuNps is in the stable range so shows combination can be used further for another *in vitro* studies.

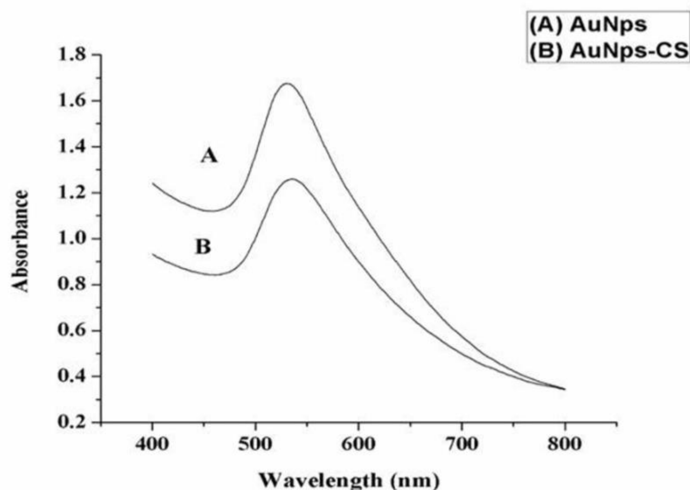

Figure 5.5) UV-Vis (Ultraviolet-Visible) Spectrum of A) AuNps(gold nanoparticles) and B) AuNps-CS(gold nanoparticles-chondroitin sulfate).

5.3.7 FTIR spectrum of AuNps and AuNps-CS

From FTIR spectrum we usually attain an infrared spectrum of absorption, emission, or raman scattering for solid, liquid, gas. The basic application is that it identifies molecular structures. When a material is exposed with infrared radiation, absorbed IR radiation excites molecules to higher vibration state (Makarov and Khmelinskii, 2011).

FTIR spectrum of AuNps was studied so in order to study the functional group present to confirm the formation AuNps. Then further the combination was also studied by FTIR to see whether the spectra are getting affected.

FTIR spectroscopy was used to confirm the presence of AuNps and CS in the combination. The IR spectrum of AuNps shows two distinct absorption bands at 2337 cm^{-1} and 1635 cm^{-1} (A), (B) which are characteristic absorption bands for AuNps, as shown in Figure 5.6(A) (Gangwar *et al.*, 2013, Narayan *et al*., 2014).

The IR spectra of AuNps-CS, as shown in Figure 5.6(B), exhibited the AuNps peaks (C) and (D) and an addition peak at 1132 cm^{-1} (E) which correspond to the stretching vibration of S=0 bond of CS (Thiruganesh *et al.,* 2011). No shift in the characteristic absorption bands of

AuNps indicates that conjugation with CS involves no significant changes to the molecular bonding of AuNps.

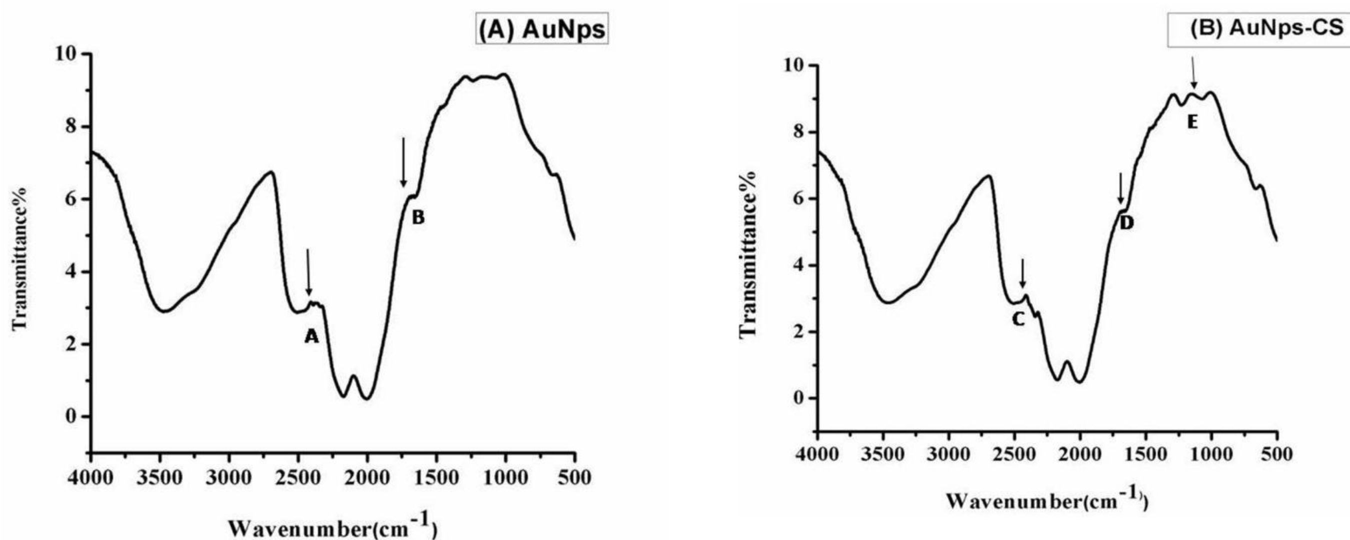

Figure 5.6) FTIR (Fourier Transform Infrared Spectroscopy spectrum)(A) AuNps and (B) AuNps-CS.

5.3.8 Zeta potential measurement

Zeta potential is one of the parameters that control electrostatic interaction in particle dispersions; overall it determines the stability of colloidal dispersions. So in order to study the long term stability this technique can be a key tool. The zeta potential of AuNps-CS was determined to check for their stability in aqueous systems. It was estimated to be -21.42 mV. The high charge is attributed to the negative sulfated polyanionic groups of CS which wrap around the AuNps and prevent their agglomeration forming stable AuNps-CS (Li *et al.*, 2011).

5.3.9 Treating Chondrocytes with AuNps, CS and AuNps-CS

Defined concentrations of AuNps, CS, AuNps-CS were selected (Table 1) for studying the synergistic effect of AuNps on CS for the bioactivity of goat chondrocytes. Goat chondrocytes were seeded at a cell density 1×10^5 cells/ml in 24 well plate with DMEM medium and allowed for cell growth and attachment. Appropriate concentrations of AuNps, CS, AuNps-CS in DMEM were added to the attached chondrocytes and the cells were incubated for further *in vitro* studies. Different concentration and combination of AuNps with CS (AuNps-CS) were tested on goat chondrocytes by MTT assay, Hoescht, GAG and collagen assay.

S.no.	AuNps	CS	Combination of AuNps-CS
1	10µM	50 µg	10µM AuNps + 50 µg CS
2	20µM	50 µg	20µM AuNps+ 50 µg CS
3	10µM	30 µg	10µM AuNps+ 30 µg CS
4	20µM	30 µg	20µM AuNps + 30 µg CS
5	10µM	10 µg	10µM AuNps + 10 µg CS
6	20µM	10 µg	20µM AuNps+ 10 µg CS

Table 1: Various concentrations of AuNps (gold nanoparticles) CS (Chondroitin sulfate) and combinations of AuNps-CS(gold nanoparticles-chondroitin sulfate) used for in vitro studies.

In vitro **studies**

5.3.10 the effect of AuNps on the activity of chondrocytes.

The effect of AuNps on growth and activity of goat chondrocytes were tested using MTT assay. AuNps (10-160µM) were added to chondrocytes seeded on 24 well plates and cell viability was analyzed. It was observed that in the concentration range of 10-80µM, AuNps supported the growth of chondrocytes (30µM-80µM) data not shown as the % viability was similar to that obtained for cultures with 10-20µM of AuNps. Cell viability gradually decreased from 90µM onwards and AuNps were found to be inhibitory above 130 µM, as shown in Figure 5.7. Then IC 50 value was also estimated in order to find out at which concentration of inhibitors or basically to check that any drug that contains 50 % of the content of inhibitor activity. Basically to find out the concentration which hampers the normal metabolic functions this might be fatal. To check the toxicity of the drug, the IC 50 value was calculated to be 157 µM, as shown in Figure 5.8.

Normally, AuNps are considered to be non-toxic due to their inherent nature. However, at higher concentrations, dose dependent cytotoxicity is expected due to the generation of ROS, as has been reported with cell lines like NIH3T3, MRC-5 (Chueh *et al*., 2014; Vijayakumar and Ganesan 2012).

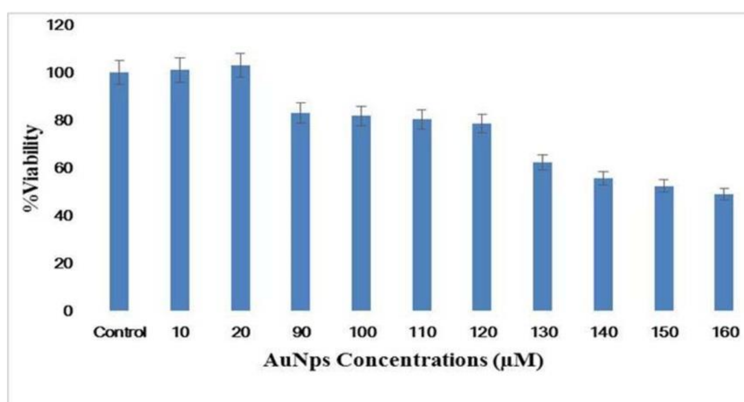

Figure 5.7) MTT(3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, assay for AuNps at various concentrations (µM). Control contains chondrocytes with no addition of AuNps or CS, all the experiments were performed in triplicates and p<0.05

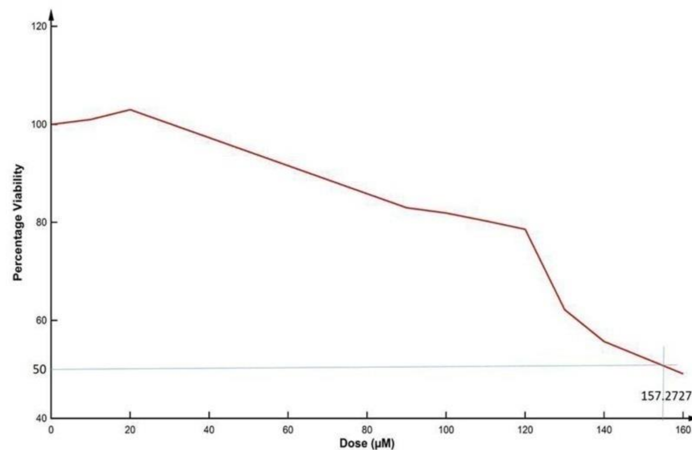

Figure 5.8) IC 50 value calculation of AuNps (gold nanoparticles)

5.3.11 Hoescht staining

In order to see the viability of chondrocytes and to confirm the MTT results hoescht staining was also performed to see how do the live cells behave when treated with AuNps at various concentration this would give us deeper insight of effect of AuNps on chondrocytes how the nuclei responds to AuNps range of concentration thus defining the viability of cells. The effect of AuNps on chondrocytes was also tested using Hoechst 33342 staining. Hoescht is a fluorescent stain that binds to adenine-thymine-rich regions of DNA in the minor groove and

allows easy visualization of the nucleus in interphase cells and chromosomes in mitotic cells. It can stain live cells as Hoechst 33342 is membrane permeant (Mathew *et al.,* 2009). On treatment with Hoechst stain, the live cells exhibit intact nuclei, while in apoptotic cells condensation and fragmentation of nuclei or stretched nuclei are observed (Latt *et al.,* 1975). Cells were incubated with AuNps in the concentration range of 10 to 160 µM for 24 hrs and stained with Hoescht dye. At lower concentrations of AuNps, the nuclear morphology was similar to that of the control (cells not exposed to AuNps). At concentration of 90 µM and above slight change in nuclear morphology and minimal stretching was observed. Fragmentation and condensation of nuclei were noted at 140, 160 µM where in the normal round shaped nuclei were deformed due to apoptosis, as shown in Figure 5.9, indicating toxicity of AuNps at high concentrations (Naqvi *et al.*, 2010). So from both of the above results it was confirmed that AuNps shows toxicity at a higher concentration so they can be used at particular dosage only. This stands correct as dosage concentration is important in determining the toxicity or non toxicity. There can be various reasons which can define toxicity of nanoparticles such as shape, size, surface chemistry, chemical composition and solubility (Kong*et al.*, 2011). Also quoted by Paracelsus the father of toxicology "*All substances are poisons and none of substance/drug is not a poison, only the dose permits something not be poisonous*". The toxicity of nanoparticles can be due to several reasons such as differential penetration, generation of oxidative stress, inflammation, or a combination of all events or any other events also for that more studies have to be done (Lanone *et al.,* 2009). Our study has confirmed that use of particular confirmation that only we can use a particular range of AuNps for combining with CS, the range which is non-toxic.

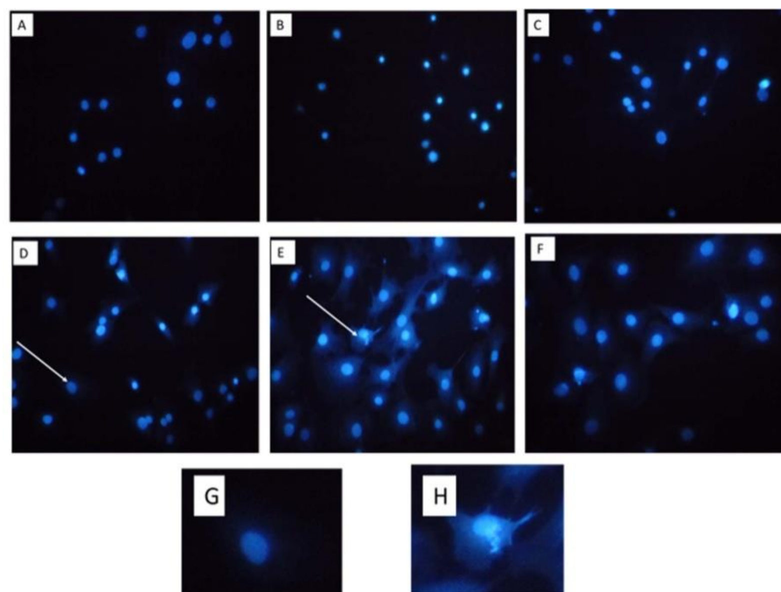

Figure 5.9) Hoescht image of chondrocytes treated with different nanoparticle concentrations (B-F) A) control B) 10μM AuNps C) 20μM AuNps D) 90μM AuNps E) 140μM AuNps F) 160μM AuNps. Apoptosis (fragmentation and condensation of nucleus) was observed at 140 and 160 μM. Deformed nucleus indicated by white arrow. G and H show the magnified image of normal nucleus and deformed nucleus

Based on the above results, concentrations of 10 and 20 μM AuNps were selected for studies on the combination of AuNps with CS (AuNps-CS). The proliferative effect of AuNps-CS on growth and biochemical activity of chondrocyte was studied.

For drug delivery application recently the therapeutic molecules can be loaded with nanoparticles or immobilized on nanoparticle surface or absorbed on the nanoparticle surface. But there can be some limitation that whether the burst drug release, lack of protection from enzymatic degradation and low stability in aqueous media. So basically after nanoparticles and drug combination there is a requirement to study the viability effect of the combination on chondrocytes to study and find out how the combination is regulating the metabolic activity of chondrocytes whether leading to toxicity or no proliferation or

increasing viability. The role of the nanoparticle can be defined to a smaller extent (Correia *et al.,* 2015). Already much work has been done to define the role of gold nanoparticles in cancer diseases. Many cancers treating drugs like doxorubicin have been tried to combine with AuNps and it has been shown that a single nanoparticles presents more than 100 DOX sites, such a high affinity could create a deadly assault on a tumor. It has been already shown that this might be due to the biocompatibility of gold nanoparticles, ease to synthesize and not only single drug but multiple drugs could be loaded, thus increasing the efficacy of the drug and ensuring better retention of drug in the blood system. So for cancer disease AuNps is already being explored and going on (Jain *et al*., 2014). In case of OA not much work has been done in trying to explore the nanoparticles or AuNps delivery efficiency which adds up the impact of this research work.

The cell viability was tested for non toxic concentration of AuNps, CS, AuNps-CS by MTT assay. A positive effect on the proliferative activity of chondrocytes was observed with AuNps-CS as compared to CS alone. Interestingly, an increase in proliferation of chondrocytes were seen at 10 µg/ml of CS added together with AuNps, whereas at this concentration no stimulatory effect was observed with CS alone. At all the three concentrations of CS (10, 30, 50 µg/ml), the proliferation of chondrocytes was more for AuNps-CS with 20 µM AuNps as compared to 10µM AuNps, as shown in Figure 5.10. The increase in proliferation of chondrocytes in the presence of AuNps-CS as compared to CS alone, with higher viability at 20 µM AuNps as compared to 10µM AuNps at a given concentration of CS, may be attributed to the effective capability of AuNps to act as a drug carrier. The role of AuNps as nano-carriers for drugs such as doxorubicin, oxaliplatin and

simadix in cancer therapeutics and treatment of heart failure has been reported earlier (Venkatpurwar *et al.,* 2011; Brown *et al.*, 2015).

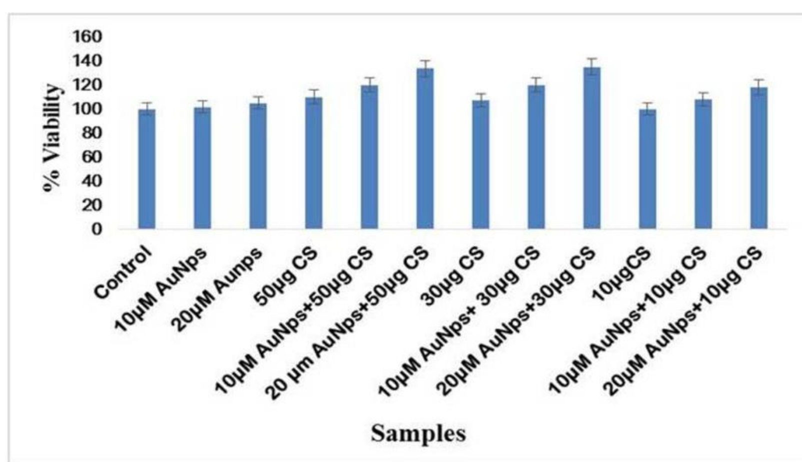

Figure 5.10) In vitro cell viability of AuNps (gold nanoparticles) CS (chondrotin sulfate) and combinations of AuNps-CS by MTT assay, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide. All the experiments were performed in triplicates and $p<0.05$. Control contains cells with no addition of AuNps or CS

5.3.12 GAG estimation

Once the cell proliferation activity was studied then further other biochemical assay was analyzed. Biochemically, native cartilage is composed of two components, GAG and collagen. GAG and collagen are important components of cartilage matrix; collagen is primarily the component which provides strength. The progression of osteoarthritis is characterized by loss of GAG component and collagen degradation. Although cartilage has poor regeneration and slow self-repair capacity as it secretes limited amounts of the extracellular matrix molecules, GAG and collagen, which in turn help in retaining functionality and regeneration (Zhang *et al.,* 2009; Chen et al., 2006; Carver and Heath, 1999). Thus, the repair response is facilitated by the increased production of matrix molecules as they provide the support for regeneration of the cartilage (Buckwalter *et al.,*

2005). CS exhibits anti-arthritic properties by suppressing the metalloproteinase and interleukin gene expression of aggrecanase. Both metalloproteinase and aggrecanase function to degrade the extracellular matrix components such as GAG, collagen and aggrecan. Suppressing their activity induces proliferation of chondrocytes and improves the availability of GAG (Wu *et al.,* 2010; Imada *et al.,* 2010; Bassleer and Franchimont 1992; Schwartz and Dorfman 1975). GAG secreted by chondrocytes when treated with AuNps-CS was estimated by DMMB (1, 9-dimethylmethylene blue) method, as shown in Figure 5.11. While 2 % GAG production was noted at 10 µg/ml of CS, GAG, production increased by 2.1 fold (3.9 %) and 2.3 fold (4.3%) when CS was added with 10 µM and 20 µM AuNps, respectively. A similar two fold increase in GAG production was observed with 30 µg and 50 µg CS added with 10 µM and 20 µM AuNps. However, AuNps in the absence of CS had no effect on GAG activity. These results indicate that AuNps act as the carrier of CS, and increase its bioavailability.

This assay also confirming the role of AuNps as drug delivery vehicle. Some work has been done in using nanoparticles as drug delivery vehicle for arthritis. Such as using PlGA nanoparticles for hylauronate uptake, where the combinations have been tried in the intra-articular region of animal models. There it has been already shown that these nanoparticles could be proposed as safe drug delivery system (Zille *et al.,* 2010).

But for AuNps which has already shown role as drug delivery vehicle in other diseases has not yet been explored in the case of OA. So this study gives us new insight into role of AuNps as drug vehicle for OA. This study where there is an increase in GAG production, which is one of the important components of cartilage, will lead us to the insight that combination can be successfully tried for intra-articular injections. There are studies showing

nanoparticles have been used for improved local retention after intra-articular injection into knee joint (Morgen *et al.,* 2013). So here using this combination which has already shown the promising result in GAG content could also be used for intra-articular treatment.

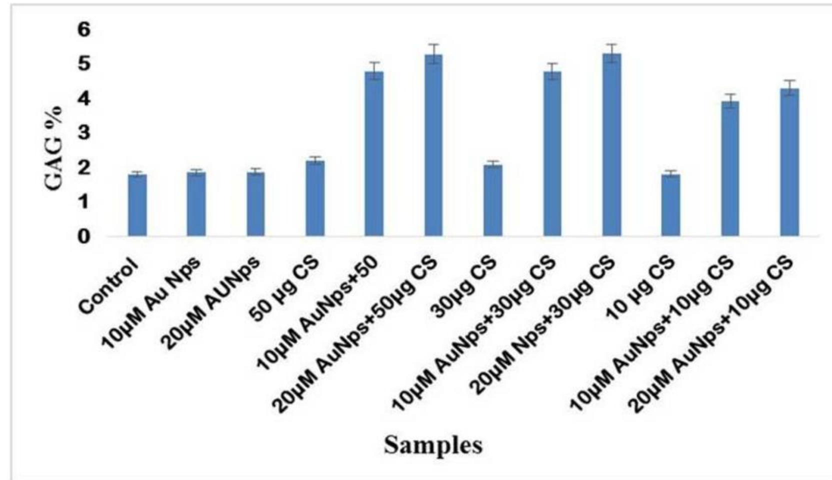

Figure 5.11) GAG analysis(glycosaminoglycan) of chondrocytes after 24 hrs of incubation with AuNps(gold nanoparticles), CS (chondroitin sulfate) and combinations of AuNps-CS estimated using the DMMB(Dimethyl methylene blue) method. Control contains cells with no addition of gold nanoparticles or Chondroitin sulfate. All the experiments were performed in triplicates and p<0.05

5.3.14 Collagen production

Another important component of native cartilage is collagen which forms 2/3 rd of the dry weight of adult articular cartilage matrix. Collagen breakdown is considered to be a critical step in the progression of OA and is used as a marker to monitor the progression of the disease. CS helps in collagen production by enhancing TIMP-1 (Tissue inhibitors of metalloproteinases) production which is inhibitors of MMPs (matrix metalloproteinases) which degrade collagen type 2(Pecchi *et al.,* 2012). Hydroxyproline assay was used to estimate collagen production.

Already the studies have been done where it has been tried to use nanoparticle to increase collagen production or regulate collagen production and to study role of silver nanoparticles in increasing collagen production or exposure to carbon black nanoparticles increase collagen type VIII expression in offspring kidney (Kwan, *et al.*, 2014; Umezawa *et al.*, 2011). Some studies where components like nitric oxide released from nanoparticle to help in wound healing process by deposition of collagen production (Han *et al.,* 2012). So already the role of nanoparticles and nanoparticles with molecule has been studied for collagen production. In this study we tried to use AuNps whose role has been already been as drug delivery vehicle for many drug molecules along with CS to find out the effect on collagen production rate.

An increase of ~2 fold in collagen production by the chondrocytes was observed when AuNps-CS was added at all the concentrations of CS. No stimulating activity was noted with AuNps alone, as shown in Figure 5.12. At a given concentration of CS, the collagen production was higher at 20µM AuNps as compared to 10µM AuNps, supporting the role of AuNps as a carrier for CS drug which in turn helps in collagen production.

In our study collagen production result supported, MTT, GAG results defining the role of AuNps as a carrier. Further combination (AuNps-CS) also can be studied for Intra-articular injection.

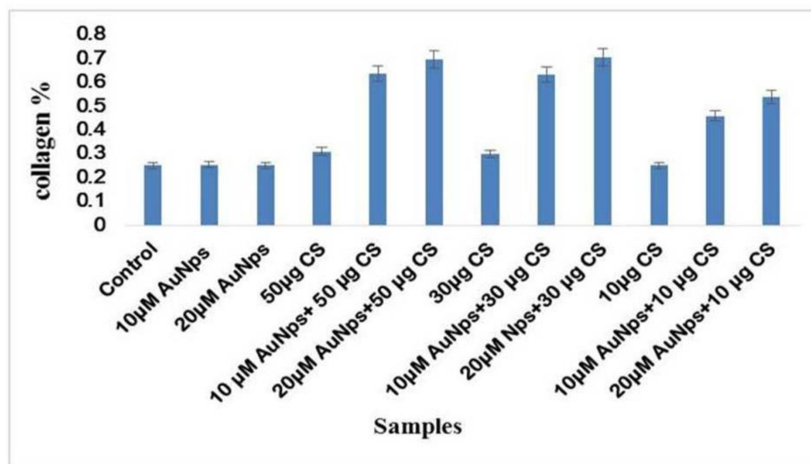

Figure 5.12) Collagen estimation of chondrocytes incubated with AuNps(gold nanoparticles), CS(chondroitin sulfate) and combinations of AuNps-CS. Control contains cells with no addition of AuNps or CS, Experiments performed in triplicates and p <0.05.

5.4 Conclusion

In this work we have tried to synthesize gold nanoparticles and then characterized by different techniques like XRD, TEM, UV-Vis and after its synthesizes they were combined with CS to study the effect of the combination (AuNps-CS), the combination was further analyzed by different techniques like UV-Vis, FTIR, Zeta potential to see whether combination doesn't alter the Gold nanoparticles basic properties. After that combination was further analyzed on chondrocytes by *in vitro* analyzes such as MTT, Hoescht, GAG, Collagen assay.

Our studies shows that AuNps formation was confirmed by wine color formation and XRD peaks which are the characteristic peak for AuNps. UV-Vis spectra also confirmed the stability of AuNps. Then the combination of different concentration when analyzed by UV-Vis it was observed that there was not much shift in wavelength when the combination was used underlying that combination is not making much change in spectral properties. Then further FTIR, Zeta potential results also supported the stability of (AuNps-CS). From our study it was clear that this combination can be used for further *in vitro* studies and its effect on chondrocytes. It was observed that in the concentration range of 10-80µM, AuNps supported growth of chondrocytes. Hoescht staining supported that range 10-20 µM showed intact nuclei and higher range 140µM, 160 µM further showed fragmentation of nuclei

Based on the above results, concentrations of 10 and 20 µM , AuNps were selected for studies on combination of AuNps with CS (AuNps-CS). AuNps-CS supported the growth of chondrocytes and enhanced the production of extracellular matrix components, GAG and

collagen, as compared to when CS or AuNps were used independently. These results suggest that AuNps can act as carriers of CS, which is a promising approach, and can be further evaluated for osteoarthritis model systems. The present study demonstrates the gold nanoparticle mediated delivery of chondroitin sulfate.

Further for future application this combination can be either tried in animal models or osteoarthritic animal models via intraarticular injections. The potential role of AuNps in drug delivery was evaluated and the positive effect of CS which has already shown the effect of treatment of osteoarthritis can also be utilized more. Thus bringing down the drug cost for treating osteoarthritis.

Summary of results and Conclusion

Articular cartilage which functions to allow the frictionless movement when damaged results in a condition known as osteoarthritis. Osteoarthritis has already affected millions of people worldwide. In this research work, we have tried to explore many different delivery modes to stimulate chondrocyte proliferation and enhance the functionality of matrix to achieve optimum regeneration. CGC scaffold was synthesized by cryogelation and scaffold showed large interconnected pores, optimum mechanical strength, elastic nature which showed the potential of these scaffold in cartilage tissue engineering. Different delivery modes were synthesized for CS which has already shown its potential in OA treatment such as incorporation in scaffold during synthesis, incorporated in microparticles for sustained release, and further these drug loaded spheres were incorporated in scaffold. Modes like CGS and CGM showed enhanced matrix production thus paving its way to OA treatment where matrix functionality is lost due to wear and tear.

Further ascorbic acid which has already shown its beneficial role in osteoarthritis was encapsulated using microparticles as drug delivery vehicle. Here in our work, we could not only provide encapsulation of ascorbic acid but also sustain release for a longer time period which is beneficial in our case as cartilage repair is a slow process, increased cell proliferation and matrix production added more advantages to this mode having potential for osteoarthritis. Another drug which we tested (collaflex) showed increase in matrix production.

In addition another delivery mode such a nanoparticle was also explored. This method is already in use as a drug delivery vehicle for cancer and heart failure though not much has

been studied for exploring potential of it in osteoarthritis. So in this study we synthesized gold nanoparticles and characterized by TEM, XRD, Uv-Vis.

After the study AuNps were combined with CS and the bioactivity of AuNps-CS (combination of gold nanoparticles- chondroitin sulfate) was evaluated. AuNps-CS supported the growth of chondrocytes and enhanced the production of extracellular matrix components, GAG and collagen, as compared to when CS or AuNps were used independently. These results suggest that AuNps can act as carriers of CS, which is a promising approach, and can be further evaluated for osteoarthritis model systems.

Future scope

In future studies, the CGC scaffold synthesized can be used for mimicking these results in the *in vivo* system, where cell material interactions and cues provided by materials should be able to help in quick recovery of the injured tissues or help in regeneration of cartilage. Further osteoarthritis model could be developed in rabbits or other animal, after development of ideal diseased model, the chondrocyte seeded CGC scaffold can be implanted at the site of lesion. These scaffolds are mechanically very stable and so can withstand the mechanical deformation during normal activities like walking, hopping etc. Further CGC having optimal biodegradability. It is presumed that while chondrocytes synthesize the ECM the cryogel matrix will degrade leaving behind natural ECM. This overall approach has the ability to be utilized for treatment of osteoarthritis after going through preclinical and clinical trials. Cartilage specific markers study can be to be performed, histology and immunohistochemistry of chondrogenic markers (collagen type 2, chondroitin sulphate, aggrecan) is desirable to validate the effect of the scaffold system on chondrogenesis.

We conclude that scaffold with microparticles can be used for other biomedical applications with some modifications to obtain desired results. Apart from this mechanism of gold nanoparticles as drug delivery can be further explored.

References

Abdelhalim, M. A. K., Mady, M. M., & Ghannam, M. M. (2012). Physical properties of different gold nanoparticles: ultraviolet-visible and fluorescence measurements. *Journal of Nanomedicine & Nanotechnology*, *2012*.

Alexis, F., Pridgen, E., Molnar, L. K., & Farokhzad, O. C. (2008). Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Molecular pharmaceutics*, *5*(4), 505

Alexopoulos, L. G., Setton, L. A., & Guilak, F. (2005). The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomaterialia*, *1*(3), 317-325.

Amanatullah, D. F., Howard, J. L., Siman, H., Trousdale, R. T., Mabry, T. M., & Berry, D. J. (2015). Revision total hip arthroplasty in patients with extensive proximal femoral bone loss using a fluted tapered modular femoral component. *Bone & Joint Journal*, *97*(3), 312-317.

Amendola, V & Meneghetti, M (2009). Size evaluation of gold nanoparticles by UV− vis spectroscopy. *The Journal of Physical Chemistry C*, *113*(11), 4277-4285.

Amrutkar, J. R., & Gattani, S. G. (2009). Chitosan–chondroitin sulfate based matrix tablets for colon specific delivery of indomethacin. *AAPS PharmSciTech*, *10*(2), 670-677.

Anandacoomarasamy, A., & March, L. (2010). Current evidence for osteoarthritis treatments. *Therapeutic advances in musculoskeletal disease*.

Aryal, S., Grailer, J. J., Pilla, S., Steeber, D. A., & Gong, S. (2009). Doxorubicin conjugated gold nanoparticles as water-soluble and pH-responsive anticancer drug nanocarriers. *Journal of Materials Chemistry*, *19*(42), 7879-7884.-515.

Astur, D. C., Arliani, G. G., Kaleka, C. C., Jalikjian, W., Golano, P., & Cohen, M. (2012). A three-dimensional anatomy of the posterolateral compartment of the knee: the use of a new technology in the study of musculoskeletal anatomy. *Open access journal of sports medicine*, *3*, 1.

Austin, K. (2007). Scaffold Design: Use of Chitosan in cartilage tissue engineering. *MMG 445 Basic Biotechnology e Journal*, *3*(1), 62-66.

Bac, L. H., Kim, J. S., & Kim, J. C. (2011). Size, optical and stability properties of gold nanoparticles synthesized by electrical explosion of wire in different aqueous media. *Reviews on Advanced Materials Science*, *28*(2), 117-121.

Barbour, K. E. (2017). Vital signs: prevalence of doctor-diagnosed arthritis and arthritis-attributable activity limitation—United States, 2013–2015. *MMWR. Morbidity and Mortality Weekly Report*, *66*

Bader, R. A. (2014). Fundamentals of Drug Delivery. *Engineering Polymer Systems for Improved Drug Delivery*, 1-28.

Bamrungsap, S., Zhao, Z., Chen, T., Wang, L., Li, C., Fu, T., & Tan, W. (2012). Nanotechnology in therapeutics: a focus on nanoparticles as a drug delivery system. *Nanomedicine*, *7*(8), 1253-1271.

BarathManiKanth, S., Kalishwaralal, K., Sriram, M., Pandian, S. R. K., Youn, H. S., Eom, S., & Gurunathan, S. (2010). Research anti-oxidant effect of gold nanoparticles restrains hyperglycemic conditions in diabetic mice. *J Nanotechnol*, *8*, 16.

Bassleer C, H. Y., & Franchimont, P. (1992). In-vitro evaluation of drugs proposed as chondroprotective agents. *Int J Tissue React*, *14*, 231-241.

Bassleer C, H. Y., & Franchimont, P. (1992). *In vitro* evaluation of drugs proposed as chondroprotective agents. *Int J Tissue React*, *14*, 231-241.

Bellamy, N., Campbell, J., Welch, V., Gee, T. L., Bourne, R., & Wells, G. A. (2006). Viscosupplementation for the treatment of osteoarthritis of the knee. *The Cochrane Library*.

Benders, K. E., van Weeren, P. R., Badylak, S. F., Saris, D. B., Dhert, W. J., & Malda, J. (2013). Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends in biotechnology*, *31*(3), 169-176.

Bernhard, J. C., & Vunjak-Novakovic, G. (2016). Should we use cells, biomaterials, or tissue engineering for cartilage regeneration. *Stem cell research & therapy*, *7*(1), 1.

Bhat, S., & Kumar, A. (2012). Cell proliferation on three-dimensional chitosan–agarose–gelatin cryogel scaffolds for tissue engineering applications. *Journal of bioscience and bioengineering*, *114*(6), 663-670.

Bhat, S., Lidgren, L., & Kumar, A. (2013). *In vitro* Neo-Cartilage Formation on a Three-Dimensional Composite Polymeric Cryogel Matrix. *Macromolecular bioscience*, *13*(7), 827-837.

Bhat, S., Tripathi, A., & Kumar, A. (2011). Supermacroprous chitosan–agarose–gelatin cryogels: in vitro characterization and *in vivo* assessment for cartilage tissue engineering. *Journal of the Royal Society Interface*, *8*(57), 540-554.

Bhatia, D., Bejarano, T., & Novo, M. (2013). Current interventions in the management of knee osteoarthritis. *Journal of Pharmacy and Bioallied Sciences*, *5*(1), 30.

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

Bhowmik, D., Gopinath, H., Kumar, B. P., Duraivel, S., & Kumar, K. S. (2012). Controlled release drug delivery systems. *The Pharma Innovation*, *1*(10).

Boyera, N., Galey, I., & Bernard, B. A. (1998). Effect of vitamin C and its derivatives on collagen synthesis and cross-linking by normal human fibroblasts. *International Journal of Cosmetic Science*, *20*(3), 151-158.

Brittberg, M. (2008). Autologous chondrocyte implantation—technique and long-term follow-up. *Injury*, *39*(1), 40-49.

Brown, K. E., Leong, K., Huang, C. H., Dalal, R., Green, G. D., Haimes, H. B., ... & Bathon, J. (1998). Gelatin/chondroitin 6-sulfate microspheres for the delivery of therapeutic proteins to the joint. *Arthritis & Rheumatism*, *41*(12), 2185-2195.

Brown, S. D., Nativo, P., Smith, J. A., Stirling, D., Edwards, P. R., Venugopal, B & Wheate, N. J. (2010). Gold nanoparticles for the improved anticancer drug delivery of the active component of oxaliplatin. *Journal of the American Chemical Society*, *132*(13), 4678-4684.

Bryant, S. J., & Anseth, K. S. (2002). Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly (ethylene glycol) hydrogels. *Journal of biomedical materials research*, *59*(1), 63-72.

Buckwalter, J. A., Mankin, H. J., & Grodzinsky, A. J. (2005). Articular cartilage and osteoarthritis. *Instructional Course Lectures-American Academy of Orthopaedic Surgeons*, *54*, 465.

Busci, L. R. (1990). Reversal of osteoarthritis by nutritional intervention. *ACA J of Chiropractic (11/90)*, 69-72.

Bush, P. G., & Hall, A. C. (2003). The volume and morphology of chondrocytes within non-degenerate and degenerate human articular cartilage. *Osteoarthritis and cartilage*, *11*(4), 242-251.

Cai, D. Z., Zeng, C., Quan, D. P., Bu, L. S., Wang, K., Lu, H. D., & Li, X. F. (2007). Biodegradable chitosan scaffolds containing microspheres as carriers for controlled transforming growth factor-beta~ 1 delivery for cartilage tissue engineering. Chinese medical *120*(3), 197.

Callahan, L. A. S., Ganios, A. M., Childers, E. P., Weiner, S. D., & Becker, M. L. (2013). Primary human chondrocyte extracellular matrix formation and phenotype maintenance using RGD-derivatized PEGDM hydrogels possessing a continuous Young's modulus gradient. *Acta biomaterialia*, *9*(4), 6095-6104.

Canto, G. S., Dalmora, S. L., & Oliveira, A. G. D. (1999). Piroxicam encapsulated in liposomes: characterization and *in vivo* evaluation of topical anti-inflammatory effect. *Drug development and industrial pharmacy*, *25*(12), 1235-1239.

Cao, Z., Dou, C., & Dong, S. (2014). Scaffolding biomaterials for cartilage regeneration. *Journal of Nanomaterials*, *2014*, 4.

Cao-Milán, R., & Liz-Marzán, L. M. (2014). Gold nanoparticle conjugates: recent advances toward clinical applications. *Expert opinion on drug delivery*, *11*(5), 741-752.

Cario, E. (2012). Nanotechnology-based drug delivery in mucosal immune diseases: hype or hope&quest. *Mucosal Immunology*, *5*(1), 2-3.

Carletti, E., Motta, A., & Migliaresi, C. (2011). Scaffolds for tissue engineering and 3D cell culture. *3D Cell Culture: Methods and Protocols*, 17-39.

Carver, S. E., & Heath, C. A. (1999). Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure. *The effects of physiological levels of intermittent pressure on the development of articular cartilage in vitro*,74.

Chan, B. P., & Leong, K. W. (2008). Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *European spine journal*, *17*(4), 467-479.

Chang, K. Y., Hung, L. H., Chu, I., Ko, C. S., & Lee, Y. D. (2010). The application of type II collagen and chondroitin sulfate grafted PCL porous scaffold in cartilage tissue engineering. *Journal of Biomedical Materials Research Part A*, *92*(2), 712-723.

Chau, D. Y. S., Agashi, K., & Shakesheff, K. M. (2008). Microparticles as tissue engineering scaffolds: manufacture, modification and manipulation. *Materials Science and Technology*, *24*(9), 1031-1044.

Chen, F. H., Rousche, K. T., & Tuan, R. S. (2006). Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. *Nature clinical practice rheumatology*, *2*(7), 373-382.

Chen, F., Wang, Y., Ma, J., & Yang, G. (2014). A biocompatible synthesis of gold nanoparticles by Tris (hydroxymethyl) aminomethane. *Nanoscale research letters*, *9*(1), 1-6.

Cheng, J., Gu, Y. J., Cheng, S. H., & Wong, W. T. (2013). Surface functionalized gold nanoparticles for drug delivery. *Journal of biomedical nanotechnology*, *9*(8), 1362-1369.

Chertok, B., Moffat, B. A., David, A. E., Yu, F., Bergemann, C., Ross, B. D., & Yang, V. C. (2008). Iron oxide nanoparticles as a drug delivery vehicle for MRI monitored magnetic targeting of brain tumors. *Biomaterials*, *29*(4), 487-496.

Cho, S. H. (2005). Estimation of tumour dose enhancement due to gold nanoparticles during typical radiation treatments: a preliminary Monte Carlo study. *Physics in medicine and biology*, *50*(15), N163.

Choy, Y. B., Park, J. H., & Prausnitz, M. R. (2008). Mucoadhesive microparticles engineered for ophthalmic drug delivery. *Journal of Physics and Chemistry of Solids*, *69*(5), 1533-1536.

Chueh, P. J., Liang, R. Y., Lee, Y. H., Zeng, Z. M., & Chuang, S. M. (2014). Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines. *Journal of hazardous materials*, *264*, 303-312.

Clark, A. G., Rohrbaugh, A. L., Otterness, I., & Kraus, V. B. (2002). The effects of ascorbic acid on cartilage metabolism in guinea pig articular cartilage explants. *Matrix Biology*, *21*(2), 175-184.

Collin, E. C., Kilcoyne, M., White, S. J., Grad, S., Alini, M., Joshi, L., & Pandit, A. S. (2016). Unique glycosignature for intervertebral disc and articular cartilage cells and tissues in immaturity and maturity. *Scientific Reports*, *6*.

Correia, A., Shahbazi, M. A., Mäkilä, E., Almeida, S., Salonen, J., Hirvonen, J., & Santos, H. A. (2015). Cyclodextrin-Modified Porous Silicon Nanoparticles for Efficient Sustained Drug Delivery and Proliferation Inhibition of Breast Cancer Cells. *ACS applied materials & interfaces*, *7*(41), 23197-23204.

Crisan, D., Roman, I., Crisan, M., Scharffetter-Kochanek, K., & Badea, R. (2015). The role of vitamin C in pushing back the boundaries of skin aging: an ultrasonographic approach. *Clinical, cosmetic and investigational dermatology*, *8*, 463.

Cucchiarini, M., & Madry, H. (2014). Overexpression of human IGF-I via direct rAAV-mediated gene transfer improves the early repair of articular cartilage defects *in vivo*. *Gene therapy*, *21*(9), 811-819.

Cui, N., Qian, J., Liu, T., Zhao, N., & Wang, H. (2015). Hyaluronic acid hydrogel scaffolds with a triple degradation behavior for bone tissue engineering. *Carbohydrate polymers*, *126*, 192-198.

Daniel, J. C., Pauli, B. U., & Kuettner, K. E. (1984). Synthesis of cartilage matrix by mammalian chondrocytes *in vitro*. III. Effects of ascorbate. *The Journal of cell biology*, *99*(6), 1960-1969.

Dash, S., Murthy, P. N., Nath, L., & Chowdhury, P. (2010). Kinetic modeling on drug release from controlled drug delivery systems. *Acta Pol Pharm*, *67*(3), 217-23.

David-Raoudi, M., Mendichi, R., & Pujol, J. P. (2009). For intra-articular delivery of chondroitin sulfate. *Glycobiology*, *19*(8), 813-815.

Davies, M. R., Ribeiro, L. R., Downey-Jones, M., Needham, M. R., Oakley, C., & Wardale, J. (2009). Ligands for retinoic acid receptors are elevated in osteoarthritis and may contribute to pathologic processes in the osteoarthritic joint. *Arthritis & Rheumatism*, *60*(6), 1722-1732.

Davoodi, P., Feng, F., Xu, Q., Yan, W. C., Tong, Y. W., Srinivasan, M. P & Wang, C. H. (2015). Coaxial electrohydrodynamic atomization: Microparticles for drug delivery applications. *Journal of Controlled Release*, *205*, 70-82.

De Jong, W. H., & Borm, P. J. (2008). Drug delivery and nanoparticles: applications and hazards. *International journal of nanomedicine*, *3*(2), 133.

Desai, K. G., Liu, C., & Park, H. J. (2006). Characteristics of vitamin C encapsulated tripolyphosphate-chitosan microspheres as affected by chitosan molecular weight. *Journal of microencapsulation*, *23*(1), 79-90.

DeYoung, M. B., MacConell, L., Sarin, V., Trautmann, M., & Herbert, P. (2011). Encapsulation of exenatide in poly-(D, L-lactide-co-glycolide) microspheres produced an investigational long-acting once-weekly formulation for type 2 diabetes. *Diabetes technology & therapeutics*, *13*(11), 1145-1154.

Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011). Polymeric scaffolds in tissue engineering application: a review. *International Journal of Polymer Science*, *2011*.

Di Stefano, A., Sozio, P., Iannitelli, A., & Cerasa, L. S. (2009). New drug delivery strategies for improved Parkinson's disease therapy. *Expert opinion on drug delivery*, *6*(4), 389-404.

Dinarvand, R., Mahmoodi, S., & Farboud, E. (2005). Effect of process variables on particle size of gelatin microspheres containing lactic acid. *Pharmaceutical Development and technology*, *9*(3), 291-299.

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*.

Dixit, N., Maurya, S. D., & Sagar, B. P. (2013). Sustained release drug delivery system. *Indian Journal of Research in Pharmacy and Biotechnology*, *1*(3), 305.

Dreaden, E. C., Austin, L. A., Mackey, M. A., & El-Sayed, M. A. (2012). Size matters: gold nanoparticles in targeted cancer drug delivery. *Therapeutic delivery*, *3*(4), 457-478.

Dreaden, E. C., Austin, L. A., Mackey, M. A., & El-Sayed, M. A. (2012). Size matters: gold nanoparticles in targeted cancer drug delivery. *Therapeutic delivery*, *3*(4), 457-478.

Dronne, N., Benel, L., Thenet, S., Larno, S., Mokondjimobe, E., Bourbouze, R., & Adolphe, M. (1989). Effects of retinoic acid on the growth of cultured rabbit articular chondrocytes: Relation with alkaline phosphatase activity and beta receptor. *Cytotechnology*, *2*(3), 233-237.

Eberhard W, N. (2008). Synthetic polymers as drug-delivery vehicles in medicine. *Metal-based drugs*, *2008*.

Elder, S. H., Nettles, D. L., & Bumgardner, J. D. (2004). Synthesis and characterization of chitosan scaffolds for cartilage-tissue engineering. *Biopolymer Methods in Tissue Engineering*, 41-48.

Engler, A. J., Sen, S., Sweeney, H. L., & Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell*, *126*(4), 677-689.

Enrione, J., Díaz-Calderón, P., Weinstein-Oppenheimer, C. R., Sánchez, E., Fuentes, M. A., Brown, D. I & Acevedo, C. A. (2013). Designing a gelatin/chitosan/hyaluronic acid biopolymer using a thermophysical approach for use in tissue engineering. *Bioprocess and biosystems engineering*, *36*(12), 1947-1956.

Enrione, J., Osorio, F., López, D., Weinstein-Oppenheimer, C., Fuentes, M. A., Ceriani, R., ... & Somoza, R. A. (2010). Characterization of a Gelatin/Chitosan/Hyaluronan scaffold-polymer. *Electronic Journal of Biotechnology*, *13*(5), 20-21.

Fleming, B. C., Hulstyn, M. J., Oksendahl, H. L., & Fadale, P. D. (2005). Ligament injury, reconstruction and osteoarthritis. *Current opinion in orthopaedics*, *16*(5), 354.

Fortier, L. A., Barker, J. U., Strauss, E. J., McCarrel, T. M., & Cole, B. J. (2011). The role of growth factors in cartilage repair. *Clinical Orthopaedics and Related Research®*, *469*(10), 2706-2715.

Fox, A. J. S., Bedi, A., & Rodeo, S. A. (2009). The basic science of articular cartilage: structure, composition, and function. *Sports Health: A Multidisciplinary Approach*, *1*(6), 461-468.

Frenkel, S. R., Saadeh, P. B., Mehrara, B. J., Chin, G. S., Steinbrech, D. S., Brent, B., ... & Longaker, M. T. (2000). Transforming growth factor beta superfamily members: role in cartilage modeling. *Plastic and reconstructive surgery*, *105*(3), 980-990.

Friedrich, K. B. A. U. K., Lu, Z. B. A. U. K., & Hager, A. M. (1995). Recent advances in polymer composites' tribology. *Wear*, *190*(2), 139-144.

Fu, Y., & Kao, W. J. (2010). Drug release kinetics and transport mechanisms of non-degradable and degradable polymeric delivery systems. *Expert opinion on drug delivery*, *7*(4), 429-444.

Gangwar, R. K., Dhumale, V. A., Gosavi, S. W., Sharma, R. B., & Datar, S. S. (2013). Catalytic activity of allamanda mediated phytosynthesized anisotropic gold nanoparticles. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, *4*(4), 045005.

Gao, Y., Liu, S., Huang, J., Guo, W., Chen, J., Zhang, L & Xu, W. (2014). The ECM-cell interaction of cartilage extracellular matrix on chondrocytes. *BioMed research international*, *2014*.

García-Carvajal, Z. Y., Garciadiego-Cázares, D., Parra-Cid, C., Aguilar-Gaytán, R., Velasquillo, C., Ibarra, C., & Carmona, J. S. C. (2013). Cartilage tissue engineering: the role of extracellular matrix (ECM) and novel strategies. *Regenerative medicine and tissue engineering. Croatia: InTech*, 365-397

Garver, M. J., Focht, B. C., & Taylor, S. J. (2015). Integrating lifestyle approaches into osteoarthritis care. *Journal of multidisciplinary healthcare*, *8*, 409.

Gothard, D., Roberts, S. J., Shakesheff, K. M., & Buttery, L. D. (2009). Engineering embryonic stem-cell aggregation allows an enhanced osteogenic differentiation in vitro. *Tissue Engineering Part C: Methods*, *16*(4), 583-595.

Grainger, R., & Cicuttini, F. M. (2004). Medical management of osteoarthritis of the knee and hip joints. *Medical Journal of Australia*, *180*(5), 232.

Gregory, P. J., Sperry, M., & Wilson, A. F. (2008). Dietary supplements for osteoarthritis. *American Family Physician*, *77*(2).

Hall, B. K., & Miyake, T. (2000). All for one and one for all: condensations and the initiation of skeletal development. *Bioessays*, *22*(2), 138-147.

Hameed, F., & Ihm, J. (2012). Injectable medications for osteoarthritis. *PM&R*, *4*(5), S75-S81.

Han, G., Nguyen, L. N., Macherla, C., Chi, Y., Friedman, J. M., Nosanchuk, J. D., & Martinez, L. R. (2012). Nitric oxide–releasing nanoparticles accelerate wound healing by promoting fibroblast migration and collagen deposition. *The American journal of pathology*, *180*(4), 1465-1473.

Heidari, B. (2011). Knee osteoarthritis diagnosis, treatment and associated factors of progression: part II. *Caspian journal of internal medicine*, *2*(3), 249.

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.

Hiraki, Y., Shukunami, C., Iyama, K., & Mizuta, H. (2001). Differentiation of chondrogenic precursor cells during the regeneration of articular cartilage. *Osteoarthritis and Cartilage*, *9*, S102-S108

Hochberg, M. C., & Dougados, M. (2001). Pharmacological therapy of osteoarthritis. *Best Practice & Research Clinical Rheumatology*, *15*(4), 583-593.

Hofmann, S., & Garcia-Fuentes, M. (2011). *Bioactive scaffolds for the controlled formation of complex skeletal tissues*. INTECH Open Access Publisher.

Hogenmiller, M. S., & Lozada, C. J. (2006). An update on osteoarthritis therapeutics. *Current opinion in rheumatology*, *18*(3), 256-260.

Holland, T. A., Bodde, E. W. H., Cuijpers, V. M. J. I., Baggett, L. S., Tabata, Y., Mikos, A. G., & Jansen, J. A. (2007). Degradable hydrogel scaffolds for in vivo delivery of single and dual growth factors in cartilage repair. *Osteoarthritis and Cartilage*, *15*(2), 187-197.

Homar, M., Ubrich, N., Ghazouani, F. E., Kristl, J., Kerč, J., & Maincent, P. (2007). Influence of polymers on the bioavailability of microencapsulated celecoxib. *Journal of microencapsulation*, *24*(7), 621-633.

http://indianexpress.com/article/lifestyle/health/india-may-have-60-mn-osteoarthritis-cases-by-2025/

https://decisionresourcesgroup.com/drg-blog/medtech-perspectives/osteoarthritis-status-quo-india/

Huglin, M. R. (1989). Hydrogels in medicine and pharmacy. *British Polymer Journal*, *21*(2), 184-184.

Ibold, Y., Lübke, C., Pelz, S., Augst, H., Kaps, C., Ringe, J., & Sittinger, M. (2009). Effect of different ascorbate supplementations on *in vitro* cartilage formation in porcine high-density pellet cultures. *Tissue and Cell*, 41(4), 249-256.

Imada, K., Oka, H., Kawasaki, D., Miura, N., Sato, T., & Ito, A. (2010). Anti-arthritic action mechanisms of natural chondroitin sulfate in human articular chondrocytes and synovial fibroblasts. *Biological and Pharmaceutical Bulletin*, *33*(3), 410-414.

Ingavle, G. C., Dormer, N. H., Gehrke, S. H., & Detamore, M. S. (2012). Using chondroitin sulfate to improve the viability and biosynthesis of chondrocytes encapsulated in interpenetrating network (IPN) hydrogels of agarose and poly (ethylene glycol) diacrylate. *Journal of Materials Science: Materials in Medicine*, *23*(1), 157-170.

Ingber, D. E., Mow, V. C., Butler, D., Niklason, L., Huard, J., Mao, J & Vunjak-Novakovic, G. (2006). Tissue engineering and developmental biology: going biomimetic. *Tissue engineering*, *12*(12), 3265-3283.

Iwamoto, M., Shapiro, I. M., Yagami, K., Boskey, A. L., Leboy, P. S., Adams, S. L., & Pacifici, M. (1993). Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. *Experimental cell research*, *207*(2), 413-420.

Izadifar, Z., Chen, X., & Kulyk, W. (2012). Strategic design and fabrication of engineered scaffolds for articular cartilage repair. *Journal of functional biomaterials*, *3*(4), 799-838.

Jabbar, S. A., Twentyman, P. R., & Watson, J. V. (1989). The MTT assay underestimates the growth inhibitory effects of interferons. *British journal of cancer*, *60*(4), 523.

Jain, E., Karande, A. A., & Kumar, A. (2011). Supermacroporous polymer-based cryogel bioreactor for monoclonal antibody production in continuous culture using hybridoma cells. *Biotechnology progress*, *27*(1), 170-180.

Jain, S., Hirst, D. G., & O'sullivan, J. M. (2014). Gold nanoparticles as novel agents for cancer therapy. *The British journal of radiology*.

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.

Janssen, M., Mihov, G., Welting, T., Thies, J., & Emans, P. (2014). Drugs and polymers for delivery systems in oa joints: clinical needs and opportunities. *Polymers*, *6*(3), 799-819.

Jayan, S. C., Sandeep, A., Rifash, M., Mareema, C., & Shamseera, S. (2009). Design and *in vitro* evaluation of gelatin microspheres of salbutamol sulphate. *Hygeia*, *1*(1).

Jerosch, J. (2011). Effects of glucosamine and chondroitin sulfate on cartilage metabolism in OA: outlook on other nutrient partners especially omega-3 fatty acids. *International journal of rheumatology*, *2011*.

Jiang, G., Wang, L., & Chen, W. (2007). Studies on the preparation and characterization of gold nanoparticles protected by dendrons. *Materials Letters*, *61*(1), 278-283.

Jiang, T., Petersen, R. R., Call, G., Ofek, G., Gao, J., & Yao, J. Q. (2011). Development of chondroitin sulfate encapsulated PLGA microsphere delivery systems with controllable multiple burst releases for treating osteoarthritis. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *97*(2), 355-363.

Jo, D. H., Kim, J. H., Lee, T. G., & Kim, J. H. (2015). Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases. *Nanomedicine: Nanotechnology, Biology and Medicine*, *11*(7), 1603-1611.

Johnson, K. A., Hulse, D. A., Hart, R. C., Kochevar, D., & Chu, Q. (2001). Effects of an orally administered mixture of chondroitin sulfate, glucosamine hydrochloride and manganese ascorbate on synovial fluid chondroitin sulfate 3B3 and 7D4 epitope in a canine cruciate ligament transection model of osteoarthritis. *OsteoArthritis and Cartilage*, *9*(1), 14-21.

Joseph, N. J., Lakshmi, S., & Jayakrishnan, A. (2002). A floating-type oral dosage form for piroxicam based on hollow polycarbonate microspheres: *in vitro* and in vivo evaluation in rabbits. *Journal of controlled release*, *79*(1), 71-79.

Junoh, H., Jaafar, J., Norddin, M. N. A. M., Ismail, A. F., Othman, M. H. D., Rahman, M. A., ... & Ilbeygi, H. (2015). A review on the fabrication of electrospun polymer electrolyte membrane for direct methanol fuel cell. *Journal of Nanomaterials*, *2015*, 4.

Kakarlapudi, T. K., & Bickerstaff, D. R. (2000). Knee instability: isolated and complex. *British journal of sports medicine*, *34*(5), 395-400.

Kang, M. L., Ko, J. Y., Kim, J. E., & Im, G. I. (2014). Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles for cartilage regeneration. *Biomaterials*, *35*(37), 9984-9994.

Kawazoe, N., Lin, X., Tateishi, T., & Chen, G. (2009). Three-dimensional cultures of rat pancreatic RIN-5F cells in porous PLGA-collagen hybrid scaffolds. *Journal of Bioactive and Compatible Polymers*, *24*(1), 25-42.

Khan, A. K., Rashid, R., Murtaza, G., & Zahra, A. (2014). Gold nanoparticles: synthesis and applications in drug delivery. *Trop J Pharm Res*, *13*(7), 1169-1177.

Kim, C. K., Ghosh, P., & Rotello, V. M. (2009). Multimodal drug delivery using gold nanoparticles. *Nanoscale*, *1*(1), 61-67.

Kim, G., Okumura, M., Bosnakovski, D., Ishiguro, T., Park, C. H., Kadosawa, T., & Fujinaga, T. (2003). Effects of ascorbic acid on proliferation and biological properties of bovine chondrocytes in alginate beads. *Japanese journal of veterinary research*, *51*(2), 83-94.

Kim, S. E., Park, J. H., Cho, Y. W., Chung, H., Jeong, S. Y., Lee, E. B., & Kwon, I. C. (2003). Porous chitosan scaffold containing microspheres loaded with transforming growth factor-β1: implications for cartilage tissue engineering. *Journal of Controlled Release*, *91*(3), 365-374.

Ko, J. Y., Choi, Y. J., Jeong, G. J., & Im, G. I. (2013). Sulforaphane–PLGA microspheres for the intra-articular treatment of osteoarthritis. *Biomaterials*, *34*(21), 5359-5368.

Kobayashi, S., Meir, A., & Urban, J. (2008). Effect of cell density on the rate of glycosaminoglycan accumulation by disc and cartilage cells in vitro. *Journal of Orthopaedic Research*, *26*(4), 493-503

Kong, B., Seog, J. H., Graham, L. M., & Lee, S. B. (2011). Experimental considerations on the cytotoxicity of nanoparticles. *Nanomedicine*, *6*(5), 929-941.

Kong, Y. Q., Li, D., Wang, L. J., & Adhikari, B. (2011). Preparation of gelatin microparticles using water-in-water (w/w) emulsification technique. *Journal of Food Engineering*, *103*(1), 9-13.

Kraus, V. B., Huebner, J. L., Stabler, T., Flahiff, C. M., Setton, L. A., Fink, C., ... & Clark, A. G. (2004). Ascorbic acid increases the severity of spontaneous knee osteoarthritis in a guinea pig model. *Arthritis & Rheumatism*, *50*(6), 1822-1831.

Kumar, M. N. V. R. (2000). Nano and microparticles as controlled drug delivery devices. *J. Pharm. Pharm. Sci*, *3*(2), 234-258.

Kuo, C. K., Li, W. J., Mauck, R. L., & Tuan, R. S. (2006). Cartilage tissue engineering: its potential and uses. *Current opinion in rheumatology*, *18*(1), 64-73.

Kvist, A. J., Johnson, A. E., Mörgelin, M., Gustafsson, E., Bengtsson, E., Lindblom, K & Aspberg, A. (2006). Chondroitin sulfate perlecan enhances collagen fibril formation Implications for perlecan chondrodysplasias. *Journal of Biological Chemistry*, *281*(44), 33127-33139.

Kwan, K. H., Yeung, K. W., Liu, X., Wong, K. K., Shum, H. C., Lam, Y. W & To, M. K. (2014). Silver nanoparticles alter proteoglycan expression in the promotion of tendon repair. *Nanomedicine: Nanotechnology, Biology and Medicine*, *10*(7), 1375-1383.

Kwok, S. K., Park, M. K., Cho, M. L., Oh, H. J., Park, E. M., Lee, D. G & Park, S. H. (2012). Retinoic acid attenuates rheumatoid inflammation in mice. *The Journal of Immunology*, *189*(2), 1062-1071.

Laar, M. V. D., Pergolizzi, J. V., Mellinghoff, H. U., Merchante, I. M., Nalamachu, S., O'Brien, J., ... & Raffa, R. B. (2012). Pain treatment in arthritis-related pain: beyond NSAIDs. *The open rheumatology journal*, *6*(1).

Lammers, T. (2013). Smart drug delivery systems: back to the future vs. clinical reality. *International journal of pharmaceutics*, *454*(1), 527-529.

Lan, M. Y., Hsu, Y. B., Hsu, C. H., Ho, C. Y., Lin, J. C., & Lee, S. W. (2013). Induction of apoptosis by high-dose gold nanoparticles in nasopharyngeal carcinoma cells. *Auris Nasus Larynx*, *40*(6), 563-568.

Lanone, S., Rogerieux, F., Geys, J., Dupont, A., Maillot-Marechal, E., Boczkowski, J., ... & Hoet, P. (2009). Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. *Particle and fibre toxicology*, *6*(1), 1.

Latt, S. A., Stetten, G. A. I. L., Juergens, L. A., Willard, H. F., & Scher, C. D. (1975). Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. *Journal of Histochemistry & Cytochemistry*, *23*(7), 493-505.

Lee, H., Lee, M. Y., Bhang, S. H., Kim, B. S., Kim, Y. S., Ju, J. H., ... & Hahn, S. K. (2014). Hyaluronate–gold nanoparticle/tocilizumab complex for the treatment of rheumatoid arthritis. *ACS nano*, *8*(5), 4790-4798.

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.

Leonavičienė, L., Kirdaitė, G., Bradūnaitė, R., Vaitkienė, D., Vasiliauskas, A., Zabulytė, D & Mackiewicz, Z. (2012). Effect of gold nanoparticles in the treatment of established collagen arthritis in rats. *Medicina (Kaunas)*, *48*(2), 91-101.

Leong, D. J., Choudhury, M., Hirsh, D. M., Hardin, J. A., Cobelli, N. J., & Sun, H. B. (2013). Nutraceuticals: potential for chondroprotection and molecular targeting of osteoarthritis. *International journal of molecular sciences*, *14*(11), 23063-23085.

Li, N., Xu, Y., Zhang, H., Gao, L., Li, J., Wang, Y & Yu, Z. (2014). Excessive retinoic acid impaired proliferation and differentiation of human fetal palatal chondrocytes (hFPCs). *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *101*(3), 276-282.

Li, W., Li, X., Su, H., Zhao, S., Li, Y., & Hu, J. (2011). Facile synthesis of chondroitin sulfate-stabilized gold nanoparticles. *Materials Chemistry and Physics*, *125*(3), 518-521.

Li, Z., & Zhang, M. (2005). Chitosan–alginate as scaffolding material for cartilage tissue engineering. *Journal of biomedical materials research Part A*, *75*(2), 485-493.

Lin, Z., Willers, C., Xu, J., & Zheng, M. H. (2006). The chondrocyte: biology and clinical application. *Tissue engineering*, *12*(7), 1971-1984.

Lohcharoenkal, W., Wang, L., Chen, Y. C., & Rojanasakul, Y. (2014). Protein nanoparticles as drug delivery carriers for cancer therapy. *BioMed research international*, *2014*.

Lozinsky, V. I., Galaev, I. Y., Plieva, F. M., Savina, I. N., Jungvid, H., & Mattiasson, B. (2003). Polymeric cryogels as promising materials of biotechnological interest. *TRENDS in Biotechnology*, *21*(10), 445-451.

Lu, H. D., Zhao, H. Q., Wang, K., & Lv, L. L. (2011). Novel hyaluronic acid–chitosan nanoparticles as non-viral gene delivery vectors targeting osteoarthritis. *International journal of pharmaceutics*, *420*(2), 358-365.

Lützner, J., Kasten, P., Günther, K. P., & Kirschner, S. (2009). Surgical options for patients with osteoarthritis of the knee. *Nature Reviews Rheumatology*, *5*(6), 309-316.

Ma, F., Xiao, Z., Meng, D., Hou, X., Zhu, J., Dai, J., & Xu, R. (2014). Use of natural neural scaffolds consisting of engineered vascular endothelial growth factor immobilized on ordered collagen fibers filled in a collagen tube for peripheral nerve regeneration in rats. *International journal of molecular sciences*, *15*(10), 18593-18609.

Machado, G. C., Maher, C. G., Ferreira, P. H., Pinheiro, M. B., Lin, C. W. C., Day, R. O & Ferreira, M. L. (2015). Efficacy and safety of paracetamol for spinal pain and osteoarthritis: systematic review and meta-analysis of randomised placebo controlled trials. *bmj*, *350*.

Madhav, N. S., & Kala, S. (2011). Review on microparticulate drug delivery system. *Int J PharmTech Res*, *3*, 1242-54.

Makarov, V. I., & Khmelinskii, I. (2011). FTIR and UV spectroscopy in real-time monitoring of S. cerevisiae cell culture. *Electromagnetic biology and medicine*, *30*(4), 181-197.

Mal, P., Dutta, S., Bandyopadhyay, D., Dutta, K., Basu, A., & Bishayi, B. (2012). Gentamicin in combination with ascorbic acid regulates the severity of Staphylococcus aureus infection–induced septic arthritis in mice. *Scandinavian journal of immunology*, *76*(6), 528-540.

Malam, Y., Loizidou, M., & Seifalian, A. M. (2009). Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends in pharmacological sciences*, *30*(11), 592-599.

Malemud, C. (2013). Repair of injury to articular cartilage with chondrocyte progenitor cells. *Rheumatol Curr Res*, *3*(2), 122.

Malmonge, S. M., Campos, D. M., Attik, N. G., Grosgogeat, B., & Gritsch, K. (2015). A chitosan-hyaluronic acid hydrogel scaffold for periodontal tissue engineering. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*.

Martin, J. A., & Buckwalter, J. A. (2002). Aging, articular cartilage chondrocyte senescence and osteoarthritis. *Biogerontology*, *3*(5), 257-264.

Martinho, N., Damgé, C., & Reis, C. P. (2011). Recent advances in drug delivery systems. *Journal of biomaterials and nanobiotechnology*, *2*, 510.

Mathew, G., Timm Jr, E. A., Sotomayor, P., Godoy, A., Montecinos, V. P., Smith, G. J., & Huss, W. J. (2009). ABCG2-mediated DyeCycle Violet efflux defined side population in benign and malignant prostate. *Cell Cycle*, *8*(7), 1053-1061.

Memon, A. R., & Quinlan, J. F. (2012). Surgical treatment of articular cartilage defects in the knee: are we winning?. *Advances in orthopedics*, *2012*.

Miot, S., Scandiucci de Freitas, P., Wirz, D., Daniels, A. U., Sims, T. J., Hollander, A. P &
Martin, I. (2006). Cartilage tissue engineering by expanded goat articular chondrocytes. *Journal of orthopaedic research*, *24*(5), 1078-1085.

Mishra, R., & Kumar, A. (2014). Osteocompatibility and osteoinductive potential of supermacroporous polyvinyl alcohol-TEOS-Agarose-CaCl2 (PTAgC) biocomposite cryogels. *Journal of Materials Science: Materials in Medicine*, *25*(5), 1327-1337.

Mishra, R., Goel, S. K., Gupta, K. C., & Kumar, A. (2013). Biocomposite cryogels as tissue-engineered biomaterials for regeneration of critical-sized cranial bone defects. *Tissue Engineering Part A*, *20*(3-4), 751-762.

Mobasheri, A., Csaki, C., Clutterbuck, A. L., Rahmanzadeh, M., & Shakibaei, M. (2009). Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy.

Mobasheri, A., Henrotin, Y., Biesalski, H. K., & Shakibaei, M. (2012). Scientific evidence and rationale for the development of curcumin and resveratrol as nutraceutricals for joint health. *International journal of molecular sciences*, *13*(4), 4202-4232.

Mogoşanu, G. D., & Grumezescu, A. M. (2014). Natural and synthetic polymers for wounds and burns dressing. *International journal of pharmaceutics*, *463*(2), 127-136.

Monfort, J., Martel-Pelletier, J., & Pelletier, J. P. (2008). Chondroitin sulphate for symptomatic osteoarthritis: critical appraisal of meta-analyses. *Current medical research and opinion*, *24*(5), 1303-1308.

Morgen, M., Tung, D., Boras, B., Miller, W., Malfait, A. M., & Tortorella, M. (2013). Nanoparticles for improved local retention after intra-articular injection into the knee joint. *Pharmaceutical research*, *30*(1), 257-268.

Mort, J. S., & Billington, C. J. (2001). Articular cartilage and changes in arthritis: matrix degradation. *Arthritis Res*, *3*(6), 337-341.

Murad, S., Grove, D., Lindberg, K. A., Reynolds, G., Sivarajah, A., & Pinnell, S. R. (1981). Regulation of collagen synthesis by ascorbic acid. *Proceedings of the National Academy of Sciences*, *78*(5), 2879-2882.

Mwangi, T. K., Bowles, R. D., Tainter, D. M., Bell, R. D., Kaplan, D. L., & Setton, L. A. (2015). Synthesis and characterization of silk fibroin microparticles for intra-articular drug delivery. *International journal of pharmaceutics*, *485*(1), 7-14.

Naqvi, S., Samim, M., Abdin, M., Ahmed, F. J., Maitra, A., Prashant, C., & Dinda, A. K. (2010). Concentration-dependent toxicity of iron oxide nanoparticles mediated by increased oxidative stress. *Int J Nanomedicine*, *5*, 983-989.

Narayan, S., Rajagopalan, A., Reddy, J. S., & Chadha, A. (2014). BSA binding to silica capped gold nanostructures: effect of surface cap and conjugation design on nanostructure–BSA interface. *RSC Advances*, *4*(3), 1412-1420.

Naskar, S., Dawn, I., Sarkar, S., De, C., & Biswas, G. (2013). A comparative study between Plasma vitamin c level and severity of knee osteoarthritis. *IOSR-JDMS*, 4, 10-14.

Natarajan, V., Madhan, B., & Tiku, M. L. (2015). Intra-articular injections of polyphenols protect articular cartilage from inflammation-induced degradation: suggesting a potential role in cartilage therapeutics. *PloS one*, *10*(6).

Nawabi, D. H., Chin, K. F., Keen, R. W., & Haddad, F. S. (2010). Vitamin D deficiency in patients with osteoarthritis undergoing total hip replacement A CAUSE FOR CONCERN?. *Journal of Bone & Joint Surgery, British Volume*, *92*(4), 496-499.

Nguyen, D. T., Kim, D. J., So, M. G., & Kim, K. S. (2010). Experimental measurements of gold nanoparticle nucleation and growth by citrate reduction of HAuCl 4. *Advanced powder technology*, *21*(2), 111-118.

Noguchi, T., Oka, M., Fujino, M., Neo, M., & Yamamuro, T. (1994). Repair of Osteochondral Defects With Grafts of Cultured Chondrocytes: Comparison of Allografts and Isografts. *Clinical orthopaedics and related research*, *302*, 251-258.

Nozaki, Y., Yamagata, T., Sugiyama, M., Ikoma, S., Kinoshita, K., & Funauchi, M. (2006). Anti-inflammatory effect of all-trans-retinoic acid in inflammatory arthritis. *Clinical Immunology*, *119*(3), 272-279.

O'brien, F. J. (2011). Biomaterials & scaffolds for tissue engineering. *Materials Today*, *14*(3), 88-95.

Oh, S. H., Park, I. K., Kim, J. M., & Lee, J. H. (2007). *In vitro* and *in vivo* characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. *Biomaterials*, *28*(9), 1664-1671.

Ohno, S., Ohno, Y., Suzuki, N., Soma, G. I., & Inoue, M. (2009). High-dose vitamin C (ascorbic acid) therapy in the treatment of patients with advanced cancer. *Anticancer Research*, *29*(3), 809-815.

Omata, T., Itokazu, Y., Inoue, N., & Segawa, Y. (2000). Effects of chondroitin sulfate-C on articular cartilage destruction in murine collagen-induced arthritis. *Arzneimittel-Forschung*, *50*(2), 148-153.

Oussedik, S., Tsitskaris, K., & Parker, D. (2015). Treatment of articular cartilage lesions of the knee by microfracture or autologous chondrocyte implantation: a systematic review. *Arthroscopy: The Journal of Arthroscopic & Related Surgery*, *31*(4), 732-744.

Pal, C. P., Singh, P., Chaturvedi, S., Pruthi, K. K., & Vij, A. (2016). Epidemiology of knee osteoarthritis in India and related factors. *Indian journal of orthopaedics*, *50*(5), 518

Parenteau-Bareil, R., Gauvin, R., & Berthod, F. (2010). Collagen-based biomaterials for tissue engineering applications. *Materials*, *3*(3), 1863-1887.

Parida, K. R., Panda, S. K., Ravanan, P., Roy, H., Manickam, M., & Talwar, P. (2013). Microparticles based drug delivery systems: preparation and application in cancer therapeutics. *Cellulose*, *17*, 18.

Park, C., Youn, H., Kim, H., Noh, T., Kook, Y. H., Oh, E. T., ... & Kim, C. (2009). Cyclodextrin-covered gold nanoparticles for targeted delivery of an anti-cancer drug. *Journal of Materials Chemistry*, *19*(16), 2310-2315.

Park, H. J., Yu, S. J., Yang, K., Jin, Y., Cho, A. N., Kim, J. & Cho, S. W. (2014). Paper-based bioactive scaffolds for stem cell-mediated bone tissue engineering. *Biomaterials*, *35*(37), 9811-9823.

Park, S. Y., Yamane, K., Adachi, S. I., Shiro, Y., Weiss, K. E., & Sligar, S. G. (2000). Crystallization and preliminary X-ray diffraction analysis of a cytochrome P450 (CYP119) from Sulfolobus solfataricus. *Acta Crystallographica Section D: Biological Crystallography*, *56*(9), 1173-1175.

Pavan Kumar, B., Sarath Chandiran, I., Bhavya, B., & Sindhuri, M. (2011). Microparticulate drug delivery system: a review. *Indian J Pharm Sci Res*, *1*(1), 19-37

Pecchi, E., Priam, S., Mladenovic, Z., Gosset, M., Saurel, A. S., Aguilar, L., ... & Jacques, C. (2012). A potential role of chondroitin sulfate on bone in osteoarthritis: inhibition of prostaglandin E 2 and matrix metalloproteinases synthesis in interleukin-1β-stimulated osteoblasts. *Osteoarthritis and Cartilage*, *20*(2), 127-135.

Peppas, N. A., Bures, P., Leobandung, W., & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European journal of pharmaceutics and biopharmaceutics*, *50*(1), 27-46.

Permyakov, E. A. (2012). The Use of UV–Vis Absorption Spectroscopy for Studies of Natively Disordered Proteins. *Intrinsically Disordered Protein Analysis: Volume 1, Methods and Experimental Tools*, 421-433.

Plajnsek, K. T., Kocbek, P., Kreft, M. E., & Kristl, J. (2012). Mechanisms of cellular uptake of nanoparticles and their effect on drug delivery. *Zdravniski Vestnik*, *81*(3).

Poole, C. A. (1997). Review. Articular cartilage chondrons: form, function and failure. *Journal of anatomy*, *191*(1), 1-13.

Poursamar, S. A., Hatami, J., Lehner, A. N., da Silva, C. L., Ferreira, F. C., & Antunes, A. P. M. (2015). Gelatin porous scaffolds fabricated using a modified gas foaming technique: Characterisation and cytotoxicity assessment. *Materials Science and Engineering: C*, *48*, 63-70.

Prades, R., Guerrero, S., Araya, E., Molina, C., Salas, E., Zurita, E & Kogan, M. J. (2012). Delivery of gold nanoparticles to the brain by conjugation with a peptide that recognizes the transferrin receptor. *Biomaterials*, *33*(29), 7194-7205.

Pramanik, N., Mishra, D., Banerjee, I., Maiti, T. K., Bhargava, P., & Pramanik, P. (2009). Chemical synthesis, characterization, and biocompatibility study of hydroxyapatite/chitosan phosphate nanocomposite for bone tissue engineering applications. *International journal of biomaterials*, *2009*

Ramanaviciene, A., Nastajute, G., Snitka, V., Kausaite, A., German, N., Barauskas-Memenas, D., & Ramanavicius, A. (2009). Spectrophotometric evaluation of gold nanoparticles as red-ox mediator for glucose oxidase. *Sensors and Actuators B: Chemical*, *137*(2), 483-489.

Ramaswamy, S., Uluer, M. C., Leen, S., Bajaj, P., Fishbein, K. W., & Spencer, R. G. (2008). Noninvasive assessment of glycosaminoglycan production in injectable tissue-engineered cartilage constructs using magnetic resonance imaging. *Tissue Engineering Part C: Methods*, *14*(3), 243-249

Ramteke, K. H., Jadhav, V. B., & Dhole, S. N. (2012). Microspheres: As carriers used for novel drug delivery system. *IOSRPHR*, *2*(4), 44-48.

Raynauld, J. P., Pelletier, J. P., Abram, F., Delorme, P., & Martel-Pelletier, J. (2016). Long-term effects of glucosamine/chondroitin sulfate on the progression of structural

changes in knee osteoarthritis: 6-year follow-up data from the osteoarthritis initiative. *Arthritis care & research*

Reddi, A. H. (1998). Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nature biotechnology*, *16*(3), 247-252.

Reddy, C. P., Chaitanya, K. S. C., & Rao, M. Y. (2011). A review on bioadhesive buccal drug delivery systems: current status of formulation and evaluation methods. *Daru*, *19*(6).

Reginster, J. Y., Neuprez, A., Lecart, M. P., Sarlet, N., & Bruyere, O. (2012). Role of glucosamine in the treatment for osteoarthritis. *Rheumatology international*, *32*(10), 2959-2967.

Richmond, J., Hunter, D., Irrgang, J., Jones, M. H., Levy, B., Marx, R & Wies, M. J. L. (2009). Treatment of osteoarthritis of the knee (nonarthroplasty). *The Journal of the American Academy of Orthopaedic Surgeons*, *17*(9), 591.

Roach, H. I. (1997). New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. *Journal of Bone and Mineral Research*, *12*(5), 795-805.

Robertson, W. V. B., & Schwartz, B. (1953). Ascorbic acid and the formation of collagen. *J. biol. Chem*, *201*, 689.

Rönn, K., Reischl, N., Gautier, E., & Jacobi, M. (2011). Current surgical treatment of knee osteoarthritis. *Arthritis*, *2011*.

Ronzière, M. C., Roche, S., Gouttenoire, J., Démarteau, O., Herbage, D., & Freyria, A. M. (2003). Ascorbate modulation of bovine chondrocyte growth, matrix protein gene expression and synthesis in three-dimensional collagen sponges. *Biomaterials*, *24*(5), 851-861.

Roth, S. H., & Anderson, S. (2011). The NSAID dilemma: managing osteoarthritis in high-risk patients. *The Physician and sportsmedicine*, *39*(3), 62-74.

Safran, M. R., & Seiber, K. (2010). The evidence for surgical repair of articular cartilage in the knee. *Journal of the American Academy of Orthopaedic Surgeons*, *18*(5), 259-266.

Sahai, N., & Tewari, R. P. (2015). Characterization of effective mechanical strength of chitosan porous tissue scaffolds using computer aided tissue engineering. *International journal of Biomedical Engineering and Science (2)*

Sahil, K., Akanksha, M., Premjeet, S., Bilandi, A., & Kapoor, B. (2011). Microsphere: A review. *Int. J. Res. Pharm. Chem*, *1*(4), 1184-1198.

Spector, M. (2002). Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. *Human Gene Therapy*, *13*(7), 791-802.

Sanghvi, D., Munshi, M., & Pardiwala, D. (2014). Imaging of cartilage repair procedures. *The Indian journal of radiology and imaging,* 24(3),249.

Saravanan, M., Bhaskar, K., Maharajan, G., & Pillai, K. S. (2011). Development of gelatin microspheres loaded with diclofenac sodium for intra-articular administration. *Journal of drug targeting*, *19*(2), 96-103.

Schneevoigt, J., Fabian, C., Leovsky, C., Seeger, J., & Bahramsoltani, M. (2016). In Vitro Expression of the Extracellular Matrix Components Aggrecan, Collagen Types I and II by Articular Cartilage-Derived Chondrocytes. *Anatomia, histologia, embryologia*

Schwartz, E. R., & Adamy, L. (1977). Effect of ascorbic acid on arylsulfatase activities and sulfated proteoglycan metabolism in chondrocyte cultures. *Journal of Clinical Investigation*, *60*(1), 96.

Schwartz, N. B., & Dorfman, A. (1975). Stimulation of chondroitin sulfate proteoglycan production by chondrocytes in monolayer. *Connective tissue research*, *3*(2-3), 115-122.

Sechriest, V. F., Miao, Y. J., Niyibizi, C., Westerhausen-Larson, A., Matthew, H. W., Evans, C. H., ... & Suh, J. K. (2000). GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. *Journal of biomedical materials research*, *49*(4), 534-541.

Selimović, A., Salkić, M., & Selimović, A. (2011). Direct spectrophotometric determination of L-ascorbic acid in pharmaceutical preparations using sodium oxalate as a stabilizer. *International Journal of Basic & Applied Sciences*, *11*(02).

Seo, S. S., Kim, C. W., & Jung, D. W. (2011). Management of focal chondral lesion in the knee joint. *Knee surgery & related research*, *23*(4), 185-196.

Sharma, A., Wood, L. D., Richardson, J. B., Roberts, S., & Kuiper, N. J. (2007). Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage. *Arthritis Research and Therapy*, *9*(4), R79.

Sharma, G., & Rathore, D. S. (2015). Potential Role of Nutraceuticals in the Management of Knee and Hip Joint Osteoarthritis. *Biomedical Science and Engineering*, *3*(1), 23-29.

Shi, M., Yang, Y. Y., Chaw, C. S., Goh, S. H., Moochhala, S. M., Ng, S., & Heller, J. (2003). Double walled POE/PLGA microspheres: encapsulation of water-soluble and water-insoluble proteins and their release properties. *Journal of controlled release*, *89*(2), 167-177

Siclari, A., Mascaro, G., Gentili, C., Kaps, C., Cancedda, R., & Boux, E. (2014). Cartilage repair in the knee with subchondral drilling augmented with a platelet-rich plasma-immersed polymer-based implant. *Knee Surgery, Sports Traumatology, Arthroscopy*, *22*(6), 1225-1234.

Silva, T. H., Moreira-Silva, J., Marques, A. L., Domingues, A., Bayon, Y., & Reis, R. L. (2014). Marine origin collagens and its potential applications. *Marine drugs*, *12*(12), 5881-5901

Singh, A. K., KAlAivAni, M., KriShnAn, A., AggArwAl, P. K., & Gupta, S. K. (2014). Prevalence of osteoarthritis of knee among elderly persons in urban slums using American College of Rheumatology (ACR) criteria. *J. Clin. Diagn. Res*, *8*(9), 9-11.

Singh, D., Nayak, V., & Kumar, A. (2010). Proliferation of myoblast skeletal cells on three-dimensional supermacroporous cryogels. *Int J Biol Sci*, *6*(4), 371-381.

Singh, D., Singh, D., & HAN, S. S. Fabrication of three dimensional macroporous matrix for cardiac regeneration. *International Journal of Applied Engineering Research*, *7*(11), 2012.

Singh, D., Tripathi, A., Nayak, V., & Kumar, A. (2011). Proliferation of Chondrocytes on a 3-D Modelled Macroporous Poly (Hydroxyethyl Methacrylate)–Gelatin Cryogel. *Journal of Biomaterials Science, Polymer Edition*, *22*(13), 1733-1751.

Singh, M. N., Hemant, K. S. Y., Ram, M., & Shivakumar, H. G. (2011). Microencapsulation: a promising technique for controlled drug delivery. *Research in pharmaceutical sciences*, *5*(2), 65-77.

Singh, M., Morris, C. P., Ellis, R. J., Detamore, M. S., & Berkland, C. (2008). Microsphere-based seamless scaffolds containing macroscopic gradients of encapsulated factors for tissue engineering. *Tissue Engineering Part C: Methods*, *14*(4), 299-309.

Singh, S., Pandey, V. K., Tewari, R. P., & Agarwal, V. (2011). Nanoparticle based drug delivery system: advantages and applications. *Indian Journal of Science and Technology*, *4*(3), 177-180.
Sinusas, K. (2012). Osteoarthritis: diagnosis and treatment. *American family physician*, *85*(1).

Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current opinion in plant biology*, *3*(3), 229-235.

Sommer, F., Kobuch, K., Brandl, F., Wild, B., Framme, C., Weiser, B., & Goepferich, A. (2007). Ascorbic acid modulates proliferation and extracellular matrix accumulation of hyalocytes. *Tissue engineering*, *13*(6), 1281-1289.

Sorice, A., Guerriero, E., Capone, F., Colonna, G., Castello, G., & Costantini, S. (2014). Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini reviews in medicinal chemistry*, *14*(5), 444-452

Spivak, M. Y., Bubnov, R. V., Yemets, I. M., Lazarenko, L. M., Tymoshok, N. O., & Ulberg, Z. R. (2013). Development and testing of gold nanoparticles for drug delivery and treatment of heart failure: a theranostic potential for PPP cardiology. *EPMA J*, *4*(1), 20.

Stephens, D., Li, L., Robinson, D., Chen, S., Chang, H. C., Liu, R. M., ... & Stultz, T. (2000). Investigation of the in vitro release of gentamicin from a polyanhydride matrix. *Journal of controlled release*, *63*(3), 305-317.

Stockert, J. C., Blázquez-Castro, A., Cañete, M., Horobin, R. W., & Villanueva, Á. (2012). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta histochemica*, *114*(8), 785-796.

Stroh, D. A., Johnson, A. J., & Mont, M. A. (2011). Surgical implants and technologies for cartilage repair and preservation of the knee. *Expert review of medical devices*, *8*(3), 339-356.

Stuart, K., & Panitch, A. (2008). Influence of chondroitin sulfate on collagen gel structure and mechanical properties at physiologically relevant levels. *Biopolymers*, *89*(10), 841-851.

Subia, B., Kundu, J., & Kundu, S. C. (2010). *Biomaterial scaffold fabrication techniques for potential tissue engineering applications*. INTECH Open Access Publisher.

Surapaneni, L., Huang, G., Bodine, A. B., Brooks, J., Podila, R., & Haley-Zitlin, V. (2014). Correlations between Chondroitin Sulfate Physicochemical Properties and its in-vitro Absorption and Anti-inflammatory Activity. *arXiv preprint arXiv:1412.5562*.

Swami, A., Shi, J., Gadde, S., Votruba, A. R., Kolishetti, N., & Farokhzad, O. C. (2012). Nanoparticles for targeted and temporally controlled drug delivery. In *Multifunctional nanoparticles for drug delivery applications* (pp. 9-29). Springer US

Sy, J. C., & Davis, M. E. (2010). Delivering regenerative cues to the heart: cardiac drug delivery by microspheres and peptide nanofibers. *Journal of cardiovascular translational research*, *3*(5), 461-468.

Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Drug Design and Discovery: Methods and Protocols*, 157-168.

Takei, T., Nakahara, H., Tanaka, S., Nishimata, H., Yoshida, M., & Kawakami, K. (2013). Effect of chitosan-gluconic acid conjugate/poly (vinyl alcohol) cryogels as wound dressing on partial-thickness wounds in diabetic rats. *Journal of Materials Science: Materials in Medicine*, *24*(10), 2479-2487.

Tantra, R., Schulze, P., & Quincey, P. (2010). Effect of nanoparticle concentration on zeta-potential measurement results and reproducibility. *Particuology*, *8*(3), 279-285.

Thiruganesh, R., Subbaih Khandasamy, U., Suresh, S., & Himabindhu, R. (2011). Formulation and evaluation of chondroitin sulphate tablets of aceclofenac for colon targeted drug delivery. *Iranian Journal of Pharmaceutical Research*, *11*(2), 465-479.

Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., & Bannerjee, S. K. (2012). Drug delivery systems: An updated review. *International journal of pharmaceutical investigation*, *2*(1), 2.

Torzilli, P. A. (1985). Influence of cartilage conformation on its equilibrium water partition. *Journal of orthopaedic research*, *3*(4), 473-483.

Tripathi, A., Sami, H., Jain, S. R., Viloria-Cols, M., Zhuravleva, N., Nilsson, G., ... & Kumar, A. (2010). Improved bio-catalytic conversion by novel immobilization process using cryogel beads to increase solvent production. *Enzyme and Microbial Technology*, *47*(1), 44-51.

Tuan, R. S. (2007). A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects. *Arthritis Research and Therapy*, *9*(5), 109.

Uddin, M. S., Hawlader, M. N. A., & Zhu, H. J. (2001). Microencapsulation of ascorbic acid: effect of process variables on product characteristics. *Journal of Microencapsulation*, *18*(2), 199-209.

Ulrich-Vinther, M., Maloney, M. D., Schwarz, E. M., Rosier, R., & O'Keefe, R. J. (2003). Articular cartilage biology. *Journal of the American Academy of Orthopaedic Surgeons*, *11*(6), 421-430.

Umezawa, M., Kudo, S., Yanagita, S., Shinkai, Y., Niki, R., Oyabu, T., ... & Sugamata, M. (2011). Maternal exposure to carbon black nanoparticle increases collagen type VIII expression in the kidney of offspring. *The Journal of toxicological sciences*, *36*(4), 461-468.

Unterman, S. A., Gibson, M., Lee, J. H., Crist, J., Chansakul, T., Yang, E. C., & Elisseeff, J. H. (2012). Hyaluronic acid-binding scaffold for articular cartilage repair. *Tissue Engineering Part A*, *18*(23-24), 2497-2506.

Van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Cancer cell culture: methods and protocols*, 237-245.

Van Vijven, J. P. J., Luijsterburg, P. A. J., Verhagen, A. P., Van Osch, G. J. V. M., Kloppenburg, M., & Bierma-Zeinstra, S. M. A. (2012). Symptomatic and chondroprotective treatment with collagen derivatives in osteoarthritis: a systematic review. *Osteoarthritis and Cartilage*, *20*(8), 809-821.

Varde, N. K. (2007). *Elucidation of drug release mechanisms in PLGA microspheres*. ProQuest.

Varde, N. K., & Pack, D. W. (2004). Microspheres for controlled release drug delivery. *Expert Opinion on Biological Therapy*, *4*(1), 35-51.

Varghese, S., Hwang, N. S., Canver, A. C., Theprungsirikul, P., Lin, D. W., & Elisseeff, J. (2008). Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biology*, *27*(1), 12-21.

Venkatpurwar, V., Shiras, A., & Pokharkar, V. (2011). Porphyran capped gold nanoparticles as a novel carrier for delivery of anticancer drug: *in vitro* cytotoxicity study. *International journal of pharmaceutics*, *409*(1), 314-320.

Venkatesha, S. H., Astry, B., Nanjundaiah, S. M., Kim, H. R., Rajaiah, R., Yang, Y., & Moudgil, K. D. (2016). Control of autoimmune arthritis by herbal extracts and their bioactive components. *asian journal of pharmaceutical sciences*, *11*(2), 301-307.

Versypt, A. N. F., Pack, D. W., & Braatz, R. D. (2013). Mathematical modeling of drug delivery from autocatalytically degradable PLGA microspheres a review. *Journal of Controlled Release*, *165*(1), 29-37.

Vijayakumar, S., & Ganesan, S. (2012). *In vitro* cytotoxicity assay on gold nanoparticles with different stabilizing agents. *Journal of Nanomaterials*, *2012*, 14.

Vilar, G., Tulla-Puche, J., & Albericio, F. (2012). Polymers and drug delivery systems. *Current Drug Delivery*, *9*(4), 367-394.

Vilos, C., & Velasquez, L. A. (2012). Therapeutic strategies based on polymeric microparticles. *BioMed Research International*, *2012*.

Vishnoi, T & Kumar, A. (2013). Conducting cryogel scaffold as a potential biomaterial for cell stimulation and proliferation. *Journal of Materials Science: Materials in Medicine*, *24*(2), 447-459.

Von den Hoff, J. W., Van Kampen, G. P. J., Van de Stadt, R. J., & Van der Korst, J. K. (1993). Kinetics of proteoglycan turnover in bovine articular cartilage explants. *Matrix*, *13*(3), 195-201.

Wang, Q., Yang, Y. Y., Niu, H. J., Zhang, W. J., Feng, Q. J., & Chen, W. F. (2013). An ultrasound study of altered hydration behaviour of proteoglycan-degraded articular cartilage. *BMC musculoskeletal disorders*, *14*(1), 289.

Wilczewska, A. Z., Niemirowicz, K., Markiewicz, K. H., & Car, H. (2012). Nanoparticles as drug delivery systems. *Pharmacological reports*, *64*(5), 1020-1037.

Wissemann, K. W., & Jacobson, B. S. (1985). Pure gelatin microcarriers: synthesis and use in cell attachment and growth of fibroblast and endothelial cells. *In vitro cellular & developmental biology*, *21*(7), 391-401.

Woodfield, T. B., Malda, J., De Wijn, J., Peters, F., Riesle, J., & van Blitterswijk, C. A. (2004). Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. *Biomaterials*, *25*(18), 4149-4161.

Wu, C. H., Ko, C. S., Huang, J. W., Huang, H. J., & Chu, I. M. (2010). Effects of exogenous glycosaminoglycans on human chondrocytes cultivated on type II collagen scaffolds. *Journal of Materials Science: Materials in Medicine*, *21*(2), 725-729.

Xu, Q., Hashimoto, M., Dang, T. T., Hoare, T., Kohane, D. S., Whitesides, G. M., ... & Anderson, D. G. (2009). Preparation of monodisperse biodegradable polymer microparticles using a microfluidic flow-focusing device for controlled drug delivery. *Small*, *5*(13), 1575-1581.

Yamada, S., & Sugahara, K. (2008). Potential therapeutic application of chondroitin sulfate/dermatan sulfate. *Current drug discovery technologies*, *5*(4), 289-301.

Yang, Y. Y., Chung, T. S., & Ng, N. P. (2001). Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *Biomaterials*, *22*(3), 231-241.

Yoshida, M., Takimoto, R., Murase, K., Sato, Y., Hirakawa, M., Tamura, F., ... & Takada, K. (2012). Targeting anticancer drug delivery to pancreatic cancer cells using a fucose-bound nanoparticle approach. *PloS one*, *7*(7), e39545.

Yoshiya, S., & Dhawan, A. (2015). Cartilage repair techniques in the knee: stem cell therapies. *Current reviews in musculoskeletal medicine*, *8*(4), 457-466.

Zhang, J., Lan, C. Q., Post, M., Simard, B., Deslandes, Y., & Hsieh, T. H. (2006). Design of nanoparticles as drug carriers for cancer therapy. *Cancer Genomics-Proteomics*, *3*(3-4), 147-157.

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).

Zhang, Z., & Huang, G. (2011). Micro-and nano-carrier mediated intra-articular drug delivery systems for the treatment of osteoarthritis. *Journal of Nanotechnology*, *2012*.

Zhang, W., Ouyang, H., Dass, C. R., & Xu, J. (2016). Current research on pharmacologic and regenerative therapies for osteoarthritis. *Bone research*, *4*, 15040.

Zhong, L., Huang, X., Karperien, M., & Post, J. N. (2015). The Regulatory Role of Signaling Crosstalk in Hypertrophy of MSCs and Human Articular Chondrocytes. *International journal of molecular sciences*, *16*(8), 19225-19247.

Zhuang, H., Zheng, J. P., Gao, H., & De Yao, K. (2007). In vitro biodegradation and biocompatibility of gelatin/montmorillonite-chitosan intercalated nanocomposite. *Journal of Materials Science: Materials in Medicine*, *18*(5), 951-957.

Zille, H., Paquet, J., Henrionnet, C., Scala-Bertola, J., Leonard, M., Six, J. L & Grossin, L. (2010). Evaluation of intra-articular delivery of hyaluronic acid functionalized biopolymeric nanoparticles in healthy rat knees. *Bio-medical materials and engineering*, *20*(3-4), 235-242.

List of publications as outcome of this research

Dwivedi, P., Bhat, S., Nayak, V., & Kumar, A. (2014). Study of different delivery modes of chondroitin sulfate using microspheres and cryogel scaffold for application in cartilage tissue engineering. *International Journal of Polymeric Materials and Polymeric Biomaterials*, *63*(16), 859-872.

Dwivedi, P., Nayak, V., & Kowshik, M. (2015). Role of gold nanoparticles as drug delivery vehicles for chondroitin sulfate in the treatment of osteoarthritis. *Biotechnology progress*, *31*(5), 1416-1422.

Dwivedi, P., Nayak, V., & Kumar, A. Encapsulation of bioactive molecule for sustained release and study effect of Nutritional supplements as a novel strategy for repair of cartilage injuries. (Manuscript in progress).

Other publications (From previous research experience in IIT Kanpur)

Kumar, A., Mishra, H. K., **Dwivedi, P.,** & Subramaniam, J. R. (2012, December). Cyclic AMP pathway independent induction of neurite exten-sion and their networking in the motor neuron cell line NSC34. In *International Journal of Developmental Neuroscience* (Vol. 30, No. 8, pp. 646-646). The boulevard, langford lane, kindlington, oxford OX 5 1GB, england: pergamon-elsevier science ltd.

Kumar, A., Mishra, H. K., **Dwivedi, P.,** & Subramaniam, J. R. (2015). Secreted trophic factors of Human umbilical cord stromal cells induce differentiation and neurite extension through PI3K and independent of cAMP pathway. *Annals of neurosciences*, *22*(2), 97.

Conferences, workshops and fellowship

Oral presentation in “International Conference on Design of Biomaterials (BIND 12), Bangalore, December 9-11, 2012.

Second prize in Poster presentation in 5th Annual International conference on Advances in biotechnology (BIOTECH2015) from 13th March-15th March 2015 at Indian Institute of Technology Kanpur.

Oral presentation in “Advances in functional materials, **Stony Brook University,** New York (USA) 29th June-3rd July 2015.

Participated in “DBT BIRAC Workshop on Bio- entrepreseurship grant-writing and intellectual property management, 18th & 19th February, 2016, BITS Pilani, K K Birla Goa Campus.

BRNS SRF

CSIR SRF

Curriculum Vitae

PRIYANKA DWIVEDI **Mobile number:08806860487**

(PhD scholar) **Residence: 09670587757**

Email address: priya.dwivedi15@gmail.com

Examination	Board/University	Year of passing	Percentage
M.Sc Biotechnology	Banasthali Vidyapith, Rajasthan	2009	77%
B.Sc Biotechnology (Hons.)	Banasthali Vidyapith, Rajasthan	2007	74%

Research experience

Current:

Pursuing P h.D. from **BITS Pilani K K Birla Goa** campus. On topic entitled "Development of novel Tissue engineering strategies to treat osteoarthritis."Under the guidance Dr. Vijayashree Nayak from BITS Pilani Goa and Co-guidance Dr. Ashok Kumar from IIT Kanpur.

Research Experience:

Worked for **1 year** as **Project Associate** in the Department of Biological Sciences & Bioengineering at the Indian Institute of Technology (IIT) Kanpur, India on the project

entitled **“Identification of Potential Strategies for Protection against Alzheimer’s Disease’’.**

Project Work:

Pursued **6 months** Project Work entitled **“Use of Recombinant DNA Technology to Generate Inducible Expression Construct of Glycogenin”** under the guidance of Dr. Ganesh Subramaniam, Department of Biological Sciences & Bioengineering (BSBE) at the Indian Institute of Technology (IIT) Kanpur, India.

Summer Training:

Carried out **One Month Summer Training** in the **Department of Biological Sciences & Bioengineering (BSBE)** at the Indian Institute of Technology (IIT) Kanpur.

Fellowship received

BRNS JRF, SRF

CSIR SRF

Technical Skills:

Tissue Engineering

Preparation of cryogel for cartilage tissue engineering, worked on primary cell culture like chondrocyte isolation,
Characterization of cryogel by various methods like rheology, mechanical testing, worked on drug delivery vehicle such as microparticle and nanoparticle and its applications

Molecular Biology:

Molecular Cloning, Transfection, Transgenic Mice Handling & Genotyping, RT-PCR, Electrophoresis (Agarose gel & SDS-PAGE), Bacterial Transformation, Colony PCR , Stable cell line generation ,Western Blot.

Cell Culture Techniques:

Primary Cortical Cultures & Astrocytes Cultures, MTT assay

Worked on Neuronal Cell lines (NSC34, NSCG37R , C6 & C6G37R), Umbilical Cord dissection and Culture

Protein Extraction, Sonication, Estimation by BSA & Lowry’s method.

Immunotechniques:

Immunocytochemistry, Immunoprecipitation, ELISA, Hoechst staining, Fluorescence Microscopy.

Computer Proficiency:

Can successfully work on Windows 98/2000/Xp**,** Knowledge of Internet, Primer designing.

Teaching experience

4 years teaching experience in Biological Laboratory, Microbiology**,** Instrumental Methods of Analysis.

Paper published: Four (2 Ph.D. tenure plus 2 in working tenure in IIT kanpur)

Additional Information:

Advanced Diploma course in **German Language**

Personal Information

Father's Name: Mr. Arun Dwivedi
Sex : Female
Nationality: Indian
Languages Known: English, Hindi
Marital Status: Unmarried
Date of Birth: 15-08-85
Permanent Address: 117/545 Pandu Nagar, Kanpur (U.P.)-208005

Declaration:

I hereby declare that all the information given above is true to the best of my knowledge and belief

Place: Goa, India **PRIYANKA**

Biography of supervisor

Personal Details

Name: Dr. Vijayashree Nayak

Designation: Associate Professor

Affiliation: Birla Institute of Technology & Science, Pilani

Email: vijayashree@goa.bits-pilani.ac.in

Contact: 0832-2580161

Dr. Vijayashree Nayak pursued her Ph.D. from MAHE Manipal University in 1998 and has been involved in teaching and research since then in various universities and colleges. Currently her lab broadly focuses on couple of aspects like molecular mechanism in Cancer and therapeutic applications of tissue engineering. Current goal of her research is to obtain a detailed molecular understanding of cell cycle regulation loss and epithelial mesenchymal transition (EMT) in certain cancers. From various known factors responsible for progression towards cancer, her work is particularly concerned with role of chronic infections and bacterial mediated carcinogenesis. Her interest also lies in developing novel scaffolds for cell culture in tissue engineering applications such as cartilage tissue engineering. She guided a student for Ph.D. Degree award in the topic related to cartilage tissue engineering and still 3 more students are pursuing their Ph.D. under her. She has received project grants from DST, BRNS, and ABSTC.

She has published 24 papers in International and National journals, and has attended and delivered several talks in National and International conferences.

Biography of Co- supervisor

Personal Details

Name: Dr. Ashok Kumar

Designation: Professor

Affiliation: Indian Institute of Technology, Kanpur

Email: ashokkum@iitk.ac.in

Contact: 0512-259-40151

Dr. Kumar pursued his Ph.D. jointly from IIT Roorkee, and the Institute of Genomics and Integrative Biology, Delhi. He has postdoctoral research experience from institutes in Sweden and Japan. He was a faculty at Lund University, Sweden. His research focuses on the design and development of new smart polymeric biomaterials and supermacroporous matrices for different applications in biology such as cartilage, bone, skin and neural tissue engineering and drug delivery. He has received several project grants from DST, DBT and HLL etc. He has guided several Ph.D. students. He has received several awards and fellowships such as TATA Innovation fellowship award by DBT (2013), Madhav Rao Scindia memorial award in science and technology (2013), Samsung-GRO International research award for health systems research (2012), International Brainpool Fellow (Korea), 2012 and Executive Board Member for Federation of Asian Biotechnology (FAOB) (2008).

He has published more than 100 papers in National and International journals and has delivered several talks in International and National conferences.

“I dedicate this thesis to my Parents, Guides and Almighty GOD”