INFLUENCE OF THE STIFFNESS OF CARBOXYMETHYL CELLULOSE BASED HYDROGELS ON THE ENCAPSULATION EFFICIENCY OF CHONDROCYTES

A THESIS SUBMITTED

BY

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DECLARATION

I, **ANJITHA S PRASAD**, hereby declare that I had personally carried out the work depicted in the thesis entitled "**Influence of the stiffness of carboxymethyl cellulose based hydrogels on the encapsulation efficiency of chondrocytes"**, under the guidance of **Dr LYNDA V THOMAS, SCIENTIST D, DTERT,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled **“Influence of the stiffness of carboxymethyl cellulose based hydrogels on the encapsulation efficiency of chondrocytes”** submitted by Ms Anjitha S Prasad in partial fulfilment for the degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Division of Tissue Engineering and Regenerative Technologies, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram.

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Of

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Anjitha S Prasad

LIST OF ABBREVIATIONS

FTIR	Fourier Transform Infrared Spectroscopy
CMC	Carboxymethyl cellulose
CMC-MA	Methacrylated carboxymethyl cellulose
PEG	Poly (ethylene glycol)
PEGDA	Poly (ethylene glycol) diacrylate
OA	Osteoarthritis
ECM	Extracellular matrix
SEMD	Spondyloepimetaphyseal dysplasia
MMP	Matrix metalloprotease
RGD	Arginylglycylaspartic acid
BMP-2	Bone morphogenetic protein 2
DMEM-HG	Dulbecco‘s Modified Essential Medium –High glucose
FBS	Phosphate buffered saline
EDTA	Ethylenediamine tetraacetic acid
DAPI	4′,6-diamidino-2-phenylindole
TEA	Triethyl amine
DCM	Dichloromethane
NMR	Nuclear Magnetic Resonance
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
AFM	Atomic Force Microscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMSO	Dimethyl sulfoxide
ABA	Antibiotic antimycotic

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

Nowadays, articular cartilage injuries and related joint illness are very common. Unlike other tissues cartilage being avascular and lymphatic, its regenerative capacity is very poor. Tissue engineering offers an appealing alternative to fully synthetic implants with a short lifespan: by combining cells and biomaterials, tissue substitutes could be created that fully integrate, change, and grow with the patient. New biomaterials could be specifically tailored to create the best possible cell-material interaction. The use of porous 3D scaffolds to offer the proper environment for tissue and organ regeneration is the mainstay of this discipline.

Hydrogels have become one of the most popular tissue engineering scaffolds over the last two decades due to their capacity to maintain a distinct 3D structure, provide mechanical support for the cells in the engineered tissues, and mimic the original extracellular matrix. Physical properties of a substrate are becoming an increasingly essential component in determining cellular behavior, in addition to soluble or immobilized biochemical variables. For example, cells actively probe the elasticity of a substrate on adhesion and adapt their behavior to it in a cell-type specific manner.

So my present study is focused on the influence of the stiffness of carboxymethyl cellulose based hydrogels on the encapsulation efficiency of chondrocytes. A methacrylated carboxymethyl cellulose / Poly(ethylene glycol) diacrylate (CMC-MA/PEGDA) hydrogel was prepared in five different ratios and physicochemically characterized using FTIR, swelling, degradation, gel fraction, surface wettability and stiffness character. Chondrocytes isolated from rabbit articular cartilage were then seeded within the hydrogels and cultured. Attachments of cells were evaluated by adhesion assay and the results showed 8:2 CMC-MA/PEGDA gel exhibits good cellular attachment. Cell viability was carried out using live dead assay for the 3rd day. Cells were viable and showed rounded morphology. Cell morphometric parameters in terms of cell spreading and cell circularity as a function of stiffness of the hydrogel substrates were determined by performing immunostaining and cell circularity was calculated. Encapsulation study was carried for 8:2 CMC

MA/PEGDA gel and the chondrocytes were seen to aggregate with spherical morphology on 3rd day retrieval.

The first chapter gives an overview about the structure and composition of articular cartilage, promise of tissue engineering in the field of cartilage regeneration, role of a scaffold, its stiffness, What role does a scaffold play in deciding cell fate, influence of scaffold stiffness on chondrocytes, hydrogels used for cartilage tissue engineering, its importance, PEG based hydrogel and CMC based hydrogel.

Second chapter deals with the materials and methods employed for the study, like preparation of poly(ethylene glycol) diacrylate (PEGDA), methacrylated carboxymethyl cellulose (CMC-MA), preparation of CMC-MA/PEGDA hydrogels, physico chemical characterization of hydrogels such as FT-IR, ^{1}H NMR, swelling, degradation and gel fraction profile, surface wettability properties and stiffness characterization. Next section comprises the isolation and culture of chondrocytes from rabbit articular cartilage, cytotoxicity assays, cell morphology analysis, encapsulation study of chondrocytes on gels after 3 days and statistical analysis.

The third chapter includes the results and discussion of the study. Poly (ethylene glycol) diacrylate (PEGDA), methacrylated carboxymethyl cellulose (CMC-MA) were prepared and characterized. Five different ratios of CMC-MA/PEGDA hydrogels were also prepared and characterized and confirmed the crosslinking. From the various physico chemical characterization studies, the difference between the 5 gels of different ratios were found out and their stiffness parameters were evaluated. It was seen that the 8:2 ratio of CMC-MA/PEGDA gel showed good properties when compared to others. Biological evaluation studies also confirmed this result since they show good adhesion on chondrocytes and good aggregation. The overall cell distribution and cell morphology was also evaluated and found that all gels maintained a spherical morphology and as the stiffness increased, cell spread area also increased.

The results from our study suggest that it is not only the composition but also the stiffness of the substrates plays an important role in determining cell fate. Findings from our study indicate that gel stiffness strongly impacts the chondrocyte microenvironment both in maintenance of phenotypic integrity and ECM production.

CHAPTER 1 – INTRODUCTION

1.1 Background

Cartilage, a type of connective tissue found in human body serves a variety of functions including bearing loads in articular joints and intervertebral discs, providing joint lubrication, forming external ears and nose, supporting the trachea, and forming the long bones during development and growth. Despite the fact that cartilage is a tough and flexible material, it is relatively easy to damage also. Diseases that affects cartilage can range from extremely common conditions such as osteoarthritis to rare genetic disorders such as Spondyloepimetaphyseal dysplasia (SEMD) aggrecan type (Krishnan 2018). Osteoarthritis (OA) is a highly prevalent cartilage disease and was estimated that it affected 303 million people globally in 2017.OA can affect any joint but mostly it affects knee, hands ,hip and spine(Kloppenburg and Berenbaum 2020). This disease can be defined as a long-term chronic disease characterized by the deterioration of cartilage in joints which results in bones rubbing together and creating stiffness, pain, and impaired movement. OA is the second most common rheumatologic problem and it is the most frequent joint disease with a prevalence of 22% to 39% in India. About 10% to 15% of all adults aged over 60 have some degree of OA, in which women are highly affected than men. It was found that overall prevalence of knee osteoarthritis in India was 28.7% (Pal et al. 2016).

Constant efforts have been undertaken to device therapies to improve care, quality of life and pain relief for OA patients. So far no effective treatments have been able to halt or delay OA progression satisfactorily or provided adequate and long-lasting symptomatic relief. Presently joint replacement with an artificial prosthesis has found to be the most effective measure to improve pain sensation and quality of life in patients. The development of novel therapeutic techniques that target the degradative and inflammatory processes of osteoarthritis in cartilage, synovium, or bone needs a thorough understanding of the disease condition of these joint tissues at the time of intervention. It is critical to use therapies in the early stages of the disease, before the osteochondral unit undergoes severe structural and functional changes; otherwise, these interventions would be ineffective (Grässel and Muschter 2020).

Tissue engineering (TE) holds a promising area in the regeneration of articular cartilage resulting from OA. Successful cartilage tissue engineering involves cells undergoing chondrogenic differentiation when treated with appropriate biochemical factors and a three-dimensional (3-D) porous scaffold. Designing a biocompatible scaffold with optimal characteristics is the main key element for successful tissue engineering. This scaffold should be capable of providing a favourable environment for chondrogenic cell growth and new cartilage-specific ECM formation. Both natural and synthetic polymers containing biological cues similar to the extracellular matrix have been used to make scaffolds for cartilage tissue engineering. Hydrogels have emerged as preferred candidates for artificial tissue matrices. It is due to their structural, compositional similarities to natural tissues, and their beneficial architecture for cellular proliferation and survival. Moreover, their ability to control the shape, size, porosity, and morphological properties, hydrogel matrices has opened up new avenues for overcoming many obstacles in tissue engineering, such as simultaneous seeding of multiple cells, vascularisation, and tissue architecture.

The ability to adjust degradation at a rate that retains gel structure while allowing tissue deposition is a major challenge in scaffold design. Slowly degrading systems limit cell growth and extracellular matrix (ECM) secretion by preventing intercellular signalling events, whereas rapidly degrading systems are incapable of localising cells and released proteins. It is possible to design a system in which one polymer contributes to general integrity while the other degrades to generate cavities that allow proliferation and protein release by combining two or more polymers with different degradation rates (Peng et al. 2017).

Cellulose is the most abundant naturally occurring biopolymer and biodegradable in nature. Biocompatibility, excellent mechanical and thermal stability, non-toxicity, and cost-efficiency are all advantages of cellulose-based hydrogel materials in biomedical and pharmaceutical application (Rusu, Ciolacu, and Simionescu 2019). Carboxymethyl cellulose is one of the most promising cellulose derivatives in the field of cartilage tissue engineering. Due to the its characteristic surface properties, low cytotoxicity, excellent biocompatibility, biodegradability, and cell viability, CMC have found to be an ideal scaffold in tissue engineering (Rahman et al. 2021). Despite favourable features, these may present some drawbacks such as low water solubility of polymer precursors and intrinsic cytotoxicity of common chemical crosslinkers (e.g., formaldehyde, glutaraldehyde, and epichlorohydrin) due to the risk of unreacted species in the system. To overcome the above mentioned drawbacks, creating hydrogels utilising chemically modified polymer derivatives such as CMC to improve water solubility, and using biocompatible and environmentally friendly crosslinkers throughout the process is preferable. To improve the properties of the hydrogels, polymers such as polyethylene glycol (PEG) have been mixed with cellulose and CMC systems as an effective network modifier. PEG is a polyether that is amphiphilic in nature and soluble in water as well in many organic solvents. It comes in a variety of molecular weights, has been determined to be nontoxic, and has been approved by the United States Food and Drug Administration (FDA). These characteristics contribute to their widespread use in biomedical research, drug delivery, tissue engineering scaffolds, and surface functionalization, and so forth (Capanema et al. 2018). PEG hydrogels have been modified with immobilised cell adhesion peptides, growth factors, and MMP-sensitive peptides to mimic the complexity of integrin-mediated cell adhesion and protease-mediated matrix remodelling. This makes the scaffolds susceptible to degradation and localised cell invasion by cell-secreted proteases. PEGDA is one such modified form of PEG and these hydrogels has been extensively studied for their ability to promote cell-mediated scaffold degradation and migration. (Sokic and Papavasiliou 2012).

In the present study CMC-MA/PEGDA based hydrogels of different combinations are studied for their surface and stiffness properties and their efficiency to affect chondroyte viability, adhesion and encapsulation.

1.2 REVIEW OF LITERATURE

1.2.1 Cartilage - Anatomy and function

Cartilage which is an avascular, aneural, alymphatic connective tissue are present throughout the body at numerous sites like ear, knee, larynx, intervertebral disc etc. Several types of cartilage are found in the human body, and their structure and relevant function depend on this variation. The three major types are hyaline, fibrous and elastic and all three have a low density of cells (chondrocytes) that synthesize and secrete the major components of the extracellular matrix (ECM).Cartilage ECM provides structural support and resistance to deformation and to carry these functions it contains a unique family of proteoglycans entangled within a highly hydrated collagen fibrillar network. The three types of cartilage differ in their abundance, distribution and types of collagens and proteoglycans and these differences make their appearance and biomechanical properties to vary from one another.

Among the three, Hyaline cartilage is the most common form in the human body. It has a glassy appearance and is found in the articulating surfaces of bones in synovial joints, in the ribs, nose, trachea, bronchi, larynx, and growth plates. Various functions of hyaline cartilage includes providing compressive strength to cartilage at the tissue level and thus enabling it to act as a load bearing tissue in the joints, plays an essential role in skeletal growth and development.

Fibrous cartilage is found primarily in the intervertebral discs and in other locations such as the menisci, bone-tendon interfaces and ligament-tendon interfaces. It has high tensile strength due to the abundant presence of type I collagen and hence can resist high degrees of tension and compression (Krishnan 2018).

Elastic cartilage is most commonly found in the larynx, ear, epiglottis, and eustachian tube and this appears in dull yellow. It is also surrounded by a perichondrium-like layer. This cartilage provides flexibility and can withstand pressure (Chang, Marston, and Martin 2021).

1.2.2 Articular Cartilage

Articular cartilage is a hyaline cartilage of about 2-4 mm thickness designed to bear and distribute loads across the diarthrodial joints. It has an organized layered structure and is divided into four zones: superficial, middle, deep zone and the zone of calcified cartilage (Akkiraju and Nohe 2015).

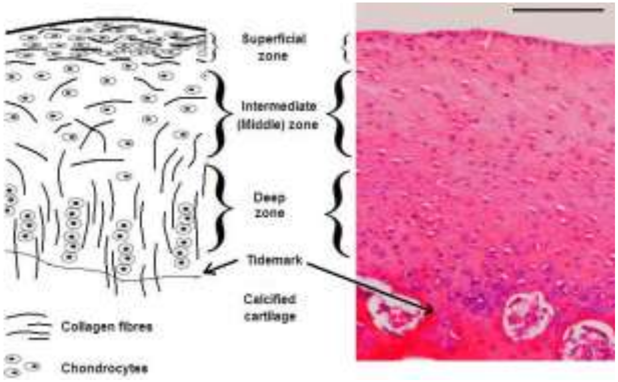

Fig1: Structural organization of articular cartilage (Matsiko, Levingstone, and O'Brien 2013)

Chondrocytes are the active cells in articular cartilage and these cells proliferate and secrete extracellular matrix.

1.2.3 Chondrocytes

Chondrocytes are produced from MSCs and account for only 1%–5% of total cartilage tissue. They are metabolically active cells that produce and cycle a huge amount of ECM components such collagen, glycoproteins, proteoglycans, and hyaluronan. Various factors that are present within their chemical and mechanical environment influence the metabolic activities of chondrocytes in which the most important ones are pro-inflammatory cytokines and growth factors that have anabolic and catabolic effects. The degradation and synthesis of matrix macromolecules are influenced by these variables. Life span of chondrocytes is controlled by the area of its residence and being an avascular tissue, in articular cartilage chondrocytes rely on diffusion of nutrients and metabolites from the articular surface. Mechanosensitive chondrocytes contribute significantly to ECM formation and give the functional and mechanical ability to bear compressional, tensile, and shear pressures across diarthrodial joints (Akkiraju and Nohe 2015).

1.2.4 Extracellular Matrix (ECM)

Chondrocytes in the superficial and mid zones of articular cartilage manufacture ECM, which is made up of collagen types II, IX, and XI, non-collagenous proteins, glycoproteins as well as proteoglycans. The collagen matrix is responsible for cartilage's shape and tensile strength. Proteoglycans and non-collagenous proteins bind to the collagenous network and aid in the stabilisation of the matrix framework as well as the binding of chondrocytes to the network's macromolecules. The ECM that surrounds the chondrocytes has been separated into zones based on their proximity to the cell. The pericellular matrix is immediately surrounding the cell, the territorial matrix is next to it, and the interterritorial matrix is the furthest away. The collagen type II, type XI, and type IX collagens in the interterritorial cartilage matrix are integrated on the fibril surface with the non-collagen domain, allowing association with other matrix components and retention of proteoglycans. These Collagens provide cartilage its shape, tensile stiffness, and strength (García-Carvajal et al. 2013).The age of the tissue affects the composition of the ECM, as well as the organisation of chondrocytes and their sensitivity to external influences like cytokines, but chondrocyte counts stay constant. The loss of chondrocytes in the superficial layers is followed by an increase in the number of chondrocytes in the deeper levels as people age. As a result, the matrix's hydration decreases, resulting in greater compressive stiffness. Proteolytic degradation to the link proteins and glycosaminoglycan chains, as well as an increase in partially degraded hyaluronan without freshly produced molecules, could explain the age-related decline in proteoglycan aggregate numbers inside the ECM. As a result, increasing mechanical pressures on the tissue cause subchondral tissue calcification. These overall structural alterations in the aging cartilage could be a contributing factor in the development of conditions like OA (Akkiraju and Nohe 2015).

1.2.5 Tissue Engineering

Tissue engineering (TE) emerged in the1980's with enormous potential due to the intricacy of human tissues. The basic goal of TE is to develop biological substitutes based on materials engineering and life sciences that restore, preserve, or increase the function of tissues and organs. This field mainly relies on the utilization of porous 3D scaffolds to provide the right environment for tissue and organ regeneration. These scaffolds effectively serve as a template for the development of new tissue and are usually seeded with cells and factors, or they're exposed to biophysical stimuli in the form of a bioreactor, which is a device or system that applies various mechanical or chemical stimuli to cells. These cell-seeded scaffolds are then either cultured in vitro to produce tissues that may then be implanted into an injured site, or they are implanted directly into the injured region, where tissue or organ regeneration is triggered in vivo, using the body's own mechanisms. Tissue engineering triad refers to this combination of cells, signals, and scaffolds (O'Brien 2011).

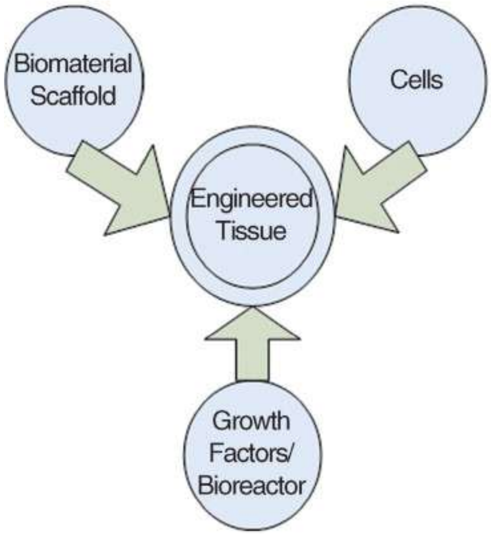

Fig 2: Tissue engineering triad of cells, signals and the scaffold (O'Brien 2011)

1.2.6 Cartilage Tissue Engineering

The cartilaginous tissue is very difficult to repair or regenerate due to its lack of vascularisation and its minimal number of cells. Scaffolds create a three-dimensional climate that is suitable to cartilaginous tissue growth. Hence it is important to develop approaches that bring the appropriate cells, biomaterials, and signalling factors to the defect site. Over the last 30 years, cartilage tissue engineering has progressed beyond treating articular cartilage defects to developing techniques to combat the osteoarthritis process. Chondrogenic cells must be combined with biomaterials and biofactors to generate cartilage tissue-engineering techniques. Primary autologous/heterologous chondrocytes, mesenchymal stem cells, and embryonic stem cells can all be used to get cells. To avoid inflammatory and immunological reactions, ideally the biomaterial used should be biocompatible and it must create a favourable environment for the maintenance of 3D chondrocyte phenotype and be adhesive to allow cell attachment within the lesion (Vinatier and Guicheux 2016).

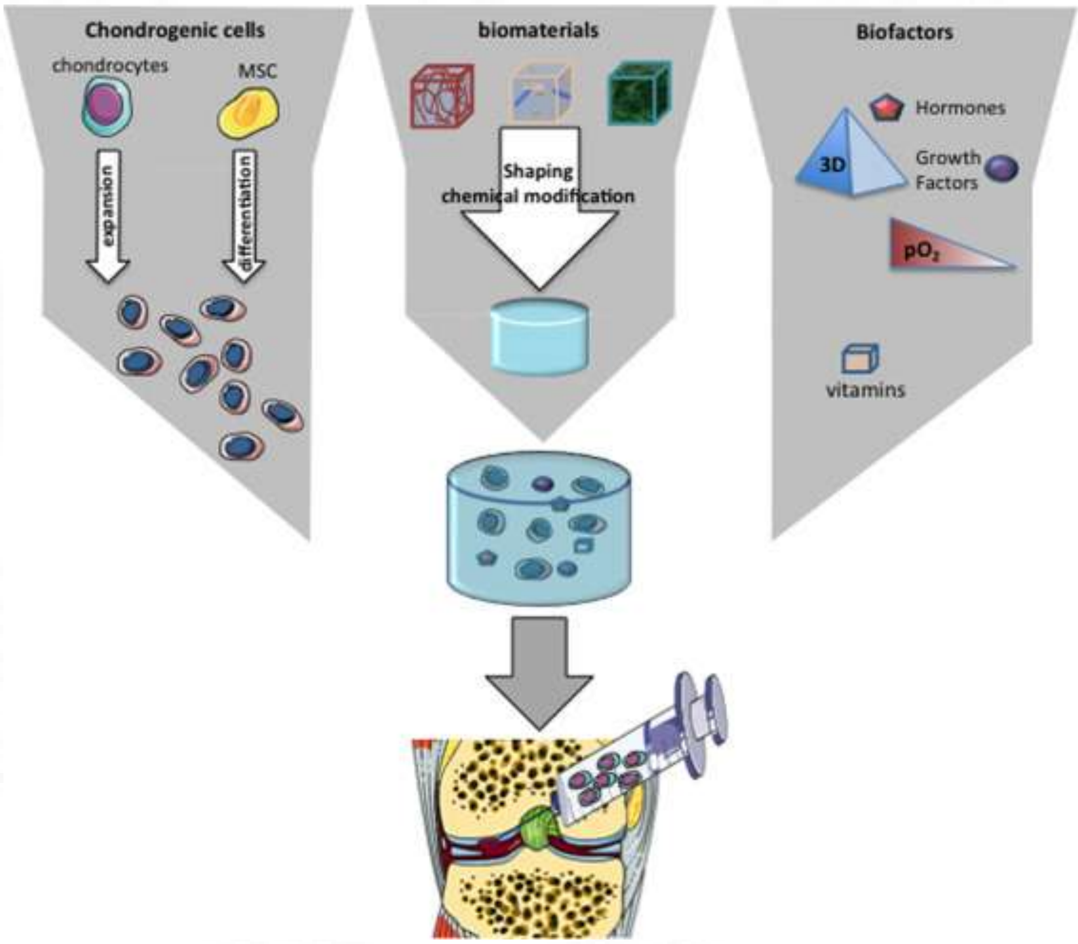

Fig 3: The tissue engineering triad used for articular cartilage repair (Vinatier and Guicheux 2016)

1.2.7 Role of Scaffold

In cartilage tissue engineering, various scaffolding materials have been used for cell delivery. Scaffolds provide a 3D environment that is appropriate for cartilaginous tissue development. An ideal scaffold should meet some properties which include directed and controlled degradation, promote cell viability, differentiation, and ECM production, allow for the diffusion of nutrients and waste products, adhere and integrate with the neighbouring native cartilage, span and assume the size of the defect, and provide mechanical integrity depending on the defect location (Chung and Burdick 2008). Porosity, pore size, interconnectivity, and permeability of scaffold are all important structural factors in articular cartilage formation and cartilage regeneration. Chondrocytes cultivated on flat surfaces lose their ability to synthesise certain proteins required for hyaline cartilage formation. Cell seeding, cell migration, cell development and tissue growth can be improved by using a highly porous membrane with an interconnected macro-pore network (Wasyłeczko, Sikorska, and Chwojnowski 2020).

1.2.8 Stiffness of Scaffold

Tissue engineering scaffolds serve as a mechanical support for tissue regeneration, particularly in load-bearing areas, at the most basic level. Mechanotransduction is the process by which a mechanical stimuli is converted into a chemical response by cells. This is an important part of probing matrix stiffness and is the focus of a lot of study right now. Even when no external forces are applied, cells receive mechanical feedback from the substrate to which they attach. Here substrate refers to a scaffold. Researchers have been looking at the significance of substrate stiffness as one of the important characteristics that affects cell behavior in recent years. The fact that cells in vivo typically face a soft environment, whereas standard tissue culture flasks are highly rigid, was a driving force for these experiments. As a result 2D *in vitro* model systems which use polymer gel substrates with tunable elastic properties that are coated with specific ECM proteins for cell attachment were developed (Breuls, Jiya, and Smit 2008).Polyacrylamide gels, in particular, have been frequently employed because they can be tailored to a wide range of stiffness to imitate in vivo tissues.

The effects of substrate stiffness and substrate biochemistry on cell behaviour may be examined independently using these in vitro ECM mimics, which is a key feature (Wang and Pelham 1998).

Substrate stiffness influence cell migration. This is necessary for the in growth of cells from host tissues, such as when a scaffold is vascularized. Apart from this scaffold stiffness also influence cytoskeletal organization, cell growth and viability. Although biochemical stimuli are definitely vital in cell behaviour regulation, it has become clear that a scaffold with an improper stiffness can hinder tissue regeneration. A scaffold with a well-chosen stiffness, on the other hand, can encourage the regeneration of new tissue and improve the efficacy of biochemical stimuli. As a result, scaffold stiffness could be a key factor in controlling cell behaviour, opening up new possibilities for tissue engineering.

1.2.9 Role of Scaffolds in determining cell fate

A tissue engineering method considers not just the scaffold's physical properties, but also biological aspects that can aid regeneration. The use of biocompatible materials and connectivity between implanted constructs/cells and host tissue are required for integration with the host tissue. One of the drawbacks of cell-scaffolds is that cells located more than 100–200 μm from the vascular network die from a lack of oxygen and nutrients (Aizawa, Owen, and Shoichet 2012). A Recent study by the Langer group demonstrated that the pre vascularised scaffolds supported survival of transplanted cells (Levenberg et al. 2005). Currently, scaffolds are used to retain cells at a desired location, serving as a template for 3D cell assembly, survival and engraftment in vivo. PEG-based hydrogels and Matrigel are two examples of 3D scaffolds that have been employed for this purpose. Scaffold characteristics influence cell behaviour via a variety of processes, influencing differentiation pathways and proliferative responses.

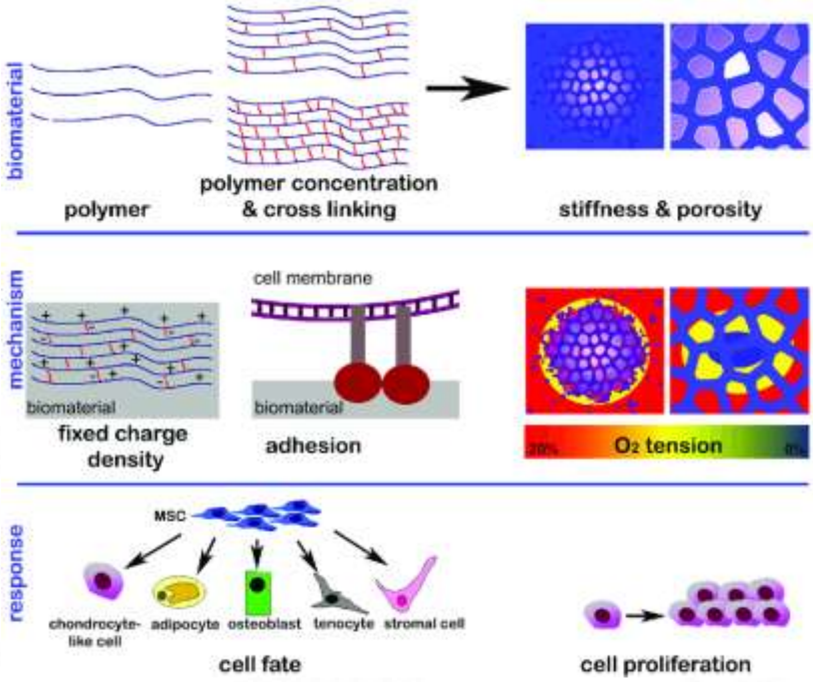

Fig 4: Scaffold characteristics determining cell behaviour and tissue regeneration (Schutgens et al. 2015)

1.2.10 Influence of Scaffold stiffness on Chondrocytes

Shear stress, stretching, compression, and matrix elasticity are all important biomechanical cues in the development of next-generation physiological *in vitro* tissue models. For instance, matrix elasticity is known to influence stem cell differentiation, healing processes, and extracellular matrix (ECM) deposition, all of which are important for tissue formation and maintenance. The resistance that cells feel in response to substrate deformation is described by the elasticity of a matrix (Bachmann et al. 2020). Despite the fact that the elasticity of physiological tissues varies from soft brain matter (1 kPa) to stiff collagenous, pre-calcified bone (100 kPa), the implications for in vitro tissue modelling are yet unknown (Discher, Mooney, and Zandstra 2009). Some studies using monolayer cultures suggest that more compliant substrates foster the occurrence and maintenance of chondrogenic phenotypes (Schuh et al. 2010a). Their findings suggest that chondrocytes detect matrix elasticity and might be exploited to develop scaffolds with mechanical properties tailored to support the chondrogenic phenotype in tissue engineering applications. Some others indicate surfaces exhibiting a cartilage-mimicking elasticity to provide chondroinductive effects (Allen, Cooke, and Alliston 2012). Only redifferentiated chondrocytes are capable of producing an in vitro cartilage analogue, which is critical for producing reliable synovial joint tissue models. There has been conflicting information published in the recent decade about the effect of matrix stiffness on chondrocytic activity, and trustworthy knowledge of 3D biomechanical signals is still in its infancy. Thereupon, the study by Bachmann and group represents an important step in elucidating physiologically relevant matrix elasticity on cellular behaviour (Bachmann et al. 2020).

1.2.11 Hydrogels used for cartilage tissue engineering

Hydrogels are three-dimensional networks of hydrophilic polymers that may absorb water from 10% to 20% to thousands of times their dry weight without dissolving the polymer because of their cross-linked structure. Hydrogels' porous structure, combined with the presence of water, enable the transport of low molecular weight solutes and nutrients into the hydrogel, and also the transport of cellular waste out of the hydrogel, both of which are essential for cellular viability. Biopolymer-based hydrogels have the ability to direct cell migration, development, and organisation during tissue regeneration by mimicking numerous aspects of the extracellular matrix. Many of these hydrogels have adequate biocompatibility and biodegradability, making them suitable candidates for scaffold development. Polysaccharides including hyaluronate, chondroitin sulphate, chitosan, alginate, and agarose, as well as proteins like silk fibroin, fibrin, and collagen, are used to make hydrogel scaffolds for cartilage tissue engineering (Balakrishnan and Banerjee 2011). Three processes are usually involved in cell encapsulation and subsequent tissue engineering within hydrogels: First, seeded cells are homogeneously suspended in the isotonic solution of gel precursors, second this cell suspension is transferred into appointed molds *in vitro* or desired sites *in vivo*, completing the gelation to obtain 3D cell/gel construct *in situ* and finally 3D cell culture *in vitro* and is followed by transplantation of the engineered constructs to target tissues, or direct neotissue development *in vivo*. This method of cell encapsulation and 3D cell culturing in hydrogels has a number of advantages. To begin with, when compared to prefabricated porous scaffolds, the cells can be disseminated more abundantly and uniformly within hydrogels by using various initiation procedures to carry out a compatible and rapid curing (few seconds to a few minutes) of cell-suspended precursor solution. Furthermore, gel precursor cell suspensions can be poured into any shaped moulds in vitro or tissue defects in vivo, assuring spatial compatibility with the host tissues. Lastly, hydrogel-based constructs may be easily manufactured and altered to satisfy practical requirements, ranging from nanogel particles to macroscopic objects. In cartilage tissue engineering, hydrogels are ideal since they can mimic the extracellular matrix (ECM) of native cartilage tissue, and its hydrophilic networks and high water content can retain the native spheroidal morphology of chondrocytes(Fan and Wang 2017).

Injectable chondrocyte-based hydrogel systems for cartilage tissue regeneration are an appealing therapeutic alternative for cartilage repair that has recently shown a lot of promise (Amini and Nair 2012) (Spiller, Maher, and Lowman 2011). Several research have shown that chondrocytes may be cultured in 3D natural and synthetic polymer hydrogel matrices such as alginate, agarose, fibrin, collagen, chitosan, hyaluronic acid, polyacrylamides, and others, in both their native and modified forms (Spiller, Maher, and Lowman 2011)(Balakrishnan and Banerjee 2011) (Kim, Mauck, and Burdick 2011) and (Peretti et al. 2006). However the development of an injectable hydrogel system also faces some major challenges such as the ease of preparation for non-invasive delivery intended for immediate use in surgical procedures and the maintenance of viability, stable phenotype, and functionality of chondrocytes contained within the hydrogel environment. These obstacles can be overcome by modifying the physical structure of these hydrogels, particularly the crosslinking density, which controls all functionalities of these gels, including stiffness and elasticity, and hence influences chondrocyte behaviour (V Thomas, Vg, and D Nair 2017).

1.2.12 PEG based Hydrogel

Polyethylene glycol (PEG) is a versatile polyether which is also known by the name polyethylene oxide (PEO).

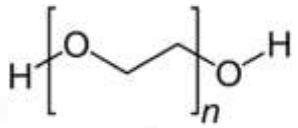

Fig 5: Chemical structure of PEG (Zarrintaj et al. 2020)

It is a synthetic bioinert polymer with no inherent cell adhesiveness or biological activity. However, it can be easily functionalized with bioactive peptide sequences or proteins, such as RGD or BMP-2, to convey biological cues and activate cell activities. Incorporating degradable crosslinking molecules catalysed by the cell process, such as matrix metalloprotease (MMP)-sensitive peptides, can aid in the remodelling of basic PEG polymeric structures and enable native cellular matrix deposition (Ruan et al. 2013). PEG hydrogels can be made utilizing a moderate, cell-compatible photopolymerization reaction with long-wave ultraviolet (UV) light, according to previous research (Fairbanks et al. 2009). This approach preserves both the survival and differentiation capability of cells encapsulated in PEG hydrogels. Photopolymerized hydrogels have been utilized in tissue engineering to change and improve tissue function, as well as being investigated for use as cell carrier materials in tissue replacement strategies.

A study in which bovine and ovine chondrocytes were encapsulated in semi-interpenetrating networks of poly(ethylene glycol)-dimethacrylate and poly(ethylene glycol), they found that cells were dispersed evenly through the scaffold material and remained viable after photopolymerization and 2 weeks of culture and also extracellular matrix production (sulfated glycosaminoglycan and collagen) was increased over the 14 days of culture. Equilibrium moduli, dynamic stiffness, and streaming potentials of these tissues increased with culture time (Elisseeff et al. 2000). All these results indicate promise for photopolymerized hydrogels for scaffolds for cartilage regeneration and replacement.

1.2.13 CMC based Hydrogel

Cellulose-based hydrogels plays an important role in tissue engineering. Hydrogels can be made from cellulose derivatives or directly from native cellulose. Cellulose derivatives which are water soluble are generally biocompatible and are widely used for various tissue engineering applications (Dutta, Patel, and Lim 2019).

Carboxymethyl cellulose (CMC), an anionic, water-soluble derivative of cellulose, is a linear polysaccharide of anhydro-glucose in which the repeating units are connected by β-1,4- glycosidic bonds.

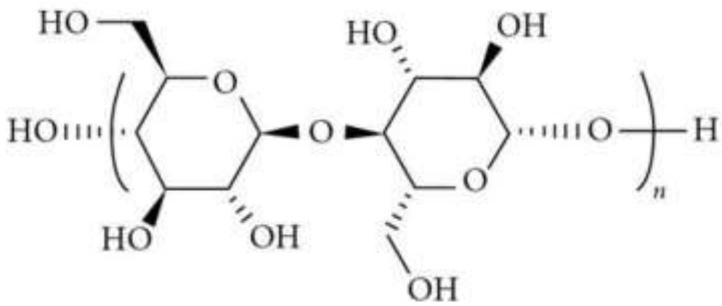

Fig 6: Molecular structure of CMC (He 2011)

CMC-based biomaterials are extensively used in tissue engineering applications. They attract water and create a hydrated environment similar to the extracellular matrix due to the highly negatively charged -COO- and -O- groups in aqueous solution. Recently, Gheysari and group fabricated a highly porous hydroxyapatite-gel/CMC nanocomposite scaffold with strong cell adhesion, non-toxicity, and good cell survival, as well as a high water uptake capacity (up to >600%).Furthermore, in vitro experimental incubations demonstrated that the synthesised scaffolds were biodegradable with satisfactory cell viability (i.e., >80% even after 48 hours) (Gheysari et al. 2019). Crosslinked CMC has also been achieved with the use of bifunctional crosslinking agents such as epichlorohydrin and dicarboxylic acid compound and these CMC that has been crosslinked absorbs a lot of water and expands to generate hydrogels with good physical characteristics and dynamic viscoelasticities (Kono 2014).

Scaffolds composed of CMC must sustain loads in joints after implantation in cartilage tissue engineering applications. One major limitation for CMC based hydrogel is their reduced mechanical strength. Hence combining CMC with more stable and controlled synthetic biomaterials will overcome the natural polymer's mechanical limitations while preserving CMC's biological features in scaffold constructs (Namkaew et al. 2021).

1.3 Significance of the study

Substrate elasticity or stiffness can influence the phenotypic and functional characteristics of chondrocytes. Some of the major challenges in the development of an a hydrogel system for chondrocytes encapsulation has been the ease of preparation for non-invasive delivery intended for immediate use in surgical interventions and the maintenance of viability, stable phenotype and functionality of the chondrocytes that are encapsulated within the hydrogel environment. These challenges can be met by altering the physical structure of these hydrogels mainly the crosslinking density which regulates all the functions including the stiffness or elasticity of these gels thereby impacting chondrocyte behaviour. In this study we will aim to evaluate the effect of the stiffness of carboxymethyl cellulose based hydrogels on the viability and functional characteristics of chondrocytes over a range of culture periods.

1.4 Hypothesis

The varying stiffness of a two component hydrogel based on methacrylated carboxymethyl cellulose and Polyethylene glycol diacrylate can influence the encapsulation efficiency of chondrocytes.

1.5 Objectives

The major objectives that involve are:

- Synthesis and physicochemical characterisation of PEGDA, methacrylated carboxymethyl cellulose and CMC-MA/PEGDA gels.
- Cytocompatibility evaluation and evaluation of the stiffness of hydrogels.
- Evaluation of stiffness mediated effects on cell viability and functionality studies of chondrocytes and encapsulation study of chondrocytes within the hydrogels.

CHAPTER 2 - MATERIALS AND METHODS

MATERIALS

Carboxymethyl cellulose (Sigma Aldrich, USA, CAS-No.9004-32-4), Polyethylene glycol (Mw~4000kDa, SDFCL, FI0Z/1310/2203/13) was used for this study. For cell culture experiments, Dulbecco's Modified Essential Medium –High glucose (DMEM-HG), Fetal Bovine serum (FBS), Phosphate buffered saline (PBS), AB-AM, and 0.25% trypsin-EDTA were procured from Invitrogen, USA. Live dead assay kit (Invitrogen), 4′,6-diamidino-2-phenylindole (DAPI) from Invitrogen-D 1306, 4% Paraformaldehyde, Collagenase II and Alexa Flour 488 Phalloidin dye from Invitrogen, USA – A 12379. All chemical reagents used in this study were of analytical grade.

METHODS

2.1. Preparation of Poly(ethylene glycol) diacrylate (PEGDA)

PEG with molecular weight 4 kDa was acrylated as previously described (S. Lee, Tong, and Yang 2016). Briefly dry PEG was reacted with acryloyl chloride (Sigma, St. Louis, MO) and triethyl amine (TEA) (Sigma) in anhydrous dichloromethane (DCM) (Sigma) under argon gas overnight. An excess of acryloyl chloride (1:4 PEG: acryloyl chloride) was used. The resulting solution was then washed thoroughly with 2M K_2CO_3 and allowed to separate into aqueous and organic phases. PEGDA which remains in the organic phase is decanted and dried using anhydrous $MgSO_4$ followed by filtration. The polymer was precipitated out using diethyl ether, filtered and dried. The final product was characterized by ^{1}H NMR and FTIR and stored at -20°C under inert gas until use. The degree of acrylation was determined from H NMR using the equation

Degree of acrylation =

$$\frac{(Vinylic\ integral/6)}{(vinylic\ integral/6)\ +\ (Oxyethylene\ integral/4)\ \times\ (44/PEG\ molecular\ weight)} \times 100$$

2.2. Preparation of methacrylated carboxymethyl cellulose (CMC-MA)

Methacrylation of carboxymethyl cellulose (CMC-MA) was performed based on previous protocols (Reza and Nicoll 2010). Briefly, to 1% wt/vol % of carboxymethyl cellulose in water at 4ºC a 20 fold excess of methacrylic acid was added and the pH adjusted to 8 and reaction carried out under stirring for 24 hours. The resulting solution dialysed against water for 3 days to remove unreacted methacrylic acid and methacrylic anhydride and then freeze dried to obtain the methacrylated CMC (CMC-MA)

2.3 Preparation of Methacrylated CMC/PEGDA hydrogels

10mg/ml Methacrylated CMC was prepared in PBS by stirring under 70C at 320 rpm. Similarly, 10(wt/vol)% of PEGDA was prepared in PBS by stirring at 110 rpm .To this prepared solution of PEGDA 0.01g/ml lithium phenyl-2,4,6-trimethylbenzoylphosphinate *(*LAP) was added and stirred at 110rpm. 10 µl gels were prepared as per the given ratios in table 1 and crosslinked by exposure to long wave UV light for 3 minutes.

Table 1: Composition of 5 different ratios of CMC-MA/PEGDA

Sample	Composition	CMC-MA(µl)	PEGDA(µl)
A	2:8 CMC-MA/PEGDA	2	8
B	4:6 CMC-MA/PEGDA	4	6
C	5:5 CMC-MA/PEGDA	5	5
D	6:4 CMC-MA/PEGDA	6	4
E	8:2 CMC-MA/PEGDA	8	2

2.4 FT-IR analysis

Fourier Transform Infrared Spectroscopy (FT-IR) provides information about the specific chemical groups of the materials. FT-IR spectrum of 3mm thickness sample were recorded at room temperature in the range of 5000-500cm^{-1} region using BRUKER optic GmbH. FT-IR was performed for the synthesized polymers and the gel ratios were also assessed..

2.5 Swelling Profile studies

Swelling profile of lyophilized gels was studied to understand the water holding capacity and stability by measuring the change in weight as a function of time. The freeze dried samples were weighed and immersed in 4ml PBS at room temperature and then kept in pre-weighed bottles for known intervals of time. At these time intervals (5 min, 20 min, 1 hr, 2 hr, 3 hr, 24 hrs and 48 hrs) the samples were withdrawn from the PBS buffer and wet weight of the samples were measured. The swelling ratio was calculated using the formula:

$$\text{Swelling \%} = \frac{\text{(Wet Weight-Dry Weight)}}{\text{Dry Weight}} \times 100$$

2.6 Degradation profile of CMC-MA/PEGDA gels

Degradation studies on 5 gels were studied by incubating in PBS at 37°C.12 samples for each ratio of gels were immersed in 4ml PBS, kept in pre-weighed bottles and then incubated at 37°C for 1 week, 2 week, 3 week and 4 week. After each week samples were retrieved by removing PBS, freeze dried and weight was taken. The % weight loss was calculated using the following formula:

$$\text{Weight loss \%} = \frac{\text{(Initial weight} - \text{Final weight)}}{\text{Initial weight}} \times 100$$

2.7 % Gel fraction of CMC-MA/PEGDA gels

To determine the percentage gel fraction, freeze dried gels (4 samples for each ratio) were kept in 4ml PBS taken in pre-weight determined bottles at room temperature for 24 hours. Samples were again freeze dried and the insoluble dried sample was again weighed (Gulrez, Al-Assaf, and Phillips 2011). Percentage gel fraction was measured by:

$$\text{\% Gel fraction} = \frac{Wd}{\text{Wi}} \times 100$$

Where W_d is the weight of dried insoluble part after extraction and W_i is the initial weight of dried sample.

2.8. Surface wettability properties using Dynamic contact angle measuring device

The surface wettability is characterized by contact angle technique that was performed on prepared dried nylon films at room temperature (approx. 23°C), using the sessile drop method, and imaging software (SCA20 software, Germany). The image was taken within 10 sec, after introduction of water droplet.

2.9. Stiffness characterization of CMC-MA/PEGDA gels using AFM

Atomic Force Microscopy (AFM, Agilent 5500) was used to ascertain the stiffness of the hydrogel samples. A POINTPROBE –PLUS silicon cantilever with a spring constant of 1 N/m and a resonant frequency of 115 kHz with a tip radius of 2nm was used to extract force-indentation curves. The force curves were collected and sensitivity constant calculated and fitted to Hertz's contact model using Mountains SPIP software to calculate the Young's modulus using the equation:

$$F = \frac{4}{3} \times \frac{Es}{1 - \nu s^2} \times \sqrt{r} \times \delta^{\frac{3}{2}}$$

Where Es is the sample modulus, νs is the Poissons ratio, r is the tip radius of curvature and δ is the opening angle.

2.10. Cytotoxicity analysis

2.10.1 Direct contact assay

To determine the cytocompatibility of the gel system direct contact assay was performed (ISO 10993 PART 5). L929 mouse fibroblast cells were seeded onto a 6 well plates. Once the cells reached 75% confluence 10µl gels of different ratios were placed on to each of the wells and observed after 24 hours in direct contact. Cells grown on a culture plate without any gel was taken as control. Images were taken using phase contrast microscope (Olympus1X71). After incubation of cells with test samples and controls at 37 ± l °C for 24h, cell culture was examined microscopically for cellular response. Cells were examined microscopically and cellular responses were scored as 0, l, 2, 3 and 4 based on the following Table 2.

Table 2: Cytotoxicity scoring for direct contact assay

Grade	Reactivity	Conditions for all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed.
4	Severe	Nearly complete or complete destruction of the cell layers.

2.10.2 MTT assay

To evaluate the viability of cells, MTT assay was performed (ISO 10993 PART 5). L929 mouse fibroblast cells were seeded on to a 96 well plate at a density of about (1×10^3 cells per well) and was incubated for 24 hr at 37 ºC with 5% CO_2.10 µl of CMC-MA/PEGDA gels of five different ratios were prepared and kept in extraction medium (DMEM) at 37º C for 24 hr. 100% and 50% of extracts was added to the wells. Cells incubated with DMEM alone were kept as the control. After 24 h of incubation, the extract was removed and incubated with 20 µl of 10mg/ml MTT reagent for 3 hr. Then MTT was completely discarded and each well was dissolved in 100µl DMSO to solubilise the formazan crystals formed and was kept in dark for 10-20 min. Optical density was measured at 530nm with the use of ASYS UVM 340. Percentage viability of cells was calculated by:

$$\text{Percentage viability} = \frac{\text{Absorbance of extract treated cells}}{\text{Absorbance of the control cells}} \times 100$$

2.11. Isolation and culture of chondrocytes from rabbit articular cartilage

Chondrocytes were isolated from rabbit articular cartilage as described earlier (V Thomas, Vg, and D Nair 2017). Articular cartilage was carefully excised from rabbit cadaver without any contamination and was thoroughly washed with 10% antibiotic antimycotic solution (ABAM).Muscles and flesh was removed and joint cavity was opened. Cartilage pieces were scrapped out from the surface of joint and washed in PBS. It was then digested with 0.2 wt/vol % collagenase II for 3-6 hrs at 37 ºC. The collagenase action was inactivated by adding FBS containing media and centrifuged at 1800 rpm for 10 min at 4 ºC. After centrifugation, the supernatant was discarded and the chondrocyte pellet was resuspended in serum containing DMEM media. It was then seeded into 25cm^2 flasks and incubated at 37ºC, 5% CO_2 and cultured to confluent monolayer. Then the cells were trypsinized using 0.25% trypsin-EDTA solution and passaged for further studies. Cells from passage 3 and 4 were used for the subsequent studies.

2.12. Cell morphology analysis

To determine the cell morphometric parameters in terms of cell spreading and cell circularity as a function of stiffness of the hydrogel substrates, immunostaining was performed after seeding cells at a density of 1×10^4 cells on 10 µl CMC-MA/PEGDA gels of varying stiffness and cultured for a period of 24 hours. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and then washed 3 times with PBS. Cells were permeabilized using 0.1% Triton X-100 (Sigma-Aldrich) in PBS. AlexaFluor-488 Phalloidin (1:200; Invitrogen) was added. Samples were washed in PBS for before taken for imaging using a fluorescence microscope(Olympus 1X71). Images were taken at 10X magnification. Multiple random fields of view were analysed from images taken from the different cell seeded constructs in triplicate in order to characterise cell morphologies and cell number. While imaging, the same procedures and parameters were used in the image analysis software and all images had the same matched exposure and contrast settings. The cell spread area and perimeter were calculated from the image analysis software Cellsens and the cell circularity was measured using the equation:

$$Cell\ circularity\ = \frac{4\pi \times Area}{Perimeter^2}$$

This formula gives a number from 0 to1, where values of 0.6 to 1 indicates rounded cells and values of 0 to 0.5 indicated elongated cells.

2.13. Encapsulation of chondrocytes in 8:2 CMC-MA/PEGDA gels

Since the 8:2 CMC-MA/PEGDA gel showed good cellular attachment, it was used for the encapsulation study. Approximately 1×10^6 chondrocytes cells were spin down and resuspended in 100µl of gel (20µl PEGDA and 80µl CMC). 10 µl cell encapsulated gels were placed on acrylated cover slips and crosslinked using higher wavelength UV for 5 minutes. The gel with cells was then transferred into chondrogenic medium and cultured for a period of 3 days at 37ºC and 5% CO_2. At the end of the culture period, samples were retrieved and assessed for viability using live dead staining with 1µl of calcein AM (4mM) to stain the live cells green and 1µl of EtBr (2mM) to stain the dead cells red for 20 minutes. The images were taken using a fluorescence microscope (Olympus 1X71). The scaffolds after 7 days were also retrieved, fixed with 4% paraformaldehyde and immunostained for specific extracellular matrix components collagen type II , aggrecan and transcription factor SOX9. The images were taken using a fluorescence microscope (Olympus 1X71).

2.14. Statistical Analysis

The arithmetic mean and standard deviations were calculated using Excel. Statistical analysis was performed using the one way Annova and Tukeys Posthoc analysis. The results were regarded as significant at a p value of < 0.05.

CHAPTER 3- RESULTS AND DISCUSSION

3.1. Preparation of Polyethylene glycol diacrylate

Polyethylene glycol needs to be acrylated in order to make crosslinking possible. The vinyl moieties on the acrylate groups once modified onto PEG can then form crosslinks on exposure to UV light. To confirm the reaction protocol, FTIR was performed. The FTIR plot of PEGDA (Fig 7) showed the C=O stretch peak at 1724 cm^{-1} and C=C stretch at 1634 cm^{-1} from the terminal acrylate group which was not evidenced in the PEG samples analyzed. The decrease in the –OH peak at 2882 cm^{-1} also confirmed the substitution of the OH group with the acrylate group. The ^{1}H NMR spectra was performed using D_2O as a solvent. The presence of vinyl peaks at δ (5.8-6.4) ppm confirmed the acrylation of PEG with the backbone peak at δ (3.3-3.8 ppm) (Fig 8). The degree of acrylation as per the equation was found to be 98.14%.

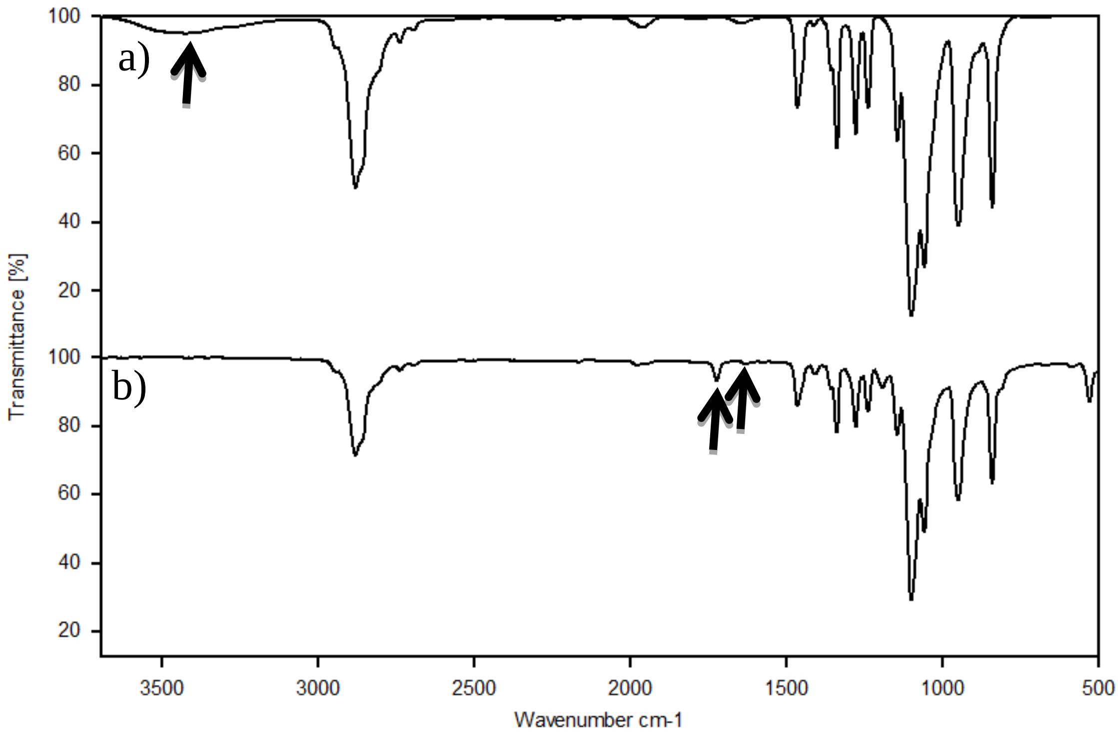

Fig 7: FT-IR showing a) PEG b) PEGDA

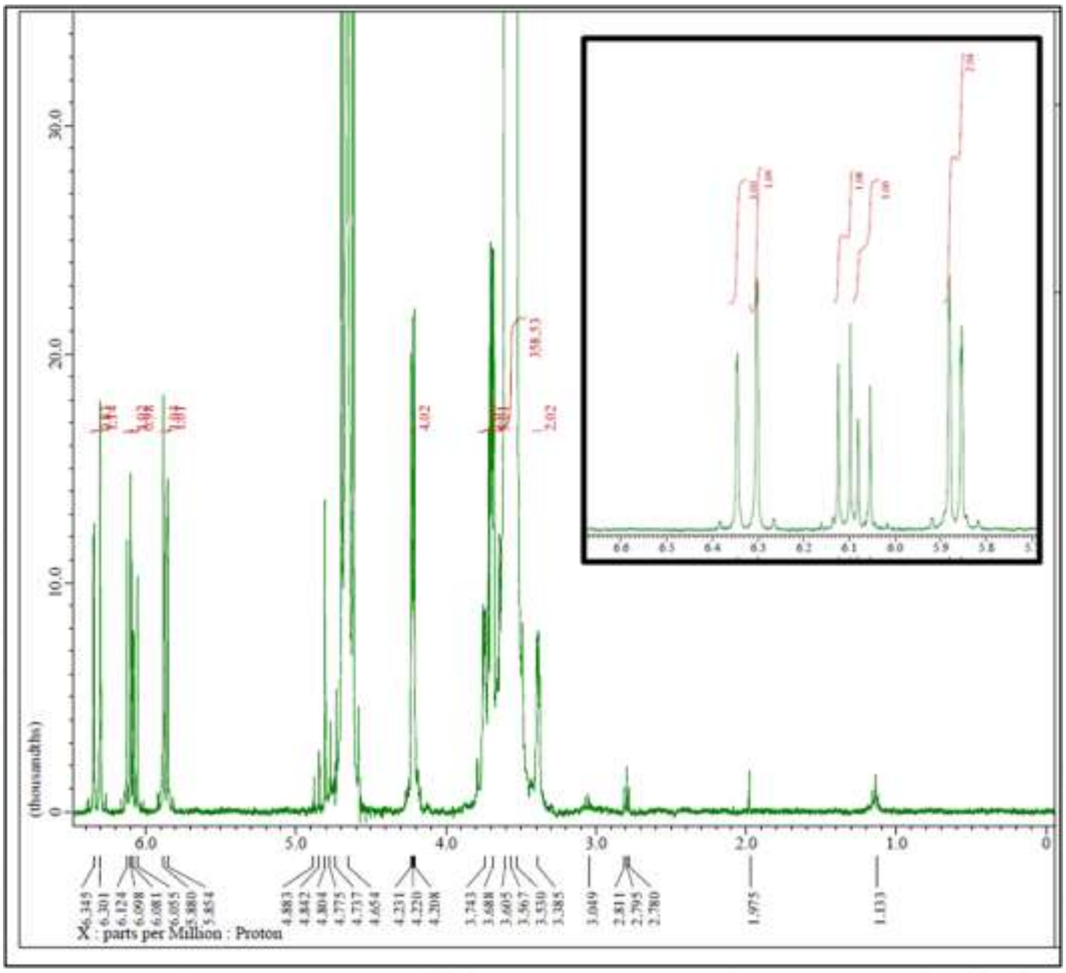

Fig 8: ^{1}HNMR spectrum showing PEGDA

3.2 Preparation of Methacrylated carboxymethyl cellulose

Carboxymethyl cellulose was modified using methacrylic anhydride to introduce methacrylate groups. The reaction was carried out at a basic pH. In this reaction, the deprotonated alcohol groups present on CMC was methacrylated with methacrylic anhydride leading to grafted methacrylated groups on the CMC backbone. The CMC methacrylation was confirmed in FTIR by the specific groups at 1730 cm^{-1} by the ester group –COO , the 1632 cm^{-1} of the –C=O and the –C-H peak at 893 cm^{-1} confirms that the methacrylation reaction has taken place (Fig 9). The ^{1}H NMR spectra of the sample was also taken using $CDCl_3$ as the solvent. The characteristic vinyl peaks at δ (6.5-7.4) ppm and the methyl peak at δ 2 ppm confirmed the methacrylation process (Fig 10).

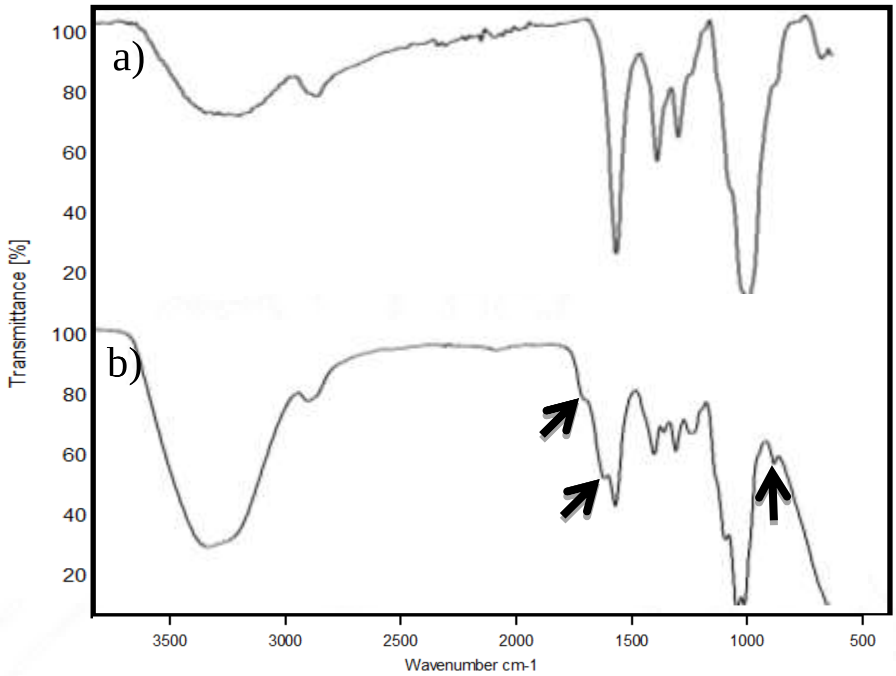

Fig 9: FT-IR showing a) CMC b) CMC-MA

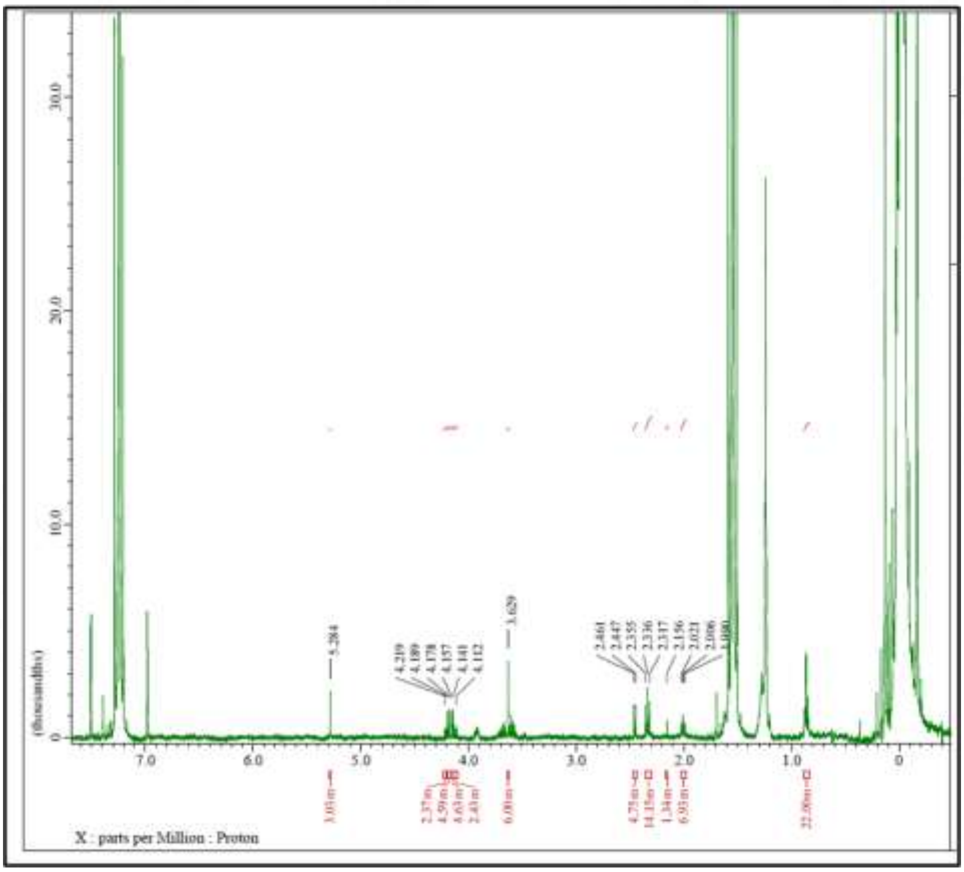

Fig 10: [1] HNMR spectrum showing CMC-MA

3.3 Preparation of Methacrylated CMC/PEGDA hydrogels

Five combinations of CMC/PEGDA gels were prepared by UV-crosslinking method. Different ratios were tried in order to see the dependency of concentration of two components. The disappearance of vinyl groups at 1634 cm^{-1} and the appearance of ester groups confirmed that crosslinking between CMC-MA and PEGDA has occurred for all the 5 ratio of gels (Fig 11).

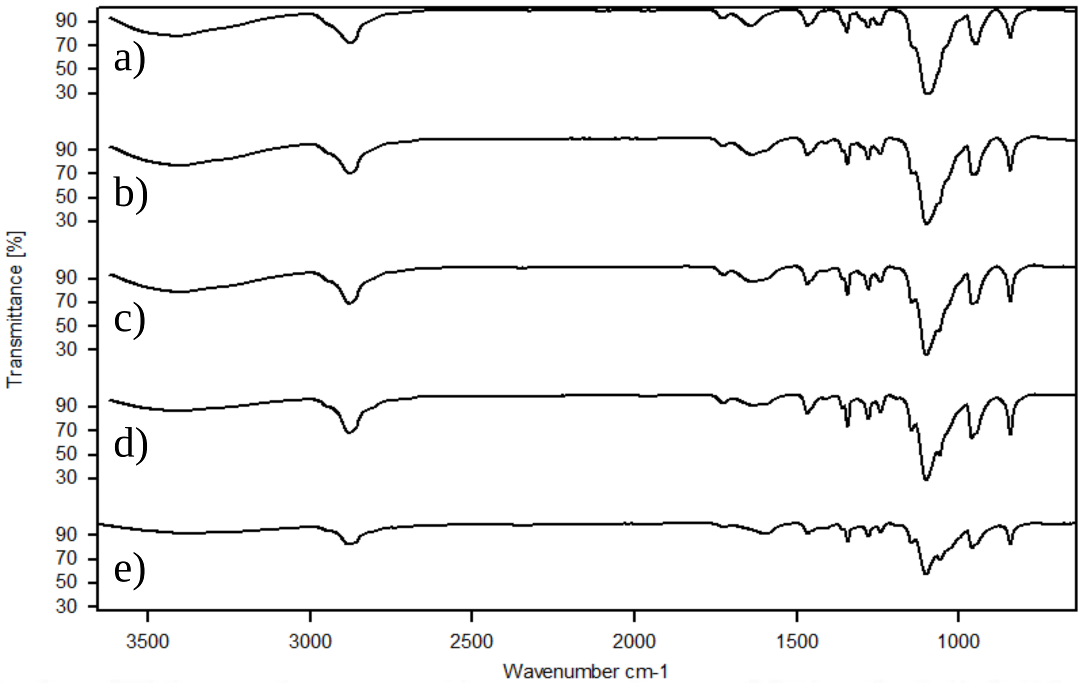

Fig 11: FT-IR peak of a) 2:8 CMC-MA/PEGDA b) 4:6 CMC-MA/PEGDA c) 5:5 CMC-MA/PEGDA d) 6:4 CMC-MA/PEGDA e) 8:2 CMC-MA/PEGDA

3.4 Swelling Profile studies

To understand the behaviour of hydrogel in retaining the amount of fluid, studying the degree of swelling of hydrogels is essential. The feature of swelling is vital in maintaining the hydrated condition of a cell for efficient nutrition and waste exchange and also for the diffusion of signalling molecules, which are critical to cellular viability when encapsulated. Maximum cell infiltration and cell adhesion is possible in highly swollen scaffolds since they have a larger surface area.

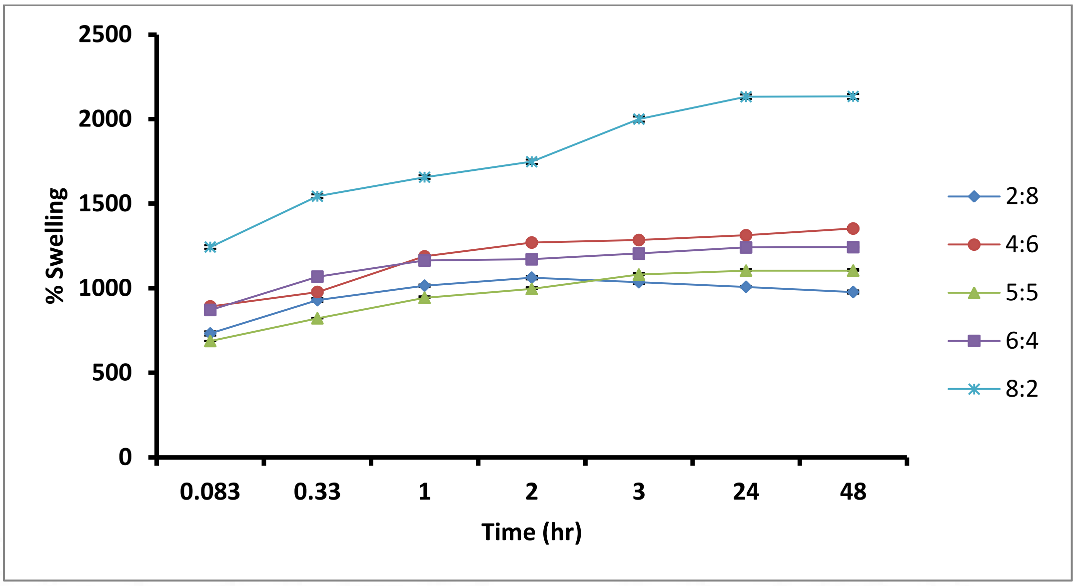

Fig 12: The degree of swelling of 5 ratios of CMC-MA/PEGDA hydrogels evaluated for a period of 48 hr (n=4)

The swelling of the freeze dried samples of five ratios was evaluated for a period of 48 hours. From Fig 12 it was shown that swelling is not much varied among the four ratios 2:8, 4:6, 5:5 and 6:4. But for 8:2 ratio a significantly high swelling profile was observed. At 0.083 hour the swelling percentage of the ratios 2:8, 4:6, 5:5 and 6:4 were in the range of 687±2.82% to 892±3.13%. Then the percentage gradually increased and reached equilibrium. According to Li et al. 2016 high cross-linking density could restrain the swelling of the hydrogels and can result in a low swelling ratio. This finding correlates with our results where the highly crosslinked gel 2:8 shows the low % swelling. For the ratio 8:2, swelling percentage increased from 1243±10.03 % to 1747±12.6% and after 2 hour it increased rapidly and then attained equilibrium. At 48 hour its swelling percentage was 2134% which was significantly high compared to the other ratios. Hence gels of this ratio should have maximum cell infiltration and cell adhesion which was further confirmed by biological evaluation studies. These results also proved that the construct of 8:2 ratio is able to absorb large amount of water which is an indication of its ability to perform a good matrix for cartilage regeneration.

3.5 Degradation profile of CMC-MA/PEGDA gels

The relative degradability of a hydrogel is critical for its performance in potential biomedical applications, as it allows for cell diffusion, nutrition flow, and integration with the host tissue. Thus *in vitro* degradation profiles of the freeze dried samples of 5 ratios were evaluated for a period of 1 week, 2 week, 3 week and 4 weeks.

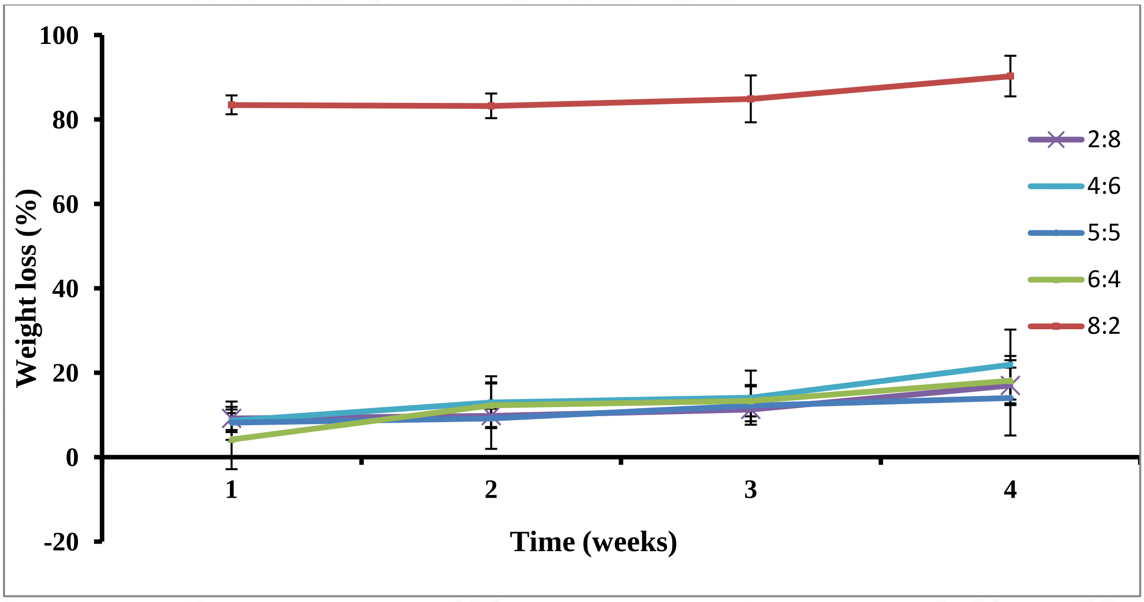

Fig 13: Weight loss % of 5 ratios of CMC-MA/PEGDA hydrogels on different time points (n=3)

From Fig 13 the degradation was found to increase significantly in a linear fashion for all the ratios as the CMC-MA concentration in the ratios is increased. 2:8 CMC-MA/PEGDA ratios showed the least degradability among the other ratios, which confirms the above finding. The p value between the weight loss % values for all ratios when compared to the 8:2 ratio at all time points were found to be significantly low ($p<0.01$). To determine the regeneration of the tissue, knowing the rate of degradation is necessary. From the above results it was evident that the ratio 8:2 having a higher swelling ratio enabled a much more hydrated gel which resulted in the increased degradation rate.

3.6. % Gel fraction of CMC-MA/PEGDA gels

To determine the fraction of crosslinked gel in 5 different ratios of CMC-MA/PEGDA hydrogels, % gel fraction was calculated.

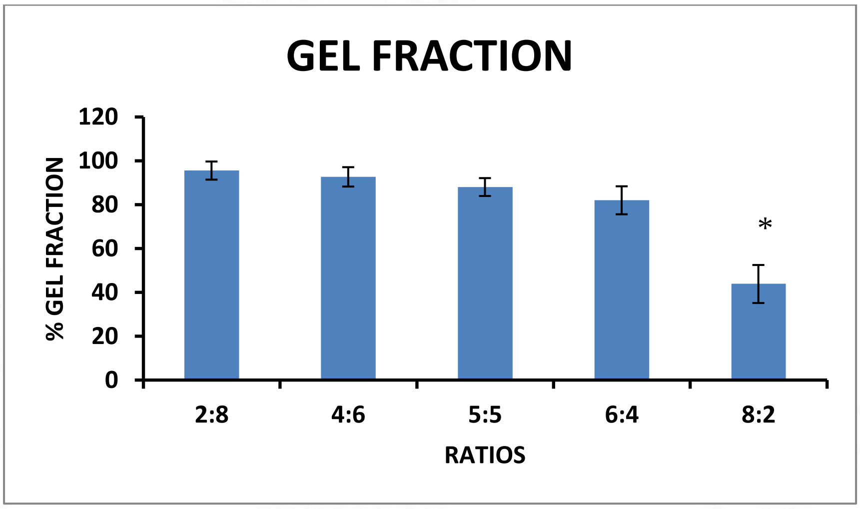

Fig 14: % gel fraction of 5 ratios of CMC-MA/PEGDA hydrogels. Data were presented as mean ± standard deviation (SD) (n = 4) (* $p < 0.01$).

For the ratios 2:8, 4:6, 5:5 and 6:4 no significant difference in % gel fraction was found and was in the range of 82±6.3% to 95±4.1% (Fig 14). A sudden decline was observed for the ratio 8:2 (43.8 ±8.67%). It is PEGDA which increases the chemical stability of the hydrogels and from these results it is evident that, as the ratio of PEGDA decreases % gel fraction also decreases. The p value between the % gel fraction values for all ratios when compared to the 8:2 ratio were found to be significantly high ($p<0.01$). A decrease in the % gel fraction in 8:2 ratio is due to the decreased amount of PEGDA since it is the contributing factor for the formation of stable hydrogels and the higher degradation profile.

3.7. Surface wettability properties using Dynamic contact angle measuring device

Surface wettability gives an idea about the surface characteristics of hydrogels and is a key factor in many properties like biocompatibility, adhesion, lubricity, selective absorption, and controlled release of molecules, etc. A hydrophilic surface has a contact angle of less than 50 degrees, while hydrophobic surfaces have contact angles greater than 90 degrees. Studies by Lee et al. 1997 and Draghi and Cigada 2007 that cells tend to adhere to surfaces that are moderately hydrophilic. Likewise in various other studies also hydrophilic substrates have been shown to stimulate chondrogenesis by increasing chondrocyte secretion and deposition of cartilage ECM [(Cui et al. 2003), (Park et al. 2005), (Ma et al. 2003)]. Results of our study (Table 3) shows that hydrophilicity increases as the concentration of CMC-MA is increased in the ratios. This highlight that the 8:2 ratio gel can get hydrated easily owing to the decrease in crosslink density in the gels due to hindrance in creating crosslinks on the CMC methacrylated backbone.

Table 3: θ_w measurements of CMC-MA, PEGDA and CMC-MA/PEGDA hydrogel of 5 ratios. Data represented as mean ± SD

Sample	**Dynamic contact angle ($\theta_{w)}$**
CMC-MA	43.08 ± 5.08
PEGDA	49.42 ± 7.12
2:8 CMC-MA/PEGDA	40.42 ±3.43
4:6 CMC-MA/PEGDA	48.80 ±9.46
5:5 CMC-MA/PEGDA	34.62 ±4.06
6:4 CMC-MA/PEGDA	38.74 ±4.10
8:2 CMC-MA/PEGDA	25.75 ±0.88

3.8. Stiffness characterization of CMC-MA/PEGDA gels using AFM

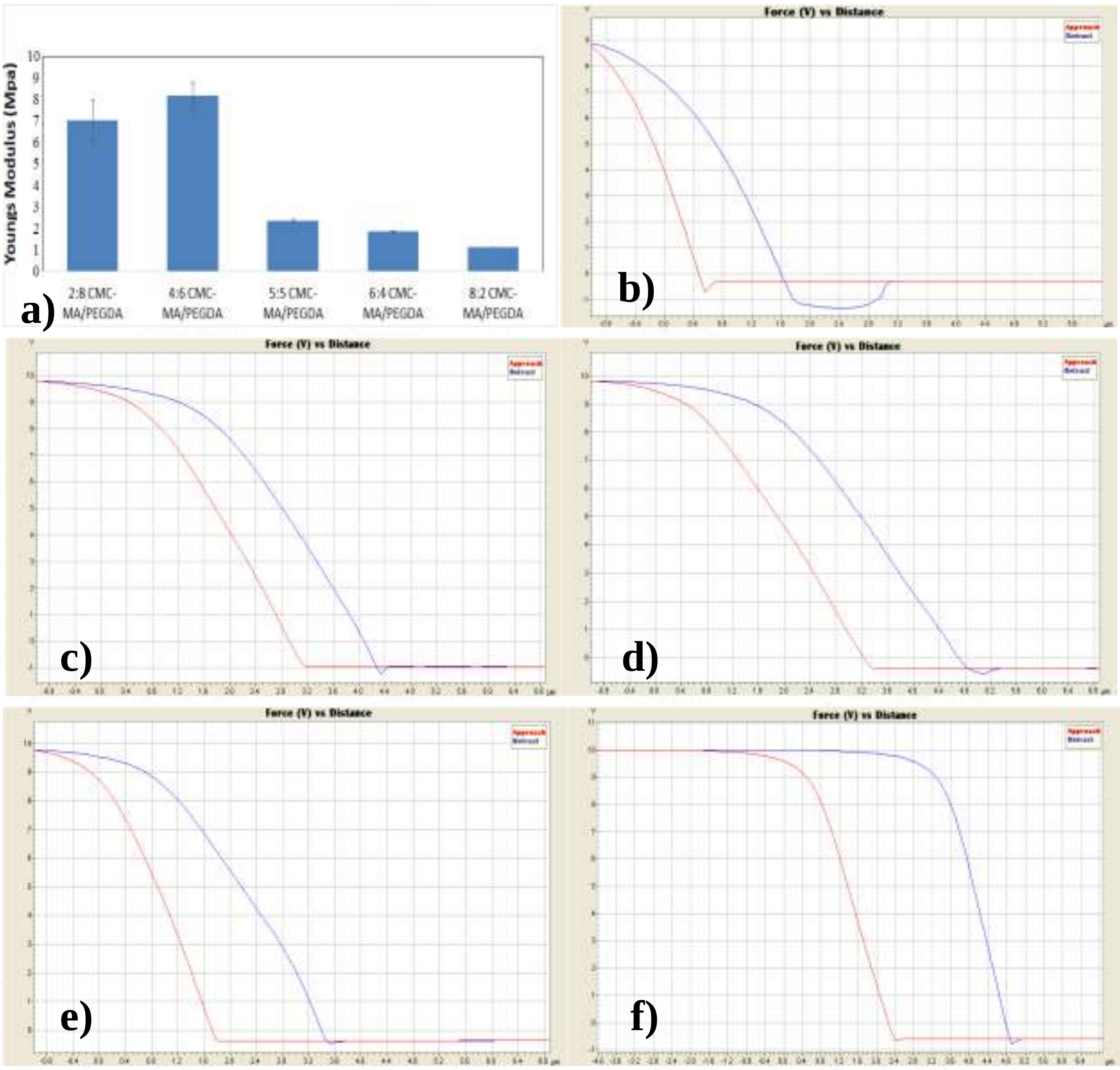

Fig 15: a) Plot showing young's modulus obtained for the five ratios. Representative force indentation curves obtained for b) 2:8 CMC-MA/PEGDA c) 4:6 CMC-MA/PEGDA d) 5:5 CMC-MA/PEGDA e) 6:4 CMC-MA/PEGDA f) 8:2 CMC-MA/PEGDA. The data was fitted to Hertz's contact model to calculate the Young's modulus and the maximum adhesion force.

To study the cellular adhesion and maintenance of cellular phenotype on the prepared gels, it is very important to study the contribution of matrix stiffness. In this study we have used the force indentation curves to ascertain the Young's modulus of the gel samples in different ratios. The Young's modulus was derived from the plot using the Hertz's contact model through the approach and retracts curves. The results (Fig 15) shows that as the concentration of CMC-MA is increased the stiffness or the Young's modulus of the gel decreases. The Young's modulus for the 2:8 CMC-MA/PEGDA was 7.03 ± 0.99 MPa and for 4:6 CMC-MA/PEGDA was 8.18 ± 0.58 MPa. A significant decrease in the stiffness was seen for the remaining ratios where 5:5 CMC-MA/PEGDA was 2.36 ± 0.06 MPa, 1.86 ± 0.03 MPa for the 6:4 CMC-MA/PEGDA and 1.14 ± 0.02 MPa for the 8:2 CMC-MA/PEGDA. The decrease in the modulus values as the concentration of CMC-MA is increased may be attributed to the decrease in the crosslinking density due to the higher molecular weight between the crosslinking methacrylate sites which are all along the backbone of the CMC chain when compared to the PEGDA moiety where the diacrylates are terminally situated and hence much efficient crosslinking efficiency is attained when PEGDA is increased in the ratio. This result is consistent with the swelling and degradation profiles that were obtained.

3.9. Cytotoxicity analysis

Direct contact test is a primary *in-vitro* cell cytotoxicity assay, performed to show an initial confirmation that the scaffold does not elicit any harmful effects on the cell viability. The five different ratios were placed on a monolayer of L929 cells for 24 hours (Fig 16). It was observed that all the five samples did not elicit any harmful leachants and the cells maintained their characteristic spindle shaped morphology and presented a score of zero and hence the gels can be considered to be non-cytotoxic. The cells were well spread and properly attached to the substratum as like control cells incubated with media alone.

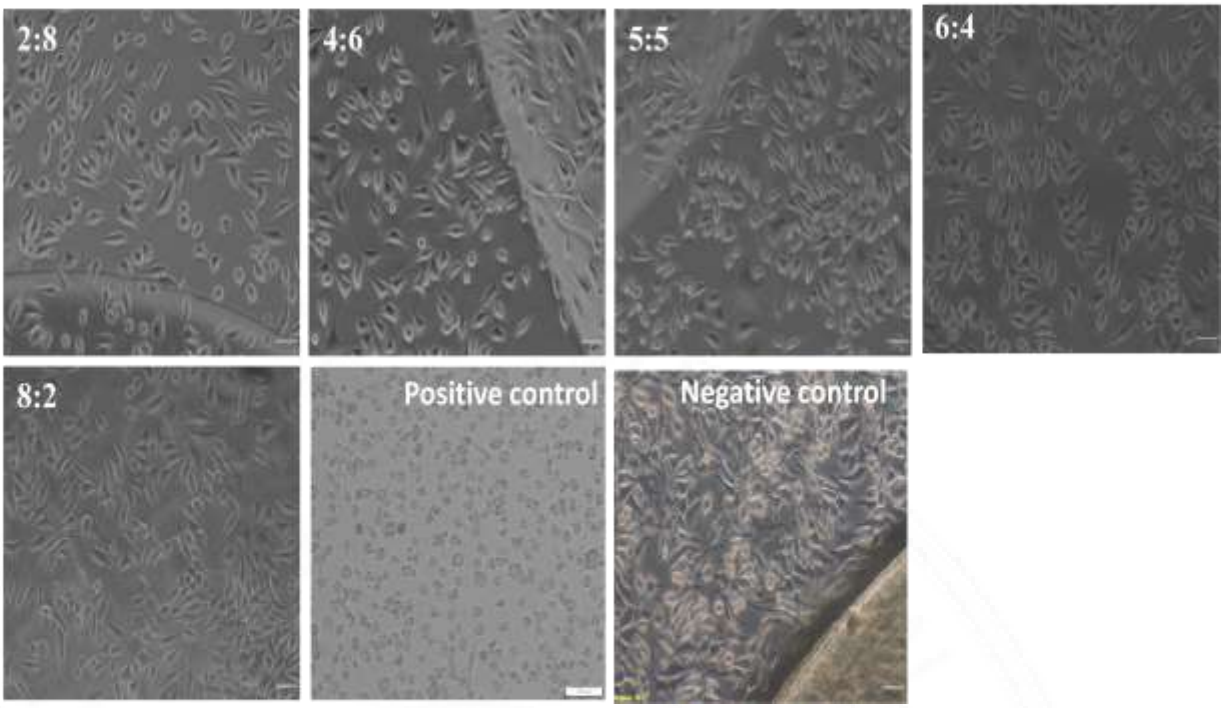

Fig 16: Microscopic images of Direct contact assay of CMC-MA/PEGDA gels. Positive control: Polyvinyl chloride, Negative Control: High molecular weight polyethylene. Scale bar: 50 µm

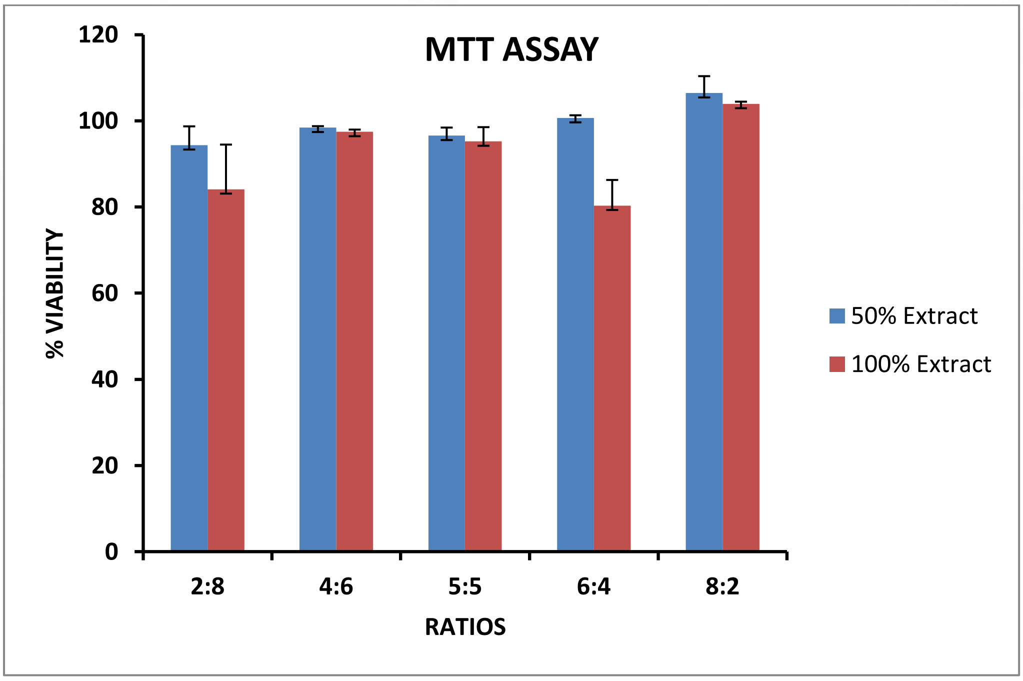

Fig 17: Assessment of mitochondrial activity by MTT assay.

The non-cytotoxicity of the scaffold was further confirmed by treating the monolayer of L929 with extracts of scaffold for a period of 24 hr (Fig 17). For all the 5 ratios, cells treated with 50 % extract showed more % viability than those treated with 100% extract. Among the five combinations, 8:2 showed maximum viability when compared to the others. This may be attributed to the increase in the cellulose content in these highly degradable gels in comparison with the inert PEG moieties which enhances the rate of cellular proliferation.

3.10. Isolation and culture of chondrocytes from rabbit articular cartilage

Chondrocytes were isolated from rabbit articular cartilage via enzymatic digestion of the tissue. A good yield of cells was obtained which were plated onto T25 flasks. The morphology of the chondrocytes had elongated fibroblast-like appearance attaining a dedifferentiated phenotype which is typical of chondrocytes grown in 2D culture (Fig 18). The first passage of cells was performed after two weeks.

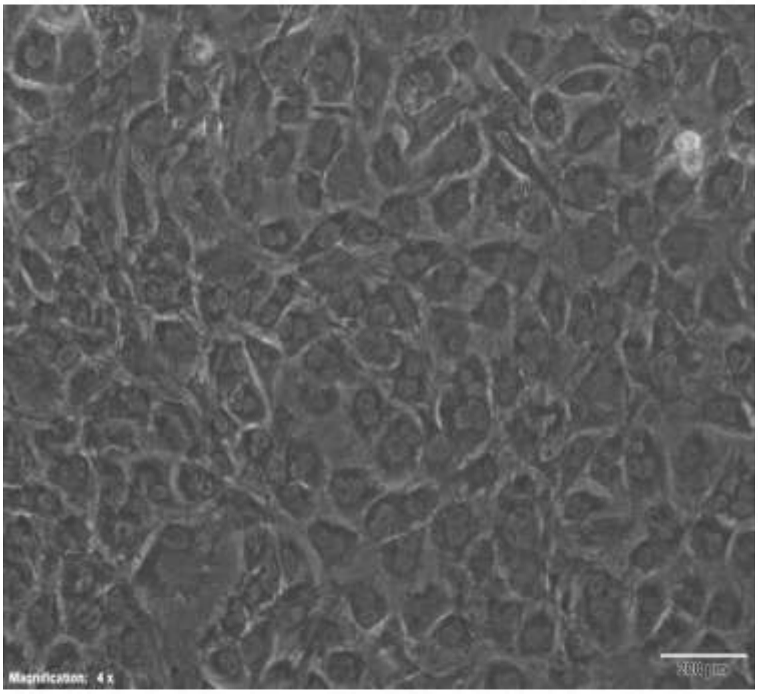

Fig 18: Phase contrast image of confluent monolayer of chondrocytes. Scale bar: 200µm

3.11. Cell morphology analysis

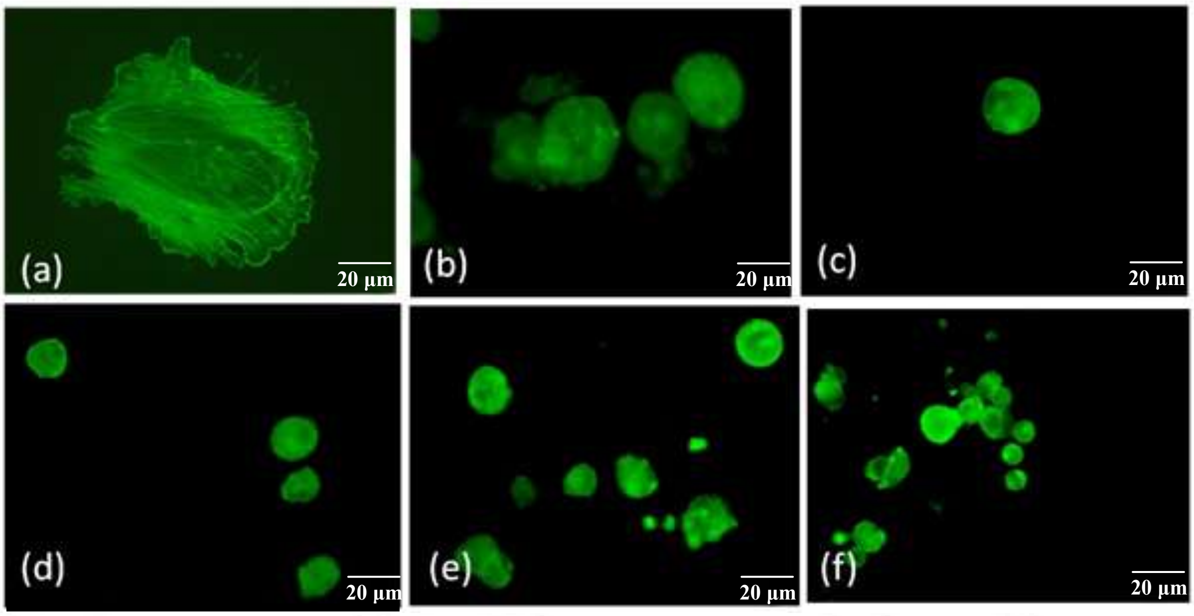

Fig 19: Digitally magnified images (10X magnification) of Immunostaining of (a) normal chondrocytes (b-f) Chondrocytes cultured in CMC-MA/PEGDA of 2:8, 4:6, 5:5, 6:4 and 8:2 ratio respectively. Scale bar: 20µm

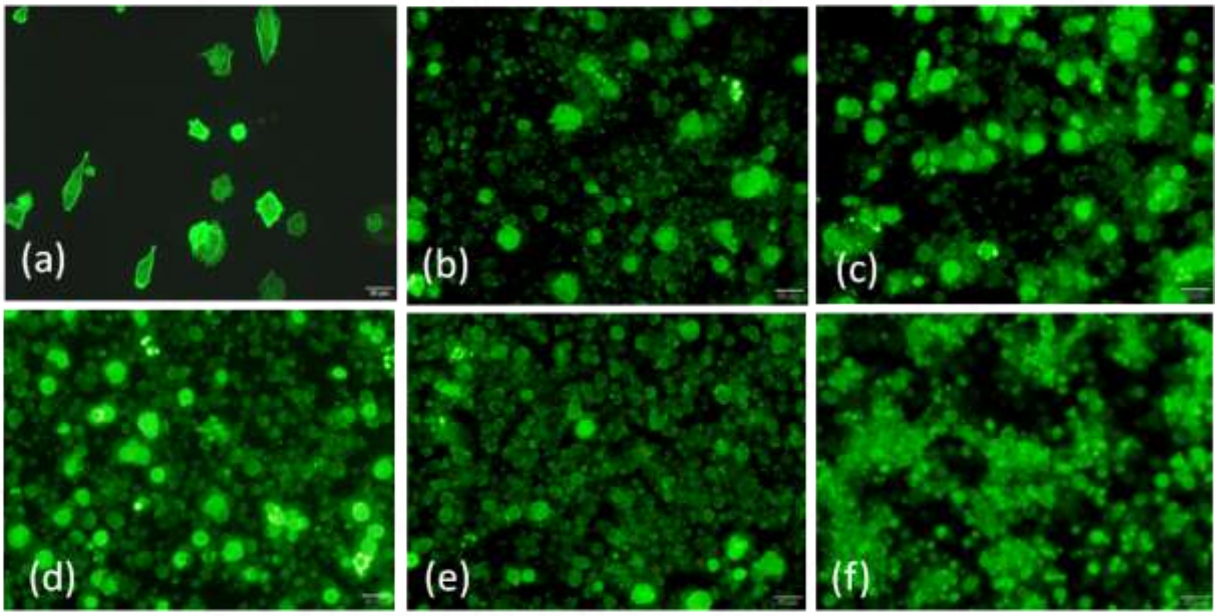

Fig 20: Zoomed out images (10X) of Immunostaining of (a) normal chondrocytes (b-f) Chondrocytes cultured in CMC-MA/PEGDA of 2:8, 4:6, 5:5, 6:4 and 8:2 ratio respectively. Scale bar: 50µm

To visualize the cellular morphology and cytoskeleton organization in the hydrogels, Immunofluorescence staining of F-actin was performed. In determining the proper cellular response, the ability to rapidly remodel the actin cytoskeleton is critically important. The results (Fig 19, Fig 20) showed that CMC-MA/PEGDA hydrogels of all the five ratios could promote chondrocytes adhesion, spreading and proliferation in which 8:2 ratio showed better attachment. Chondrocytes cultured in the less stiff 8:2 CMC-MA/PEGDA gel showed a better spherical morphology than others. Schuh et al. 2010 showed that the less stiff substrate of collagen-coated polyacrylamide (PA) gel system promoted the maintenance of the chondrogenic phenotype and also reported that chondrocytes cultured on stiff substrates had developed spread morphology. The results from our study can also be correlated with another study by (V Thomas, Vg, and D Nair 2017) in which they showed that spherical morphology was observed in less stiff 10:1 chitosan-hyaluronic acid dialdehyde hydrogels. The results of our study suggest that substrate stiffness also play an important role in determining cell fate.

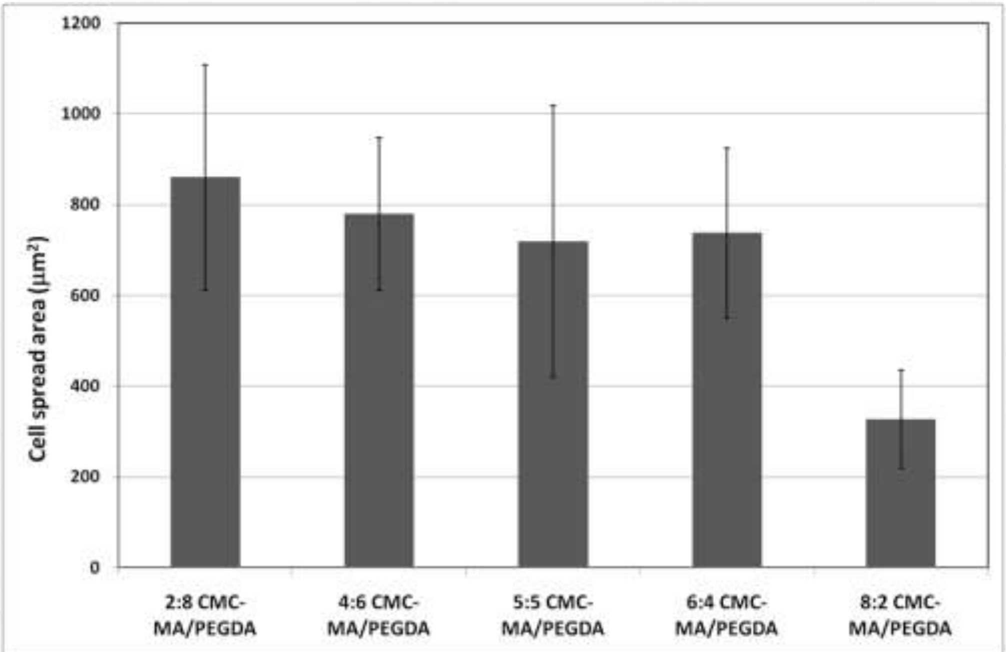

Fig 21: Graph showing cell spread area of chondrocytes cultured in 5 ratios of CMC-MA/PEGDA gels. Data were presented as mean ± standard deviation (SD)

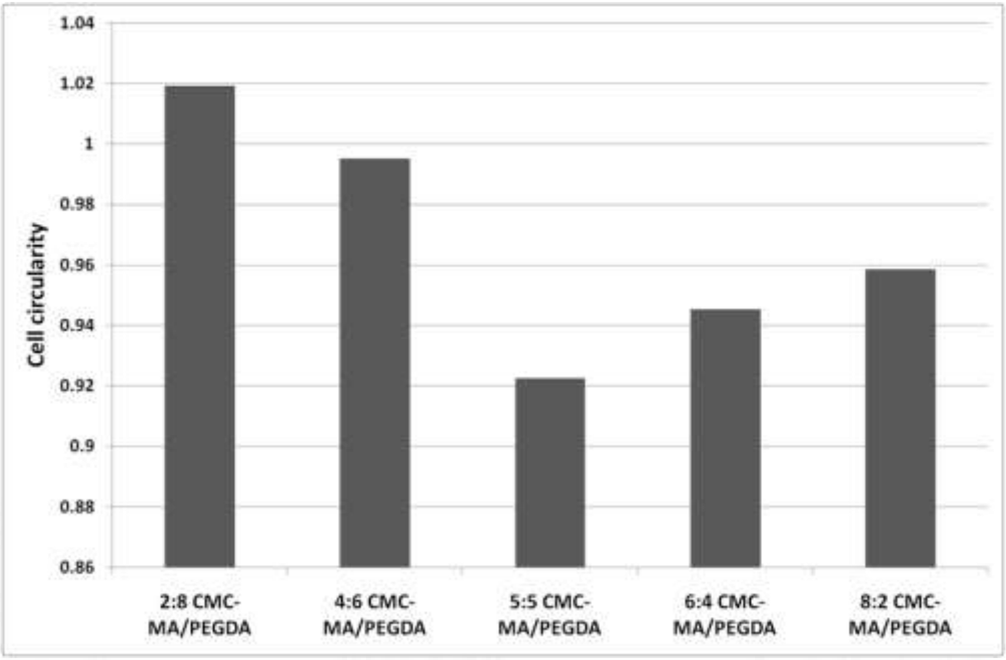

Fig 22: Graph showing cell circularity of chondrocytes cultured in 5 ratios of CMC-MA/PEGDA gels.

Table 4: Cell spread area and circularity calculated for chondrocytes cultured in 5 ratios of CMC-MA/PEGDA gels.

Ratios	**Cell spread Area**	**Std Dev**		**Ratios**	**Circularity**
2:8 CMC-MA/PEGDA	**860.33**	**248.12**		**2:8 CMC-MA/PEGDA**	**1.01**
4:6 CMC-MA/PEGDA	**779.05**	**168.96**		**4:6 CMC-MA/PEGDA**	**0.99**
5:5 CMC-MA/PEGDA	**718.73**	**298.80**		**5:5 CMC-MA/PEGDA**	**0.922**
6:4 CMC-MA/PEGDA	**736.65**	**187.41**		**6:4 CMC-MA/PEGDA**	**0.94**
8:2 CMC-MA/PEGDA	**326.95**	**108.51**		**8:2 CMC-MA/PEGDA**	**0.95**

Cell spreading (Fig 21) and cell circularity (Fig 22) was also calculated as a function of substrate stiffness. Cell spread area was found to be almost similar for the chondrocytes cultured in CMC-MA/PEGDA gels of 2:8, 4:6, 5:5 and 6:4 ratios. The least spread area was found in 8:2 ratio (Table 4).This may be due to the less stiffness of this gels. Sunyer et al. 2012 reported that cell spreading strongly correlates with hydrogel stiffness. They showed increased cell spreading with increased hydrogel stiffness. Our result also suggests that as the stiffness of hydrogels increased, cell spread area also increased. 2:8 being the stiffer gel of CMC-MA/PEGDA showed the maximum cell spread area compared to others. Cell circularity results showed that chondrocytes cultured in all the 5 ratios of CMC MA/PEGDA gels have a rounded morphology since the values are between 0.6 to 1.

3.12. Encapsulation of chondrocytes in 8:2 CMC-MA/PEGDA gels

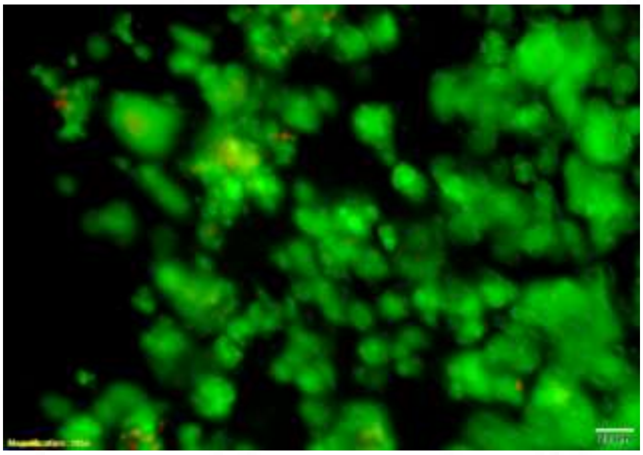

Fig 23: 8:2 CMC-MA/PEGDA with Calcein AM/ethidiumbromide (LIVE/DEAD assay) for ascertaining the viability of the cells that were encapsulated.
Scale bar: 20μm

After confirming that the hydrogel was non-toxic and the ratio 8:2 showed better attachment than the others, it was used for the chondrocyte encapsulation study. Chondrocytes were encapsulated on 8:2 ratio of CMC-MA/PEGDA gels and cultured for a period of 3 days. To assess the viability of encapsulated cell within the hydrogel live dead assay was performed on 3rd day retrieved samples. To distinguish live cells, the presence of intracellular esterase activity was determined by the enzymatic conversion of non-fluorescent cell permeant calcein AM to intensely fluorescent green calcein. In dead cells, ethidium bromide enters through damaged membranes and bind to the nucleic acid to produce bright red fluorescence. Few dead cells were observed, however it did not affect the culture. The chondrocytes were seen to aggregate with spherical morphology which shows increased extracellular matrix production (Fig 23). According to Liu, Zhou, and Cao 2017 formation of aggregation is very important in the initial stage of chondrogenic differentiation because it can facilitate extracellular matrix condensation. Studies by Moreira Teixeira et al. 2012 and Fang et al. 2015 also reported that spherical aggregation was beneficial for chondrogenesis due to the increased intercellular contacts. Findings from this study suggest that CMC-MA/PEGDA based hydrogel of 8:2 ratio could produce ECM of articular cartilage and promotes cell adhesion and growth.

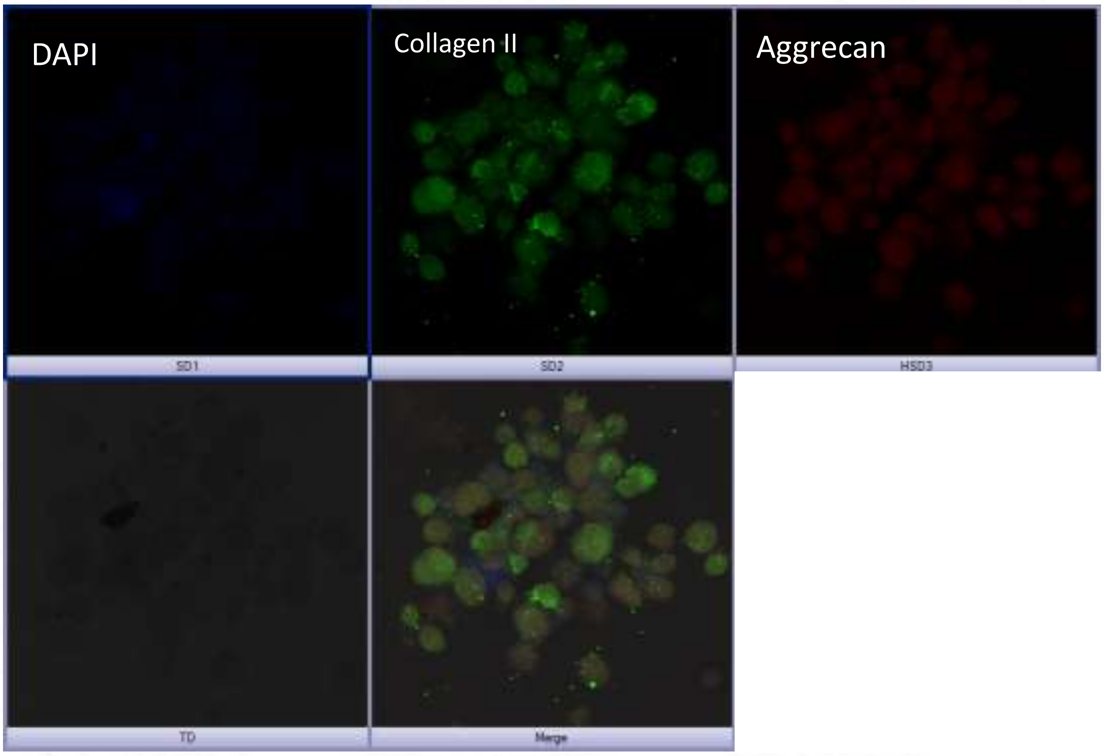

Fig 24: Confocal laser microscopy images showing Type II Collagen, Aggrecan staining of chondrocytes encapsulated in 8:2 CMC-MA/PEGDA gels in a 7 day culture period. Magnification: 10X

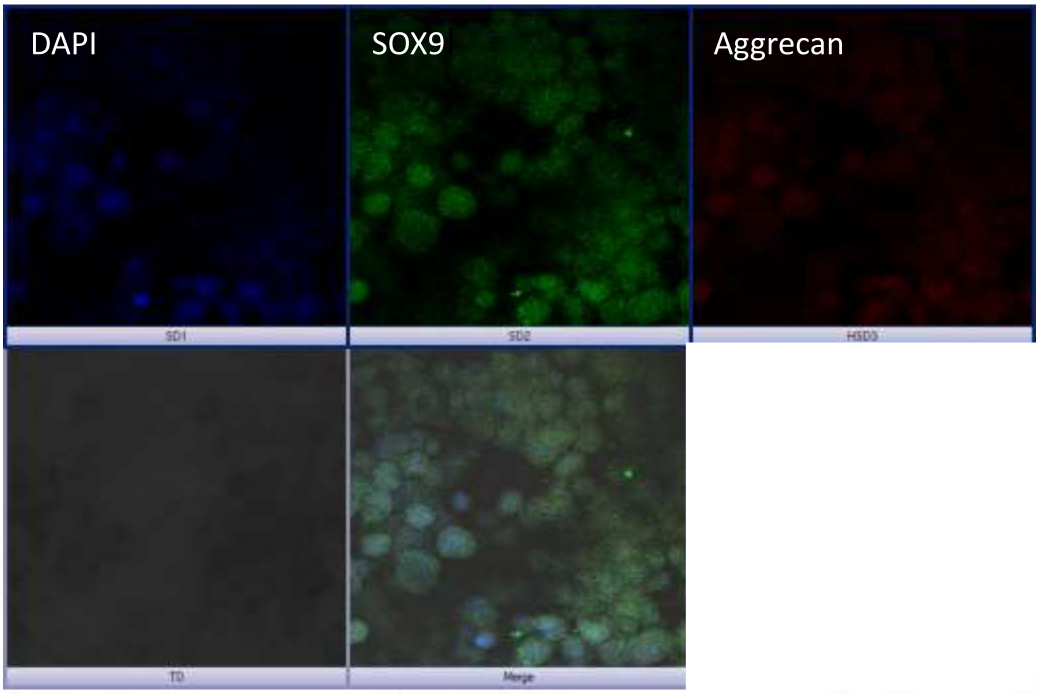

Fig 25: Confocal laser microscopy images showing SOX9, Aggrecan staining of chondrocytes encapsulated in 8:2 CMC-MA/PEGDA gels in a 7 day culture period. Magnification: 10X

Immunostaining of collagen type II, the characteristic ECM marker of functional chondrocytes and aggrecan, major proteoglycan of articular cartilage ECM on the chondrocyte encapsulated hydrogels were performed and it was found that in 7-day culture time point, the hydrogel 8:2 CMC-MA/PEGDA showed an enhanced production of both of these (Fig 24).This can be attributed to the less stiffness of these gels. A study by Zhang et al. 2016 showed that when rat chondrocytes cultured on polydimethylsiloxane (PDMS) materials of various stiffness, Type II collagen and aggrecan expression were higher when substrate stiffness was decreased. Hyaline-cartilage specific genes SRY-related high mobility group-box gene 9(SOX 9) was also assessed and an enhanced production of SOX 9 was found in 7-day culture period of cells encapsulated in 8:2 CMC-MA/PEGDA gels (Fig 25).The results of our study suggests that CMC-MA/PEGDA based hydrogel of 8:2 ratio could be a good matrix for ECM of articular cartilage.

CONCLUSION

In this study we have explored the effect of varying stiffness compositions of a two component hydrogel based on methacrylated carboxymethyl cellulose and Polyethylene glycol diacrylate on the growth and functionality of encapsulated chondrocyte. Of the selected five ratios, the less stiff 8:1 CMC-MA/PEGDA gel showed maximum swelling and degradation hence proved its ability to perform a good hydrated matrix for cartilage regeneration. This assumption was confirmed by biological evaluation studies in which chondrocytes cultured in this ratio of gels retained their characteristic spherical morphology with sufficient extracellular matrix production. However the long term stability of the gel is an issue that needs to be addressed. Nevertheless the gel can be used as a matrix for delivering of redifferentiated chondrocytes within the defect size for regenerating the hyaline cartilage tissue. The knowledge and understanding gained in this study, of chondrocyte behaviour encapsulated within the hydrogel system with varying stiffness will help us to design an appropriate matrix that can manipulate and control cell behaviour to engineer hyaline cartilage tissue.

FUTURE PERSPECTIVE

Future works that need to be done in this area include:

- A detailed study on the rheological properties of the substrate.
- Encapsulation study for a long term culture periods and assessment of ECM secretion.
- To extend the study to *in vivo* animal models to ascertain the efficiency and functionality of the gel system as a delivery vehicle or a matrix for developing an injectable gel system for cartilage tissue regeneration.

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