PRODUCTION AND CHARACTERIZATION OF 3D PRINTED METHYLCELLULOSE-GELATIN TISSUE SCAFFOLDS FOR ARTICULAR CARTILAGE TREATMENT

by

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

I, Berk Acarkan, hereby certify that I am aware of the Academic Ethics and Integrity Policy issued by the Council of Higher Education (YÖK) and I fully acknowledge all the consequences due to its violation by plagiarism or any other way.

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ABSTRACT

PRODUCTION AND CHARACTERIZATION OF 3D PRINTED METHYLCELLULOSE-GELATIN TISSUE SCAFFOLDS FOR ARTICULAR CARTILAGE TREATMENT

Articular cartilage is a highly specialized tissue that plays a crucial role in movement. However, its avascular nature severely limits its regenerative capacity, often resulting in degenerative diseases. Current treatment methods face challenges such as prolonged recovery times, inconsistent outcomes, and high costs. This study aims to develop scaffolds that mimic the properties of natural cartilage, as to be an alternative. For this purpose, Methylcellulose(MC)-Gelatin-based tissue scaffolds were fabricated with varying MC(10%, 12.5%, and 15%) and constant gelatin(20%) concentrations using a 3D printer. Characterization studies were performed to evaluate scaffold performance. Fourier Transform Infrared (FTIR) spectroscopy was employed to analyze the chemical interactions between MC and gelatin, particularly the effects of MC concentration. Contact Angle Measurement (CAM) results indicated that increasing MC concentration enhanced hydrophobicity due to the amphiphilic nature of MC. Mechanical tests revealed that increasing MC concentrations led to higher elasticity but reduced stiffness. Swelling and degradation tests revealed that higher MC concentrations reduced degradation rates and enhanced scaffold durability. In conclusion, the produced tissue scaffolds were able to mimic natural cartilage tissue closely, especially 10MC scaffolds stand out as a promising candidate to be a treatment alternative.

Keywords: Articular Cartilage, Cartilage Defects, 3D Printing, Methylcellulose, Gelatin, Tissue Scaffold, Tissue Engineering.

ÖZET

EKLEM KIKIRDAĞI TEDAVİSİ İÇİN 3B METİLSELÜLOZ - JELATİN DOKU İSKELELERİNİN ÜRETİMİ VE KARAKTERİZASYONU

Eklem kıkırdağı, hareket için hayati öneme sahip bir dokudur. Ancak, avasküler yapısı nedeniyle kendini yenileme kapasitesi sınırlıdır ve bu dejeneratif hastalıklara yol açmaktadır. Mevcut tedavi yöntemleri, uzun iyileşme süreleri, tutarsız sonuçlar ve yüksek maliyetler gibi zorluklarla karşı karşıyadır. Bu çalışma, doğal kıkırdağın özelliklerini mimikleyen ve potansiyel bir tedavi olarak kullanılabilecek iskeleler geliştirmeyi amaçlamaktadır. Bu amaçla, farklı Metilselüloz (MC) (%10, %12.5 ve %15) ve jelatin (%20) konsantrasyonlarında Metilselüloz-Jelatin doku iskeleleri 3D yazıcı kullanılarak üretilmiştir. İskelelerin özellikleri, karakterizasyon çalışmalarıyla analiz edilmiştir. Fourier Dönüşümlü Kızılötesi (FTIR) spektroskopisi, MC ve jelatin arasındaki kimyasal etkileşimleri, özellikle de MC konsantrasyonunun etkilerini ortaya koymuştur.Temas Açısı Ölçümu (CAM) sonuçları, MC konsantrasyonunun artmasının, MC'nin amfifilik yapısı nedeniyle hidrofobikliği artırdığını göstermiştir. Mekanik testler, MC konsantrasyonunun artmasının elastisiteyi artırırken, sertliği azalttığını ortaya koymuştur. Şişme ve bozunma testleri, daha yüksek MC konsantrasyonlarının bozunma oranlarını düşürdüğünü ve iskele dayanıklılığını artırdığını göstermiştir. Sonuçlara göre üretilen doku iskeleleri, doğal kıkırdağı dokunun özelliklerini büyük öçüde mimiklemeyi başarmakta ve özellikle %10 MC iskeleleri alternatif bir tedavi stratejisi olarak öne çıkmaktadır.

Anahtar Kelimeler: Kıkırdak Doku, Kıkırdak Doku Defektleri, Metilselüloz, Jelatin, Doku Mühendisliği, 3B Yazıcı, Doku İskelesi.

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LIST OF SYMBOLS

MC10	Hydrogel with 10% Methylcellulose ratio
MC12.5	Hydrogel with 12.5% Methylcellulose ratio
MC15	Hydrogel with 15% Methylcellulose ratio
θ	Contact Angle
-OH	Hydroxyl Group
$-NH_2$	Amine Group
$-OCH_3$	Methoxy Group

LIST OF ABBREVIATIONS

MC	Methylcellulose
Gel	Gelatin
CAD	Computer Aided Diagnosis
AC	Articular Cartilage
OA	Osteoarthritis
WHO	World Health Organization
PBS	Phosphate Buffered Saline
CAM	Contact Angle Measurement
kPA	Kilopascal
FTIR	Fourier Transform Infrared
SPSS	Statistical Package for the Social Sciences
ANOVA	Analysis of Variance
TEC	Tissue-Engineered Construct
MRI	Magnetic Resonance Imaging

1. INTRODUCTION

1.1 Motivation

In the definition of vitality, things that possess certain qualities; such as growth, metabolism, energy conversion and reproduction are characterized as living things [1].

In the evolutionary process, the complexity of these functions increases along with the structure of these living things. When it is moved up in the phylogenetic tree, the "movement" feature is an ability that has emerged as necessary for the functionality of these mechanisms. As organisms develop, the potential for movement and the mechanisms that provide the movement have gained differences. In prokaryotic organisms, movement is actuated by simple structures such as flagellum, and pili [2]; while in vertebrate organisms by limbs and joints.

Although joints are inspected under different categories, those responsible for mechanical movement are generally categorized as synovial joints, which are the most common joint types in the body. These joints allow to perform a wide range of movements such as walking, running, writing and more. Synovial joints are flexible and can move, slide over each other, rotate, and so on. These joints can be found in the shoulder joint, neck joint, knee joint, wrist joint, etc[3]. They bear great mechanical stress while participating in movement. The effect of friction and gravity, wear and damage will inevitably occur in these joint types over time. Abnormalities in these structures can lead to pain or loss of function [4].

Articular cartilage covers the tips of the bones and provides the major load-bearing function in the joint with its outstanding frictional properties. It also ensures that one end of the joint forms a highly wear-resistant surface. It allows joints to move effectively over the other with little or no wear.

Most arthritic changes start as little lesions on the surface of the cartilage and progress to total wear of the tissue and arthritis [5]. However, the avascular and aneural nature of this tissue significantly limits its capacity to repair itself. Therefore, injuries of articular cartilage, often caused by trauma, aging and genetic predisposition, often progress to degenerative diseases such as osteoarthritis [6]. Osteoarthritis alone affects more than 500 million people worldwide, not only limiting mobility but also reducing quality of life [7, 8]. Thus, there is a growing need for new approaches aimed at regeneration of cartilage tissue.

Current clinical treatments for articular cartilage, such as microfracture surgery, graft transplantation, and autologous chondrocyte implantation (ACI), face limitations such as variable success rates, limited regenerative capacities, long recovery times, and potential complications [9]. In this context, tissue engineering has emerged as a promising approach to developing scaffolds that mimic tissues by combining the principles of biology, engineering, and materials science [10]. An ideal scaffold should be biocompatible, biodegradable, and able to mimic the mechanical and biochemical properties of natural cartilage [11].

In recent years, advances in biomaterials and 3D printing technologies have facilitated the development of more sophisticated scaffold designs and increased the potential of hydrogel-based scaffolds in particular. By combining biocompatible and biodegradable polymers such as gelatin and methylcellulose, it's aiming to

create scaffolds with cartilage-like properties.

Gelatin, derived from the partial hydrolysis of collagen, is often preferred in tissue engineering applications for its biocompatibility and biological activity-promoting properties. Its protein-based, hydrophilic structure promotes the adhesion and proliferation of cells to the scaffold, thus supporting tissue regeneration [12]. The heat reversibility of gelatin due to its modest binding energy enables the development of biomedical systems that require responsive material properties [12, 13, 14].

Water-soluble cellulose derivative Methylcellulose obtained by partial substitution of the hydroxyl groups (-OH) of cellulose with methoxy groups (-OCH_3). This chemical modification makes methylcellulose a material with an amphiphilic structure, capable of interacting with water and exhibiting temperature-variable properties [15, 16]. Methylcellulose, which forms a thermally stable structure depending on temperature changes, increases the mechanical stability of the hydrogel and provides the advantage of long-term use. This property creates a suitable environment for the proliferation of cells by maintaining the scaffold [15, 17].

Both methylcellulose (MC) and gelatin have elastomeric properties and are capable of forming stable network structures at room temperature [16]. These two components form strong hydrogen bonds with water molecules due to their hydrophilic nature. This property can increase the water holding capacity and hydrophilicity of the hydrogel to be formed. The physical interactions between the methoxy groups of MC and the amino and carboxyl groups of gelatin may support the structural stability of the hydrogel [18, 19]. In this context, these two materials together have the potential to form a hydrogel scaffold that will support cartilage

regeneration and comply with the biological and mechanical requirements.

Considering all these factors, a study in this field is desired to be conducted due to the ease of producing structures that were previously impossible or difficult to apply, using 3D printers. Although gelatin is frequently used in cartilage treatment studies due to its accessibility and cost-effectiveness, there is a notable absence of studies involving 3D scaffold studies that incorporate gelatin with methylcellulose.

This study aims to focus on the development and characterization of methylcellulose and gelatin-based hydrogel scaffolds. For this purpose, hydrogel scaffolds with varying methylcellulose concentrations are produced using pneumatic 3D printing technology, are aimed to be biocompatible, biodegradable and close to the mechanical properties of native cartilage. The findings from the study may contribute to the development of cartilage repair strategies and have the potential to reduce the social and economic burden of cartilage-related diseases.

1.2 Objective

This study aims to develop Gelatin-methylcellulose-based tissue scaffolds as a potential alternative for articular cartilage treatment. The objectives of this study are as follows:

- To be able to produce Methylcellulose-Gelatin-based scaffolds with a 3D printer.
- To investigate the effect of Methylcellulose concentration on the physical, chemical and mechanical properties of the scaffolds.
- Evaluate the results and determine the optimum composition for potential application as a treatment method.
- To establish a foundation for future cell studies based on the data obtained.

1.3 Outline

After explaining the structure and function of cartilage tissue in the background section, the thesis provides an overview of cartilage tissue-related disorders, its prevalence, economic impact, and treatment methods and then mentions the tissue scaffolds produced with 3D printers, which served as the inspiration for this research. Subsequently, detailed information about the methodology to be used in production and the materials to be used in the tissue scaffolds are presented. In Chapter 3, the production process of the scaffolds and the characterization studies are explained. In Chapter 4, the results of the study are presented. In Chapter 5, the characterization results are evaluated. In the last part, Chapter 6, the conclusion and possible future works are mentioned.

2. BACKGROUND

2.1 Structure of Cartilage

Cartilage tissue can be categorized according to the area where it is located and its functions there. Hyaline cartilage is the most abundant type of cartilage in the body, and articular cartilage is a specialized form of hyaline cartilage [20]. Hyaline cartilage plays an important role in skeletal growth and development. Initially, during the development of endochondral bones in embryonic and fetal stages, and secondarily as growth plates that result in bone elongation during childhood and adolescence [6, 20].

Articular Cartilage is composed of hyaline cartilage with a dense extracellular matrix (ECM) and scattered specialized cells of cartilage cells called chondrocytes. The extracellular matrix is composed mainly of collagen, proteoglycans and glycoproteins that help hold water molecules in the matrix [21].

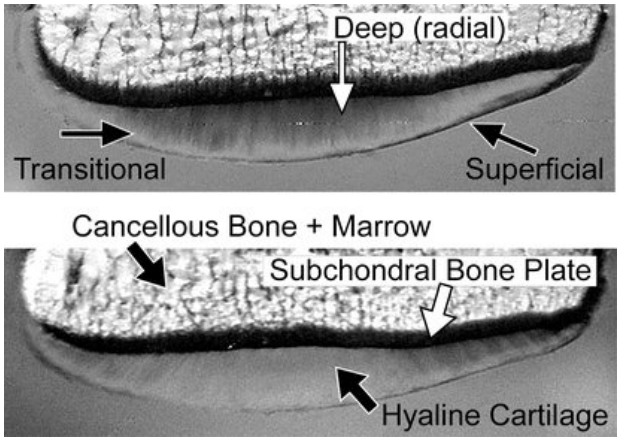

Figure 2.1 Structure of Articular Cartilage by MRI Imaging [22].

Collagen is arranged as forms of fibrils. The fibres are positioned to react to shear forces, traction and pressure. Also, the fibrils position defines the layers within the cartilage. The collagen fibrils are parallel to the articular surface and are closely arranged. Collagen bundles are dispersed randomly in the adjacent layer. The third layer is called the radial zone. This is the thickest layer of the tissue and the fibrils in this layer are positioned perpendicular to the joint surface. The deepest layer is called as calcification zone and next to the subchondral bone, which is also the thinnest layer [21, 22]. Even though the cartilage doesn't have a lymph or blood vessels in between layers; synovial vessels and the synovial fluid that synoviocytes provide supply the cartilage [23].

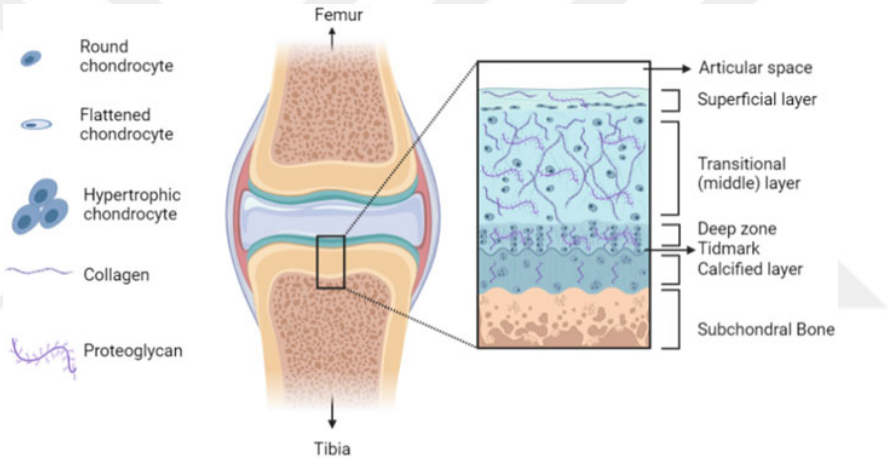

Figure 2.2 Layers of Articular Cartilage[24].

The primary cell type in articular cartilage are chondrocytes, which consist of less than 10% of the cartilage volume in total and are in charge of organizing, synthesizing, and maintaining all ECM components. The articular cartilage ECM is mainly composed of water (68-85% of total wet weight, the major fluid phase), collagen (60-86% of dry weight), proteoglycan (15-40% of dry weight) and some other small non-collagenous proteins and glycoproteins [25].

Amount of water in layer varies and directly affects the structure. Water and the extracellular matrix (ECM) are the primary components of articular cartilage (AC), providing the high elasticity and strength, required for sustained and repetitive movement. The movement of water over the cartilage delivers nutrients to the chondrocytes and also lubricates the joint surface which is crucial for movement [24].

There are different types of collagens in articular cartilage. However, Type II collagen, which makes up 90-95% of the collagen in articular cartilage, is most prevalent there and forms collagen fibers that are interlaced with microfibrils, fibrils and eventually proteoglycan aggregates. Together with the type II collagen fiber network, other collagens including types I, IV, V, VI, IX, and XI are also present in tiny amounts and may improve the structural and elastic strength of cartilage [25, 26].

The osmotic pressure that proteoglycans exert, drives the high-water content in the extracellular matrix which is essential for cartilage's resistance to mechanical stresses. By creating an expansion pressure that opposes the tension in the collagen fibers, this osmotic pressure counters the mechanical forces that push water out of the cartilage. Articular cartilage's specialized constitution and high percentage of water allow it to move nearly frictionless the joint and tolerate intense mechanical forces [21, 26].

Articular cartilage is an extremely specialized connective tissue found in synovial joints. Its primary purpose is to present the transmission of loads with a low friction coefficient by creating an articulation surface that is slick and smooth. The amount of coefficient of friction between 0.001-0.01 [26, 27].

Articular cartilage is subjected to a severe biomechanical environment and has no blood vessels, nerves or lymphatics. Due to these inadequacies, the ability of articular cartilage to be treated and repaired internally is limited [6]. Its main objective is to create the most suitable surface for the movement of the joints. More precisely, it lessens friction between the bones and makes stresses to be transferred to the bone easier [26].

Articular cartilage is structurally specialized to perform these functions and has acquired a characteristic structure. Articular cartilage is usually 2 to 4 mm thick [28, 29] and exhibits a viscoelastic structure due to its biphasic or triphasic nature. Under load, it shows both elastic deformation (instantaneous return) and viscous deformation (slow return over time). This viscoelastic behavior contributes greatly to the load-bearing capacity of cartilage [29].

Three major methods for determining the mechanical properties of articular cartilage are shear, tensile, and compressive testing. Shear testing is used to measure the viscoelastic properties of cartilage, while tensile testing measures how cartilage responds when a tensile force is applied, and it is used to understand elasticity and stiffness [30]. Compressive modulus for articular cartilage refers to how the cartilage behaves under load, that is, how much it resists deformation when compressed. This is a critical parameter in understanding the resistance of cartilage to pressure in joints. A high compressive modulus indicates that the cartilage has greater resistance to deformation under compression and thus it has a stiffer structure [28, 29, 30]. A native articular cartilage tissue that belongs to a healthy human has a compressive modulus ranging from 240-1000 kPa [30, 31].

Articular cartilage has great clinical importance because its injury can cause significant musculoskeletal dysfunction. Furthermore, due to cartilage's limited ability to heal and repair, preservation of cartilage's integrity throughout life has a key importance for health [23].

Over time, repetitive mechanical loading and other factors such as genetics can lead to localized damage to collagen fibres. When the damage reaches a critical point, the fibres can no longer withstand the swelling pressure of hydrophilic proteoglycans, resulting in localized oedema and cartilage swelling. It's biomechanical and structural properties make articular cartilage tailored for load bearing and joint lubrication during movement but also cause difficulties in repairing and regenerating this tissue when damaged [23].

2.2 Cartilage Defects: Causes, Types and Impacts

Many cartilage-related diseases are associated with changes in the ECM. These changes can influence the progression of diseases. For instance, disorders like osteochondritis dissecans and inflammatory arthropathies can result from secondary damage in pathological processes occurring in other surrounding tissues. Also, might induce the main symptoms of dwarfism caused by genetically inherited abnormalities. In addition to exterior damage, genetic abnormalities can impact the growth plate cartilage, which can interfere with normal skeletal growth. Genes encoding extracellular matrix molecules or regulatory proteins might undergo mutations. Collectively, hereditary disorders are called chondrodysplasia, and while each one is an uncommon disorder, they affect about 1 in 4,000 births [6, 23, 32].

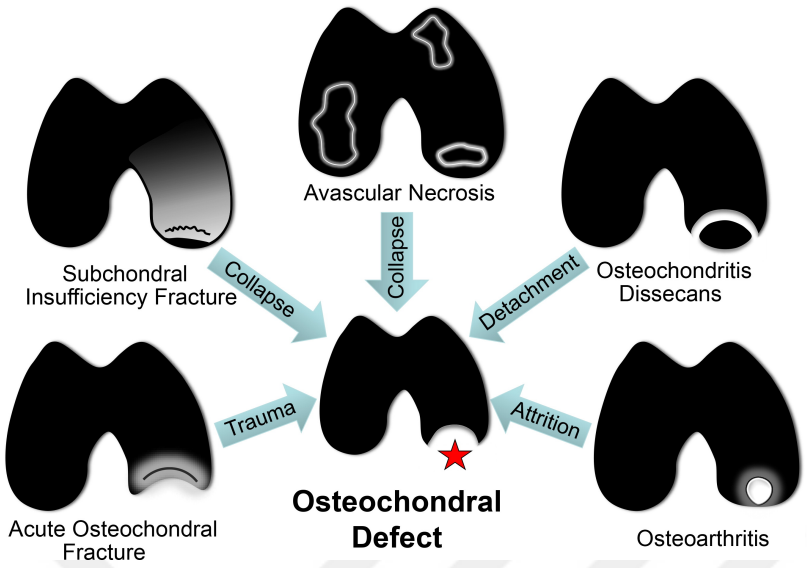

Figure 2.3 Pathological conditions leading to osteochondral defects[22].

Osteochondritis dissecans (OCD) is a joint disorder, particularly in children and adolescents, and usually affects joints under intense stress, such as the knee, elbow and ankle. The cause of the disease is not known for certain, but it is thought that factors such as repeated microtraumas, genetic predisposition and ischemic events may lead to OCD. Symptoms may present as pain, stiffness and swelling in the joint [33].

Rheumatoid arthritis (RA) is an autoimmune disease that affects between 0.5% to 1% of the world's population, and it is defined as an autoimmune disorder marked by chronic inflammation, primarily affecting joints but also potentially involving other tissues and organs. This systemic inflammation results in joint pain, stiffness, swelling, and can lead to joint damage and deformity over time [6, 34].

Achondroplasia is a disorder in which chondrocytes do not proliferate in the cartilage and the epiphyseal plate of long bones near joints is especially affected. These plates play a crucial role in bone growth and development. so achondroplasia may cause conditions like dwarfism [35].

Relapsing polychondritis (RP) is a rare autoimmune disease characterized by relapsing cartilage inflammation that can affect all forms of cartilage as well as proteoglycan-containing organs such as ears, nose and respiratory tract. This disease is caused by the immune system's misdirected attack on cartilage, which results in chondrocyte loss, ECM disintegration, and chronic inflammation. As a result, cartilage gradually breaks away, resulting in the disease's typical symptoms and complications. Every year 3.5 million people are diagnosed with RP in the USA [6, 36].

Chondrocalcinosis is the aggregation of calcium pyrophosphate dihydrate (CPPD) crystals in joints [6]. The deposition of CPPD crystals in the cartilage causes mechanical disruption, inflammatory responses, and matrix degradation. These may lead to progressive deterioration of cartilage and may cause joint pain, swelling and joint dysfunction eventually [37].

Cartilage tissue tumors are a significant subclass of bone cancers. Chondromas are known as benign or non-cancerous cartilage tumors. It includes osteochondroma, enchondroma, periosteal chondroma, multiple chondromatosis or enchondromatosis, chondroblastoma, and chondromyxoid fibroma are examples of benign cartilage tumors. There are also, chondrosarcomas are tumors of the cartilage that are malignant or cancerous [6, 38].

Osteoarthritis is one of the most common cartilage defects, which is affecting hundreds of millions of individuals globally. The disorder mostly affects the articular cartilage of load-bearing joints, including the hands and feet, hips, shoulders and most commonly knees. It refers to a disorder in which the cartilage covering the bones of the joints becomes thinner and occasionally wears completely down. This causes friction and erosion of the ends of the bone, causing bone damage. In this instance, bones that move against each other without protective cartilage can also cause movement limitations, joint inflammation along with severe pain and swelling [6, 39].

Osteoarthritis is the most prevalent cartilage-related disease because the disease is not only associated with genetic tendency but also with external factors. Obesity, gender, age, genetic anatomical abnormalities of the joints, and acute joint trauma are among the many risk factors for osteoarthritis. Patients may not exhibit any symptoms until the disease reaches later stages and there is substantial and irreversible joint damage [7, 23].

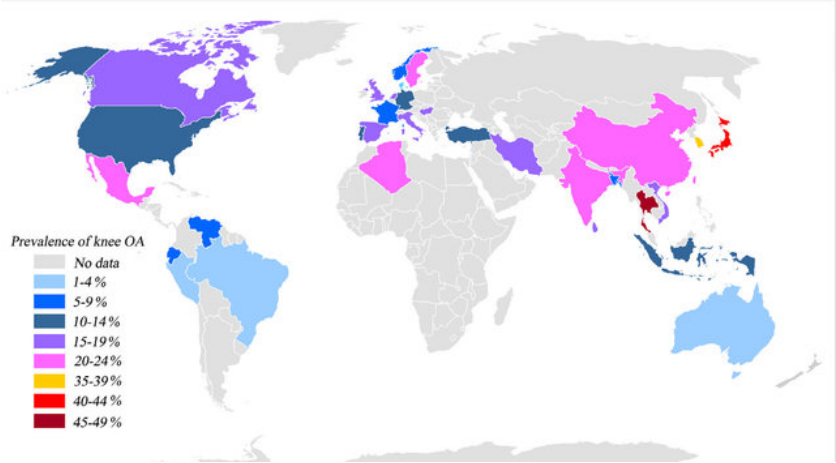

Figure 2.4 Prevalence of knee OA around the world [40].

According to WHO[7] and OA Prevalence & Burden[8]:

- Approximately 528 million individuals globally suffer from osteoarthritis and its prevalence has increased by 113% from 1990 to 2019.
- About 73% of people suffering from osteoarthritis are over 55 years old and 60% of them are women.
- With an estimated number of 365 million people globally, the knee is the most usually affected joint, followed by the hip and hand.
- Globally, 344 million individuals suffer from moderate to severe osteoarthritis, which may benefit from rehabilitation.
- 1/3 of osteoarthritis sufferers over the age of 45 experience anxiety or depression.
- After dementia and diabetes, it is the third disability that is growing at the fastest rate.
- It is known that OA patients are 50% more prone to having heart disease and that 1/3 of them have five or more chronic illnesses. Because OA restricts exercise, patients with OA are also more likely to have conditions including diabetes and hypertension.
- The prevalence of osteoarthritis is predicted to rise worldwide because of the aging population, increasing rates of obesity, and an increase in injuries.

In 2019, arthritis caused an estimated damage of 136 billion dollars in the USA. Over the past ten years, this amount has more than doubled. To provide a context, the yearly economic burden of arthritis is greater than that of diabetes,

cancer, and health problems associated with tobacco use. The annual cost of direct medical care needed because of arthritis is $65 billion [41].

Poor knowledge of the etiology and pathogenesis of cartilage disorders, delayed diagnosis since the tissue is aneural and challenges with drug administration because adult cartilage is avascular are some of the challenges related to cartilage diseases [6]. All of these cons limit the body's ability to heal itself and lower the probability that current treatments will be effective. Repair of damaged cartilage is a high priority issue that needs to be solved, yet so far little progress has been made. The need for innovative treatments and techniques for cartilage treatment has been increasing over the years. .

Advances in biotechnology, material science and regenerative medicine are able to create a whole new approach for cartilage regeneration. The advancements in these fields are expanding the possibilities for the treatment of injured cartilage, including biomaterial implants, tissue engineering and cell-based treatments. Also, stem cell research shows great potential in using the body's healing abilities, it promises a whole new route for cartilage regeneration.

Additionally, developments in 3D bioprinting and scaffolding techniques make it possible to construction of unique structures that closely mimic the cartilage, thereby increasing the precision and effectiveness of treatment techniques. Furthermore, optimization of treatment strategy and prediction of treatment outcomes have become simpler with the integration of computer modeling and artificial intelligence.

2.3 Current Treatment Strategies for Cartilage Defects

The choice of therapy depends majorly on the degree or depth of cartilage injury. Numerous methods are used to define the size and depth of damage while determining the degree of damage. The Outerbridge classification is the most extensively used approach, which evaluates the size and depth of the damage [42].

Table 2.1
Outerbridge classification of articular cartilage injuries [42].

Grade of Damage	**Description**
Grade 0	Healthy Articular Cartilage with a smooth surface.
Grade I	Soft and swollen AC with decreased proteoglycan and high water content.
Grade II	The cartilage surface fracture, and extending up to half its thickness. Swelling or fraying can be detected through MRI. The damaged area is confined to less than 1.25 (<50%) of the surface. It indicates intermediate-thickness damage.
Grade III	The damage extends beyond half the cartilage thickness and may expose the subchondral bone. The damaged surface area exceeds 1.25 cm. The deep defect involves over 50% of the thickness.
Grade IV	Full-thickness defects. Subchondral bone is completely exposed by destruction.

For the damages graded as I and II, conservative treatment methods, such as education of the patient, BMI reduction, rehabilitation, or the use of medication and diets, typically have positive outcomes. It is advised to use surgery for grade III or IV damages. The most popular surgical techniques are osteochondral transplantation, mosaicplasty, chondroplasty surgery, and the microfracture (MF) procedure. Also, cell-based therapies like autologous chondrocyte implantation (ACI) or matrix-derived autologous chondrocyte implantation (MACI), are aimed specifically for articular cartilage treatment [42].

Periosteal transplantation is a surgical procedure performed for treating chondral defects in which cartilage injury is repaired by using the periosteum, a layer of tissue that contains many cells which are capable of producing new cartilage. Periosteum, is placed directly above a defect with its chondrogenic cells that promote cartilage regeneration. Even though this approach takes advantage of the regenerative potential and reduced immunogenicity of tissues, its outcome is variable and it has to overcome aspects such as a limited amount of available periosteum [43].

Perichondral transplantation is a similar procedure to periosteal transplantation to treat chondral defects. Yet, perichondrium is a more similar tissue to native articular cartilage. Perichondrium is inserted into the defect of the injured joint after being removed from the cartilaginous covering of the bones like ribs [44].

Autologous chondrocyte implantation (ACI) is a treatment technique that has been used in clinics. Small amounts of cartilage are collected from the damaged joint. It is then taken to the laboratory for proliferation. The damaged cartilage area is then prepared for implantation by cleaning the cartilage down to the healthy surrounding cartilage. Cultured chondrocytes are injected under the periosteal flap and sealed with fibrin glue [45]. Thanks to breakthroughs in technology, many variations of the ACI technique, such as Matrix-Assisted Autologous Chondrocyte Implantation (MACI) have been developed. Additionally, a novel method derived from ACI uses a sponge-like scaffold made of hyaluronan, which is seeded with autologous chondrocytes and used to cover the defected cartilage site [46].

The minced cartilage technique is considerably one of the newest techniques. In this procedure, the patient's cartilage tissue harvests, and mincing them into tiny fragments. These fragments are placed on a scaffold and reimplanted into the damaged area of the joint. In comparison to ACI, a greater surface area may be created and implanted for cartilage repair in the same surgical session by chopping and equally distributing the tissue on the scaffold. This technique benefits from the natural ability of the body to regenerate cartilage and may lead to more natural repair [47, 48].

Microfracture is one of the marrow stimulation techniques among abrasion arthroplasty, drilling to treat cartilage defects. It is a very frequent procedure, which involves creating small holes in the underlying bone so that blood, bone marrow, and stem cells produced from the marrow may reach the defect location. This process aims to propagate self-regeneration in the damaged cartilage [48, 49].

Mesenchymal stem cells that have been obtained from bone marrow and other sources can offer another alternative and a plentiful supply of cells for cartilage treatment methods. By adopting tissue-engineering, gene therapy, and cell therapy approaches, adult marrow stromal cells are being studied as potential therapies for abnormalities in connective tissues, such as articular cartilage [48, 49].

Embryonic stem cells are less differentiated than are less differentiated than mesenchymal stem cells. They exhibit a greater near-infinite potential for self-renewal and can be directed to create every type of cell found in the body [48]. However, the use of these cells for cartilage treatment is not yet as widespread as other stem cell types due to their rarity, high acquisition costs and the need for specialized expertise to administer them [50, 51].

Gene therapies use viral or non-viral vectors to insert genes that encode growth factors (such as TGF-b), anti-inflammatory cytokines, or matrix proteins, aiming to enhance chondrocyte function and fix cartilage structure. Protein-based therapies, like using TGF-b in the culture medium can be challenging, due to the short half-lives of proteins, need to be administered often, being costly and challenging and posing risks such as high doses that may cause undesired side effects in other tissues [48, 52]. Gene editing methods like CRISPR/Cas9 are also being investigated as promising alternatives for defects such as osteoarthritis [52, 53]. These methods have a potential to improve cartilage repair and fix genetic defects. However, there are still many unanswered questions about the safety, effectiveness, and application of these gene treatments [54].

2.4 Tissue Scaffolds: A Novel Treatment Approach

The treatment of articular cartilage defects has remained one of the largest challenges in health sector for a long time [55]. Conventional methods that aim to repair the defects, usually either using tissues taken from the patient or adding different material on the defected tissue. However, these treatments have significant disadvantages such as limited regenerative capacity, long recovery times and low success rates. Specifically, the limited ability of articular cartilage to regenerate itself is the effectiveness of conventional methods [55, 56].

In this context, the need for novel treatment methods is increasing undoubtedly and tissue engineering applications are emerging as an innovative strategy with a promising approach in the treatment of articular cartilage injuries. Tissue engineering majorly focuses on repairing and regeneration to treat defected tissues

in the body by using biomaterial, cells or biomolecules [10]. This approach aims to build synthetic structures that mimic the structural, functional, and biological features of native tissue. These structures are named tissue scaffolds. The use of tissue scaffolds plays a critical role for cells to organize correctly and form functional tissue in the native environment [11].

Tissue scaffolds are aimed to provide the physical support that is necessary for cells to adhere, proliferate and differentiate. These scaffolds are based on biocompatible and biodegradable materials that can heal the defected tissue while allowing cells to behave as they would in the native environment [11]. By using different materials and manufacturing techniques, different types of tissue scaffolds can be designed and optimized according to the specific requirements of the targeted area and scope of treatment. For a successful application of scaffolds into a host, scaffolds must carry some properties as shown in Table 2.2 [57].

Table 2.2
Summary of desirable scaffold properties[57].

Scaffold Characteristics	**Desirable Properties**
Bioactivity	Interaction and binding of scaffold material with host tissue.
Biodegradability	Controlled scaffold degradation which can complement tissue ingrowth while maintaining sufficient support.
Scaffold Architecture	Interconnected pores allow diffusion and cell migration, and a large surface area for cell-scaffold interaction.
Mechanical Properties	Compressive, elastic and fatigue strength comparable to host tissue.
Biocompatibility	Non-toxic and non-inflammatory scaffold components and break down products avoiding immune rejection.

Scaffolds for cartilage tissue treatment in order to promote cell adhesion, cell migration, and development should have a suitable architecture, controlled

degradation, proper mechanical properties and adequate biocompatibility to provide a suitable environment that is close to the native tissue. Numerous structural characteristics, such as permeability, interconnectivity, porosity and pore size are also crucial for cartilage regeneration [42, 58].

In monolayer cultures, chondrocytes are tend to dedifferentiate, so the tissue scaffolds must be designed in three dimensions in order to prevent chondrocytes from dedifferentiating [49]. As grown on flat surfaces, chondrocytes are unable to synthesize certain proteins required for the formation of cartilage. It is possible to enhance tissue ingrowth, cell migration, seeding and development by using a highly porous membrane with an interconnected macropore network. It is also necessary to ensure the diffusion of oxygen and delivery of nutrients and products of metabolism. The formation of regenerated cartilage tissue is related to regulated and controlled biodegradation, which is mostly dependent on the materials that are used in production of tissue scaffolds. The byproducts of breakdown must be easily removed and should be non-toxic to the body. It is essential that scaffolds preserve suitable stiffness, strength, and flexibility properties to facilitate integration and tissue growth. All these variables are crucial for cell cultivation of scaffolds and particularly following implantation into the body. All these parameters of scaffolds should be tailored for different cell types [42].

Countless types of biomaterials have been used in tissue engineering applications, natural and synthetic polymers are the most significant ones. Additionally, ceramic materials can be used, especially when paired with polymers to create composite materials that have better mechanical and biological qualities [59].

Natural polymers possess several beneficial properties that make them ideal

for use as scaffolds. They typically exhibit excellent adhesion, good biocompatibility, and reduced toxicity during the degradation process of the scaffold [58]. On the other hand, there are also some drawbacks about natural polymers. First of all, it is difficult to manage the rate of degradation. Since the body is a system in constant motion, there are many different parameters. Therefore, adjusting the rate of degradation is very critical and challenging. Additionally, natural polymers generally have low breaking loads. However, if the polymer is cross-linked, mechanical characteristics increase significantly [59, 60].

Synthetic polymers can also offer significant advantages over natural polymers. Their porosity, degradation rate and mechanical properties can be customized for particular applications which is their main advantage [59]. Generally, they are less expensive than the natural ones and they also can be produced in vast amounts under controlled conditions. Moreover, the majority of synthetic polymers show nearly independent hydrolytic degradation from the host tissue. Although synthetic polymers offer advantages over natural ones in some aspects, inevitably there are some aspects where they are inferior. One of the significant drawbacks of synthetic polymers is the degradation process of synthetic polymers into unwanted byproducts, like acids that can provoke inflammation at higher concentrations [59, 60].

2.5 Fabrication Techniques for Tissue Scaffolds

Tissue scaffolds can be produced from various materials, such as 3D membranes (sponge-like), hydrogels, nanofibers or combinations of these and many others. In order to produce a tissue scaffold for a specific purpose, the architec-

tural, mechanical and biochemical properties of the scaffold are crucial aspects. Additionally, selecting an appropriate production method is essential to achieve a scaffold with the desired properties [48, 57, 58].

Solvent casting/particulate leaching is one of the traditional methods of scaffold production that starts with the dissolving of a polymer in an organic solvent. The process makes use of porogens, which are materials that may be mixed into a mold and then dissolved after the mold has hardened to produce pores in a specified shape and size [57, 61].

Gas foaming is a great alternative to avoid the use of harsh solvents employed in solvent casting/particulate leaching methods. This process forms a highly porous structure by using carbon dioxide; a gas bubble to nucleate and expand within the polymer [57, 62].

Electrospinning is a widely used scaffold fabrication method that is capable of producing nanofibrous interconnected porous scaffolds. In this method, a generated electric field to pull charged polymer solutions as fine thread from a capillary tube or a needle toward a negatively charged collector plate. Fibers ranging from micro to nanometers can be sequentially created and deposited to form a scaffold that may contain composite materials and biomolecules [57, 62].

Freeze drying starts with a polymer solution freezing, which creates solvent ice crystals coated in polymer aggregates. A vacuum is then used to lower the pressure in the environment until it is lower than the frozen solvent's equilibrium vapor pressure. This causes the solvent to sublimate from the solid phase straight into gas. There remains a dry polymer scaffold with an interconnected porous

structure when the solvent has entirely sublimated [57, 61].

Phase separation involves, forcing a solution to de-mix into two phases, one of which has a high polymer concentration (or polymer-rich phase) and the other of which has a lower polymer concentration (or polymer lean phase) [59]. There are several methods for phase separation. Thermally induced phase separation (TIPS) is one of the most common methods on use. This method relies on altering the system's free energy through temperature changes. After the solution separates, the solvent is eliminated via extraction, evaporation, or freeze-drying [59, 62]

2.6 3D Printing

The conventional techniques for fabricating scaffolds, which have been briefly reviewed thus far, often provide restricted control over the dimensions, pore size and interconnectivity [57]. Advances in computer-aided design and modeling programs, as well as breakthroughs in 3D printing technology tissue scaffold fabrication methods, have entered a new era.

3D Printing method makes possible to produce scaffolds with small-scale and large-scale designs. It is a method of manufacturing that may create controlled interior structures and geometries of 3D objects, including pore size. This manufacturing method has been extensively used in tissue engineering and regenerative medicine to create scaffolds or cell-laden structures with improved control over material and cell localization in 3D technology [63]. The scaffold provides cells with a 3D structure and mechanical support for their attachment and pro-

liferation, ultimately growing into a functional tissue-engineered construct (TEC) [30].

3D printing technology offers the possibility to precisely control the properties of the biomaterial and structure produced. This control plays a critical role in creating a suitable microenvironment for cells to attach, proliferate and migrate. For example, pore size, density and structure accelerate the integration of cells into the scaffold and tissue regeneration [64]. Compared to traditional methods, mass production of scaffolds with 3D printing technology can be realized more quickly and cost-effectively. Furthermore, this technology enables the use of various biomaterials and allows scaffolds to be detailed at both micro and macro scales[63].

In 3D printing, a wide range of materials can be used, including: natural polymers such as collagen, gelatin, hyaluronic acid (HA), alginate, chitin and chitosan; synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA), polycaprolactone (PCL), polyethylene glycol (PEG); bioceramics such as Hydroxyapatite (HAp), Tricalcium Phosphate (TCP), bioactive glass and inorganic materials; composites where more than one type of material is used together such as natural and synthetic polymer mixtures and ceramic-polymer mixtures.[64]. These materials can be used according to the mechanical and biological needs of the tissue to be studied.

3D printers can be customized based on the properties of the materials to be used as well as the requirements of the material wanted to be produced. Different printing technologies come with various advantages and disadvantages.

Inkjet bioprinters create three-dimensional structures by spraying bioinks layer by layer onto the targeted surface. In these printers, the bioink is sprayed in small droplets by a piezoelectric or thermal mechanism and these droplets come together to form a structure. The advantages of these printers include high resolution, low cost and the ability to precisely place biomolecules. It also makes it possible to print cells with biomaterials [63].

Extrusion printers extrude mixtures that contain polymers, hydrogels or cells by applying pressure through a needle or nozzle, and this material is deposited layer by layer to form a 3D structure. The flow rate through the nozzle is adjusted according to the viscosity of the material used and parameters such as printing speed. The extrusion method is ideal for scaffold production in tissue engineering by controlling the mechanical properties and pore structures of the biomaterial [63].

Selective Laser System (SLS) technology is based on the principle of sintering powdered biomaterials layer by layer using a laser. This type of printer is ideal for the production of complex internal structures and scaffolds with high mechanical strength. Although it provides high resolution, it is more costly to use because it is laser-assisted [64].

Fused Deposition Modelling (FDM) technology is generally used for prototyping purposes. Thermoplastics such as PLA and PETG are melted and printed layer by layer in specified shapes [61]. This method is especially suitable for structures that require low resolution and mechanical strength [65].

Stereolithography (SLA) Printers create 3D structures by hardening photopolymer resins with UV light. This method is preferred for high-resolution and fine-detailed structures. It is suitable for the polymerization of biomaterials, although it has some limitations in terms of the placement of cells and providing biological functionality [65].

For instance, if a whole osteochondral tissue is to be mimicked, in addition to the cartilage tissue, the cancellous bone, which has a completely different composition and properties, must also be mimicked. For this, either an extrusion printer with multiple extrusion features or the production of each segment separately and combining them using various chemical methods is needed [66, 67].

2.7 Polymers in the Fabrication of Tissue Scaffolds

A polymer mixture of Gelatin and Methylcellulose was chosen to produce tissue scaffolds for 3D printing using a pneumatic extrusion-based printer, aiming to mimic articular cartilage tissue.

2.7.1 Gelatin

Gelatin is a naturally occurring macromolecular protein derived from the partial hydrolysis of collagen found in connective tissues, bones and skin of animals [12]. It contains a high content of amino acids, including glycine, proline and 4-hydroxyproline, and forms a structure favorable for gel formation. It also contains a large amount of carbonyl (C=O), amine (-NH) and hydroxyl (-OH) groups[16, 19].

The strength of the gel depends on the content of proline and hydroxyproline, which increase hydrogen bonding with water and contribute to the triple-helical structure [14, 68, 69].

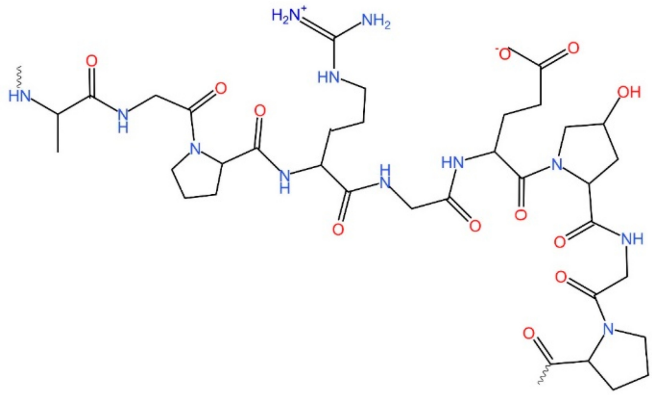

Figure 2.5 Chemical Structure of Gelatin [12].

Gel strength, a critical property for evaluating gelatin, is influenced by factors such as the extraction process, amino acid composition and molecular weight. Higher temperatures during extraction can disrupt the α and β chains of gelatin, weakening its network and reducing gel strength [12, 69]. Furthermore, during extraction, gelatin can be produced as Type A if it is obtained by acidic processing and Type B if it is obtained by alkaline processing, and this type of processing determines the pH range and general properties of the gelatin produced [14].

Despite its favorable properties, gelatin has inherent limitations such as poor mechanical strength and brittleness and is often crosslinked, usually with substances such as EDC-NHS, for greater stability. This approach improves mechanical properties without compromising biocompatibility as EDC is non-toxic and does not remain in the final product. Crosslinking also prevents rapid degradation in aqueous environments, thus enhancing the performance of the scaffold in tissue engineering applications [70, 71].

Gelatin solutions exhibit temperature-dependent behavior, sol at about 40°C and gel upon cooling due to the reformation of collagen triple helices. The heat reversibility of gelatin due to its modest binding energy enables the development of biomedical systems that require responsive material properties. [12, 13, 14].

Gelatin is a natural polymer and is highly biocompatible and promotes cell adhesion and proliferation, making it ideal for tissue engineering applications, including scaffolds for wound healing [19, 71]. Its biodegradability ensures safe use in a variety of medical and non-medical fields [12, 72].

Gelatin is a versatile biopolymer frequently used in tissue engineering studies due to its biocompatibility, biodegradability, thermally reversible structure, easy production, easy processing, low cost and wide accessibility despite its mechanical limitations. For all these reasons, Gelatin was determined as one of the polymers to be used in the study.

2.7.2 Methylcellulose

Methylcellulose (MC) is a water-soluble cellulose derivative obtained by replacing some of the hydroxyl groups (-OH) of cellulose with methoxy groups ($-OCH_3$) through a methylation process. This chemical modification makes methylcellulose a material with an amphiphilic characteristic, capable of interacting with water and exhibiting temperature variable properties; exhibiting thermally-reversible crosslinking behavior [15, 16].

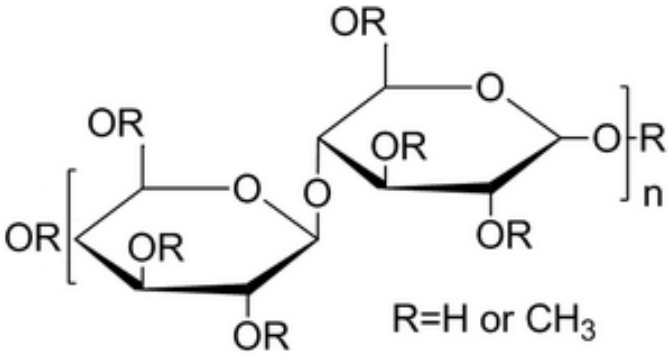

Figure 2.6 Chemical Structure of Methylcellulose [73].

Although MC is widely recognized on paper and less favored in practice, its potential in this area is attracting increasing interest [16]. Unlike proteins, MC form a strong gel at temperatures above 50°C [16]. Methylcellulose undergoes a physical sol-gel transition when it reaches a temperature (30-80°C) known as the low critical solubility temperature (LCST). The gelation process is shaped by hydrogen bonds and intermolecular interactions of hydrophobic groups. DSC (Differential Scanning Calorimetry) analyses at different MC concentrations revealed that MC has a multi-step thermal reversible gelation mechanism. [15, 74]. The chemical structure of methylcellulose presents a unique feature by providing a balance between hydrophilic and hydrophobic regions. Hydrophobic regions in the structure of MC form water cages surrounded by water molecules, and as the temperature increases, these cages are disrupted, hydrophobic interactions between methylcellulose molecules are strengthened and gel structure is formed [15, 75]. Solutions of MC in water form temporary bonds between hydrophobic regions as the temperature increases, which increases the physical strength of the gel. While the viscosity of the solution decreases at low temperatures, the viscosity increases when the gelation temperature is approached. This thermogelation process is reversible and the gel returns to its solution when cooled again [74, 75].

Gelation mechanism which is known to be influenced by intermolecular hydrophobic relationships, is complex and not yet fully understood. Numerous studies have investigated the thermal gelation process, but the application of scaling laws to methylcellulose has not been sufficiently investigated [74]. In addition, there is a significant increase in porosity when the MC content increases in polymer solutions. This may be due to the hydrophilic character of MC, which leads to the formation of a spongy structure between open pores [17].

The ability of MC to transform into a thermoreversible hydrogel in water makes it attractive for use in biomedical fields, particularly in tissue engineering, cell or growth factor transport and drug delivery systems. As the mechanical properties of the gel can be tuned by temperature, it can transition to a solid hydrogel form at body temperature, enabling it to function in the biological environment [15, 17]. Furthermore, MC shows degradation at high temperatures (200°C and above) and this thermal stability makes it ideal for temperature-sensitive applications [74].

Overall, both polymers are considered promising candidates for tissue scaffold materials due to their ease of processing, cost-effectiveness, and their similar mechanical and structural properties.

3. MATERIALS AND METHODS

3.1 Preparation of Hydrogel

The hydrogel solution is prepared with DI water, Sigma-Aldrich M7140 Methylcellulose and Sigma-Aldrich G1890 Porcine Gelatin, by setting Methylcellulose concentration (w/v) to 10%, 12.5% and 15% while setting Gelatin concentration at 20% (w/v). Methylcellulose is added into a beaker on a stirrer which is set 42°C at 1500 rpm, for 15 minutes. The beaker's mouth is sealed with parafilm to prevent evaporation. Then, gelatin is added and stirred for another 15 minutes. Temperature may increase to encourage dissolution. After that, the prepared hydrogel is taken from the beaker with the aid of a syringe to be cast in a Teflon mold and also transferred into the syringe of the 3D printer.

3.2 3D Printing

Scaffolds were fabricated using Axo A1 pneumatic 3D printer, operated through Repetier Host software, which served both as a slicer and a printer control program. Firstly, The scaffold shape was designed as a mesh with dimensions of 11.25 mm x 11.25 mm x 2 mm. The infill density was set at 20%, as native cartilage's porosity is approximately 80% [76]. After various trials, the speed of the nozzle was set to 8mm/s and the temperature was fixed at 42°C, to prevent denaturation of Gelatin [13]. Printing was performed using 21-gauge (0.83 mm) needles. The pressure that pushes the hydrogel through the nozzle was manually adjusted to maintain consistency in the scaffold's shape and flow characteristics.

Table 3.1
Printing Parameters of Tissue Scaffolds.

Concentration	**10 MC**	**12.5 MC**	**15 MC**
Pressure (psi)	8-10	15-20	20-30
Temperature (°C)	42	42	42
Printing Speed (mm/s)	8	8	8
Infill Density (%)	20	20	20

3.3 Crosslinker Preparation and Application

Sigma-Aldrich 03450 N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, also known as EDC, was used as the crosslinker. To prepare a 50mM solution, in 10 mL of 90% ethanol, 0.095g of EDC was dissolved. 1 mL of prepared solution was used to fully immerse each scaffold in 15-well plates. The scaffolds were then incubated in the EDC solution at 4°C for 1 hour, followed by washing with deionized (DI) water.

Figure 3.1 Preliminary Printing of MC-Gelatin Hydrogel.

3.4 Characterization Studies

3.4.1 Printing Accuracy

To assess the printing accuracy of the scaffolds that were produced (n=6), they were photographed in the same frame with a ruler to calculate the dimensions and porosity later ImageJ software. The area of produced scaffolds is calculated as Ai. The theoretical value, represented as A, is calculated by taking the sliced image directly from the slicer program Repetier Host. To calculate the printing accuracy Eq. 3.1 was used [77].

$$\%Printing \quad Accuracy = [1 - \frac{Ai - A}{A}] * 100 \tag{3.1}$$

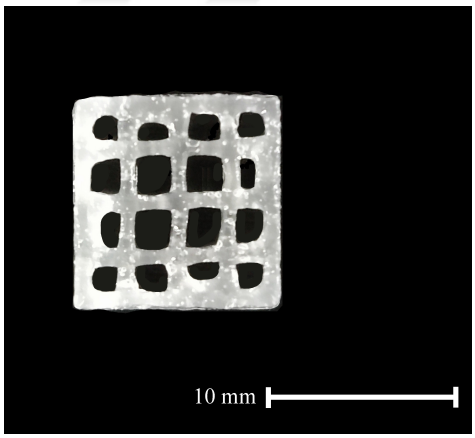

Figure 3.2 A scaffold used for measuring Printing Accuracy.

3.4.2 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

To assess the chemical interactions between Methylcellulose and Gelatin at determined concentrations Thermo Scientific Nicolet 380 FTIR spectrometer was used (n = 3). The spectrum of the samples was recorded between 4000-400 cm^{-1} at room temperature. FTIR analysis was conducted at the Chemistry Department of Boğaziçi University. The results were assessed and arranged in OMNIC software. The data obtained from the software were processed and visualized in Microsoft Excel.

3.4.3 Mechanical Evaluation - Compression Test

Methylcellulose-Gelatin based scaffolds containing 10%, 12.5%, and 15% MC respectively, were prepared with 5 samples per set (n=5). The tests were performed at Boğaziçi University Biomedical Engineering Institute, where the specimens were tested on Universal Test Machine with Nexygen Plus software. Each sample was loaded starting from 2N until they completely torn apart. The data obtained from the software were processed in Microsoft Excel and Stress-Strain graphs and Compressive modulus values at each concentration were calculated.

3.4.4 Contact Angle Measurement

Contact angles of Methylcellulose-Gelatin blend hydrogel in a bulk form which were cast in Teflon molds, were measured by KSV Instruments LTD CAM101 at room temperature at Boğaziçi University Chemistry Laboratory. Samples were photographed every second for 15 seconds. Three samples were tested for each

study group (n=3). The photos were processed using the Drop Analysis plugin installed on ImageJ to calculate the contact angles. In the 15-second measurement period, it was decided to make a comparison based on the photos at the 6th second.

3.4.5 Swelling and Degradation Studies

To understand the characteristics of the scaffolds better, swelling-degradation studies were conducted. 3 samples were prepared for each MC concentration (n=3) and placed into 24-well plates.

The weight of each scaffold was recorded both before and after it was dried for 24 hours at 37°C. Following that, 1.5 ml of PBS solution was added to each well for the experiment, and they were incubated for 1, 4, 7, 10, and 14 days. PBS solution changed every other day.

After the specified incubation time, the samples were placed on tissue paper and dried on both sides before their final weight was measured as Mf. Using Mi and Mw, where Mi represents the initial dry mass, Mf the final dry mass, and Mw the wet weight, the %Water Uptake was calculated according to Eq. 3.2 [78].

$$\%Water\ \ Uptake = 100 * \frac{Mw - Mi}{Mi} \tag{3.2}$$

In parallel with the swelling tests, degradation tests were also conducted. To determine the degradation by calculating %Weight Loss, Eq. 3.3 was used [78].

$$\%Weight\ \ Loss = 100 * \frac{Mi - Mf}{Mi} \tag{3.3}$$

3.4.6 pH Study

Along with degradation and swelling tests, pH measurement was also performed. pH measurements of the scaffolds(n=3) which were again immersed in PBS solution each time before changing the PBS solution until they were completely dissolved in PBS. Measurements taken by Thermo Scientific-Orion Star A211 pH meter.

3.4.7 Statistical Analysis

Statistical evaluation of the results was done by One-Way ANOVA with Tukey's Post-Hoc Test through IBM SPSS (Statistical Package for the Social Sciences, version 28.0.1) software. One-Way ANOVA allows comparing the means of more than one group at the same time, while Tukey's Post-Hoc test allows pairwise comparisons between groups to determine which groups have statistically significant differences. In the results, P value is less than 0.05 ($p \leq 0.05$) was considered statistically significant.

4. RESULTS

4.1 Printing Accuracy

Dimensional changes occurred in tissue scaffolds printed with a pneumatic 3D printer depending on the methylcellulose concentration. The printing accuracy was calculated according to the amount of deviation from the theoretical value and resulted as 10MC 92.33% (±0.61), 12.5MC 90.09% (±1.62), 15MC 89.55% (±4.61) and visualized in Figure 4.1. According to One-Way ANOVA results, no significant difference was found between the groups (F= 1.411, p = 0.232). In addition to that, in the pairwise comparisons of groups conducted using Tukey's Post-Hoc Test, no significant differences were found too.

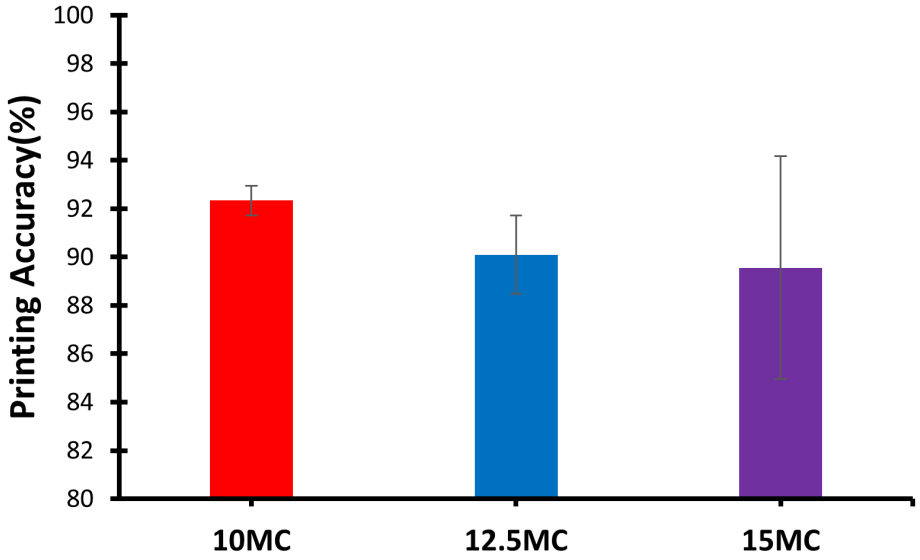

Figure 4.1 Printing Accuracy of Scaffolds.

4.2 Fourier Transform Infrared Spectroscopy Analysis

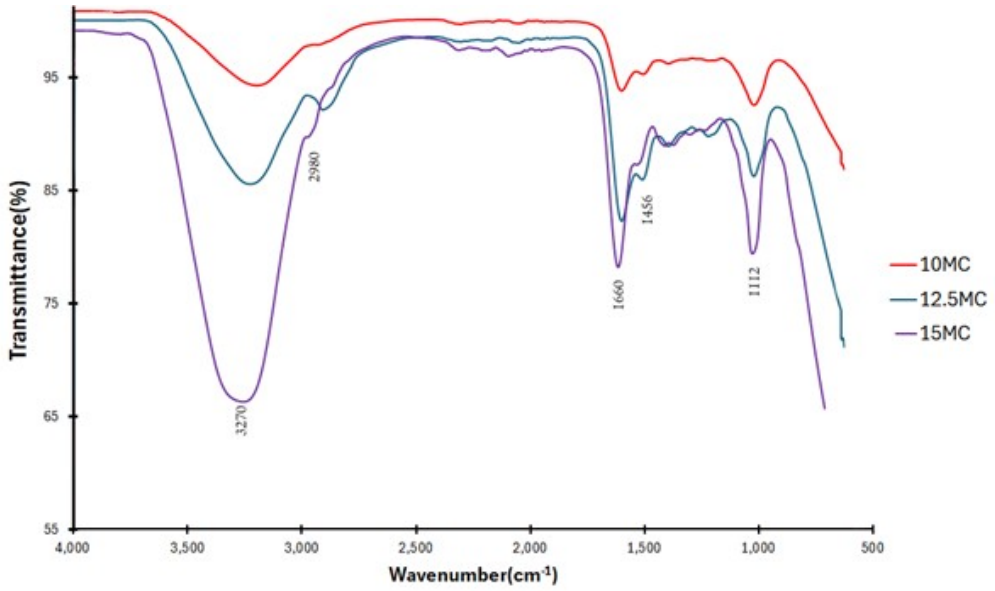

Figure 4.2 FTIR spectrum of Methylcellulose - Gelatin scaffolds.

According to the results, there is a peak between 3100-3300 cm^{-1} band which indicates a -OH stretching in Gelatin-Methylcellulose scaffolds. Also, it may referred to as -NH stretching vibrations. Both groups are commonly found in gelatin and methylcellulose [17, 79, 80].

The peaks around 2980 cm^{-1} attributed to the asymmetric and symmetric stretching vibration of C-H respectively, primarily from the methyl (-CH_3) and methoxy (-OCH_3) groups [17]. The spectra also illustrate absorption bands around 1600 cm^{-1} which correspond to the amide I band, mainly due to C=O stretching vibrations in gelatin [79, 80].

Beyond the functional region, in the fingerprint region:

Peaks around 1456 cm^{-1} this band is associated with (-CH) bending vibrations, reflecting the methyl and methoxy groups in methylcellulose. Also, another peak can be seen between the 1050-1150 cm^{-1} band, which is likely related to C-O-C stretching from the ether linkages, which can be attributed to the polysaccharide backbone of methylcellulose. The peaks beyond 1000 cm^{-1} are related to methoxy (-OCH_3) groups, which are functional groups consisting of a methyl group (-CH_3) attached to an oxygen atom [17, 79]. The region between also 1,000 and 1500 cm^{-1} shows bending and stretching vibrations of various bonds such as C-H, C-O, C-N, and ether bonds [80, 81].

The amount of absorption is almost always changed by the MC concentration. As the MC concentration increases, the amount of absorption increases as well.

4.3 Mechanical Evaluation - Compression Test

Compression tests were conducted to understand the mechanical properties of the scaffolds and to assess their similarity with native tissue.

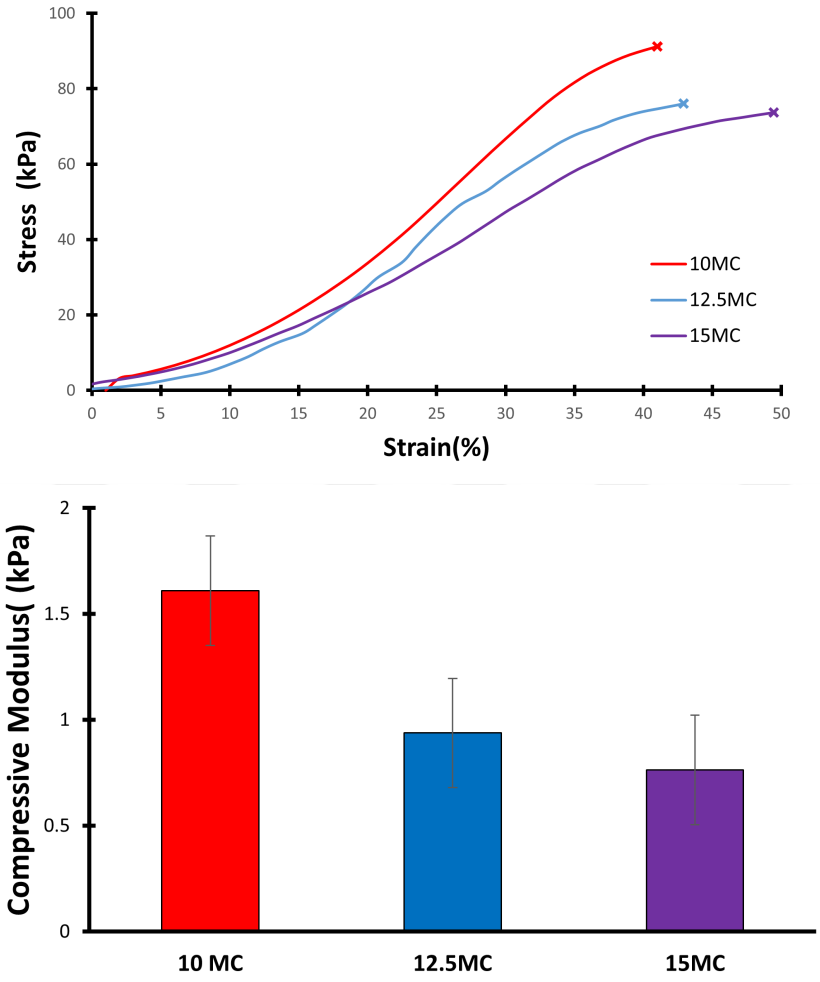

Figure 4.3 Stress-Strain values (above) and Compressive Modulus (below) of scaffolds.

Stress-Strain values are obtained by compression tests on Universal Test Machine, by these results each composition's compressive modulus were calculated. The compressive modulus is calculated on the ratio of the stress values to the strain % values [82]. According to the Compressive Modulus values of scaffolds, 10 MC (1.609 ± 0.264 kPa) is significantly higher than 12.5 MC (0.915 ± 0.537 kPa) and 15 MC (0.748 ± 0.150 kPa).

The One-Way ANOVA results revealed a statistically significant difference between groups ($F = 58.27$, $p \leq 0.001$). However, with regards to the pairwise comparisons of groups conducted using Tukey's Post-Hoc Test, there's a significant difference ($p < 0.001$) between 10MC and other groups, yet the difference between 12.5MC and 15MC is not significant($p = 0.107$). It can be concluded that, concentration has a critical role in the differences between compressive modulus according to these results.

Also, during the compression tests, it was qualitatively observed that the samples that were torn apart, crystallized and changed color.

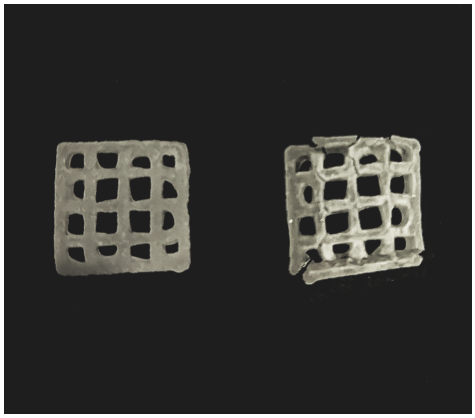

Figure 4.4 Normal vs. Color-Changed Scaffold due to Compression.

4.4 Contact Angle Measurement

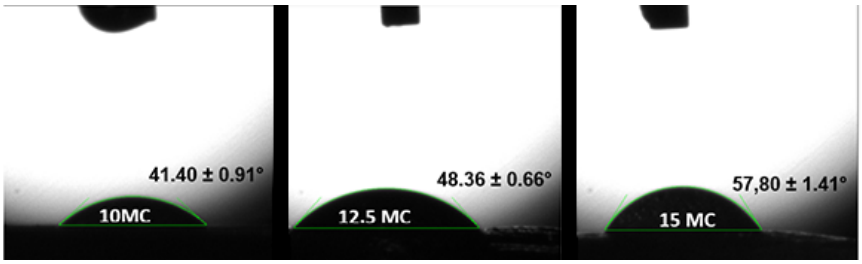

Figure 4.5 Images taken during Contact Angle Measurement.

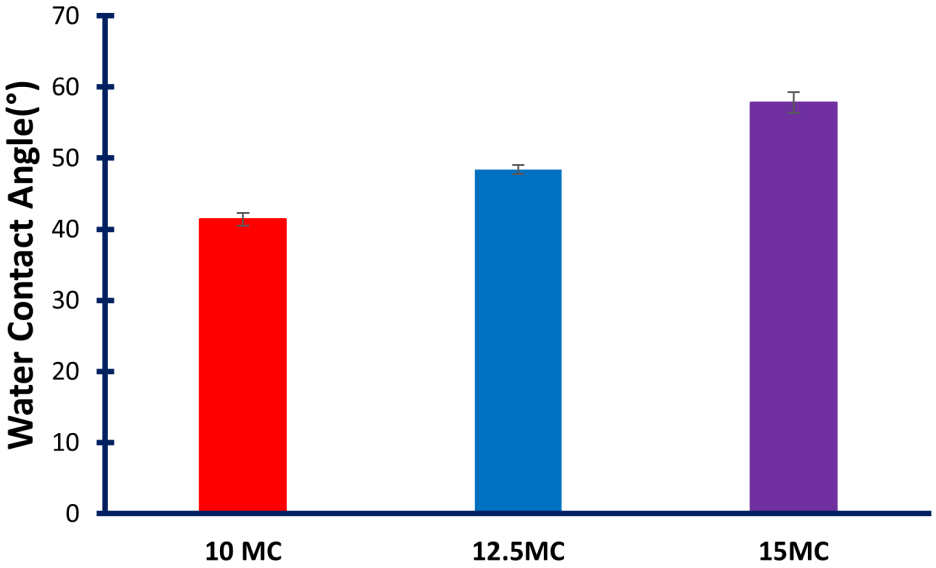

Figure 4.6 Water Contact Angle of samples containing different Methylcellulose concentrations.

The effect of concentration on the contact angle in the samples is shown in Figure 4.6. 15MC (57.80 ±1.41) shows a greater contact angle value than 12.5MC (48.36 ±0.66) and 10MC (41.40 ±0.91). As a result of One-Way ANOVA, it was observed that there was a significant difference between the groups (F = 186,441, $p < 0.001$). In the pairwise comparisons done by Tukey's Post-Hoc Test, the differences between the groups were statistically significant ($p < 0.001$).

4.5 Swelling & Degradation Study

Swelling and Degradation characteristics of the scaffolds were observed by the study which was continued for 14 days by keeping the scaffolds in PBS solution at 37°C, and their results were visualized in Figure 4.7 and 4.8

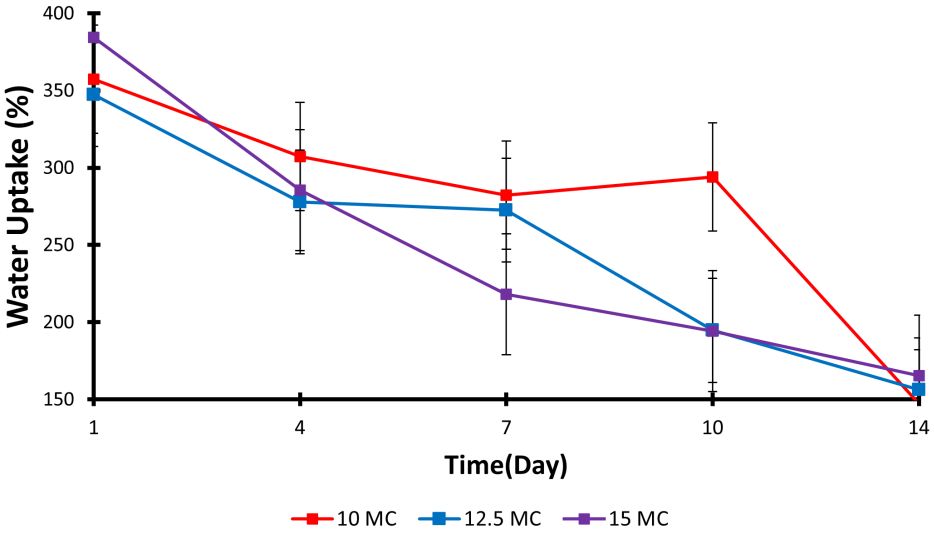

Figure 4.7 Swelling results of scaffolds after 1, 4, 7, 10 and 14 days of incubation in PBS.

According to the One-Way ANOVA performed on the results of the swelling tests, no significant difference was found. In addition, Tukey's Post Hoc Test applied as a pairwise comparison has showed that there was no significant difference between the groups. It was observed that the samples were able to absorb water up to almost 4 times their weight, and a decrease in this ratio was observed, although not linearly over time. A steady decrease can only be observed in the 15MC group.

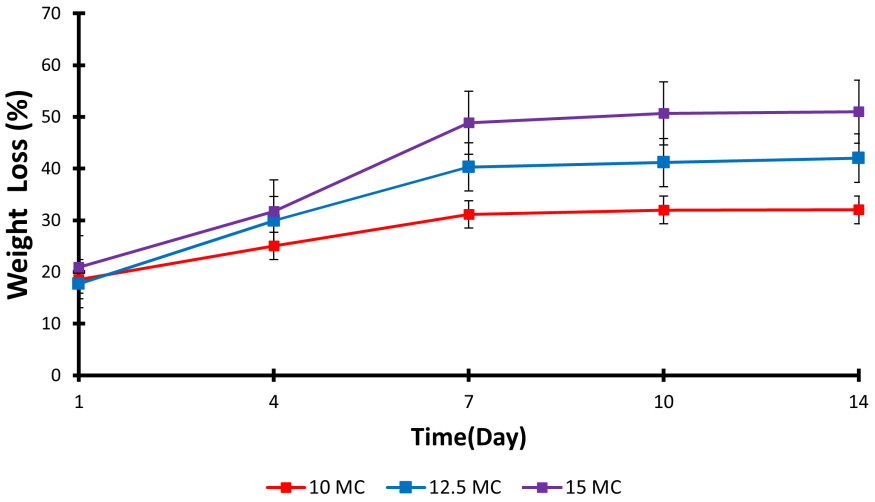

Figure 4.8 Degradation rate of scaffolds after 1, 4, 7, 10 and 14 days of incubation in PBS.

According to the graph, weight loss was correspondingly higher in groups with higher methylcellulose concentrations. However, the One-Way ANOVA performed on the results of the degradation tests, no significant difference was found ($p = 0.187$). Similarly, there was no significant difference between the groups according to the results of multiple comparisons made with the Tukey's Post Hoc Test method.

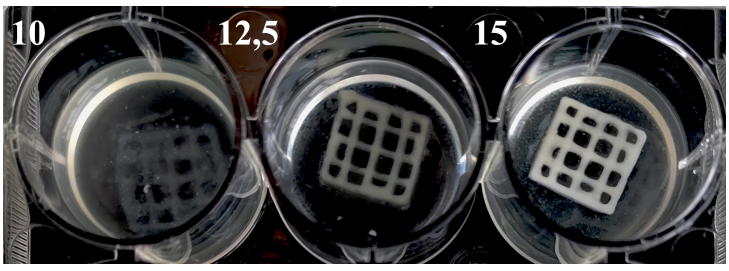

Figure 4.9 Degradation Process of Scaffolds at the Day 32.

4.6 pH Studies

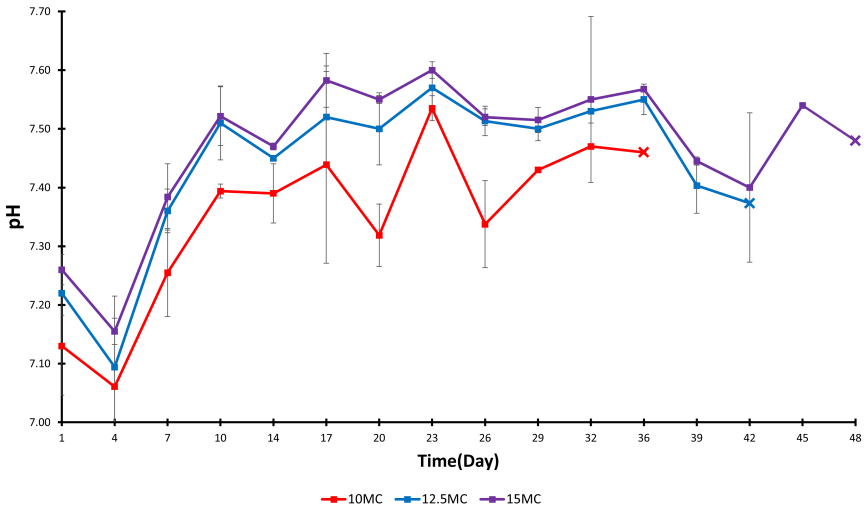

Figure 4.10 pH measurement of scaffolds immersed in PBS until dissolved.

Changes in the PBS solution which is scaffolds immersed, are demonstrated in Figure 4.10. After the first day of immersion, pH tends to decrease by around 2%. After the fourth day, it increased in each group and a fluctuating graph was formed but remained above the initial values. On day 36, the 10MC group was completely dissolved in PBS, followed by 12.5MC on day 42 and 15MC on day 48. The mean values of groups were calculated as 10 MC 7.35 $\pm$0.14, 12.5MC 7.43 $\pm$0.136, 15MC 7.47 $\pm$0.12.

According to the One-Way ANOVA output, no significant difference was observed between the groups ($p = 0.058$). Additionally, Tukey's Post Hoc Test revealed no significant differences in the pairwise comparisons between the groups.

5. DISCUSSION

5.1 Printing Accuracy

Printing Accuracy is a method that is used to measure the efficiency of a print which is done by a 3D printer. Adjusting the pore size is important to optimize cell infiltration [77]. According to the calculations on different Methylcellulose concentrations, an inverse correlation was observed between Methylcellulose concentration and printing accuracy. However, according to statistical analysis, these differences were not significant, they were not substantial enough to create statistically distinguishable differences.

When the standard deviations of the groups were analyzed, it was seen that as MC concentration increased, the variability in printing increased as well. Accordingly, although a high MC concentration provides advantages in many aspects, it is prone to creating problems in printing efficiency and accuracy.

According to the results obtained here, the parameters used during the printing of the scaffolds need to be optimized. In addition to parameters such as printing speed, temperature, pressure, nozzle width, and ambient temperature; measuring and adjusting the viscosity of the prepared mixture will also be advantageous in obtaining a more optimal printing accuracy.

5.2 Fourier Transform Infrared Spectroscopy Analysis

Fourier transform infrared (FTIR) spectroscopy is one of the most used analytical techniques. It can be employed to characterize samples in various forms, including liquids, solutions, pastes, powders, films, fibers, and gases. This analysis allows for the simultaneous determination of organic components, including chemical bonds and organic content [81].

Since -OH groups and -NH groups between 3100 - 3330 cm^{-1} form hydrogen bonds which enhance intermolecular interactions. These bonds also increase the attraction between polymer chains. This contributes to scaffolds maintaining their structural integrity under compression and it also can contribute to slowing down their degradation. Also, -OH groups are known to increase hydrophilicity. As hydrophilicity increases, cell adhesion and biocompatibility are also known to increase [83].

Although C-H bonds around 2980 cm^{-1} and 1450 cm^{-1} are less directly involved in hydrogen bonding, they affect the overall molecular packing and hydrophobic interactions in the polymer matrix. Stronger packing can slightly increase the compressive strength and stiffness of the scaffold by reducing free volume and limiting chain mobility. Methyl groups are associated with increase flexibility [84]. This peak remains relatively constant between MC groups, indicating minimal effect on mechanical properties due to changes in C-H content.

The amide band observed around 1660 cm^{-1} is usually associated with the presence of protein. These groups are found in gelatin, especially in amino acid residues that form the protein structure. This may be due to the presence of C=O

(carbonyl) stretching that may correspond to gelatin in the scaffold [79]. The presence of amide groups is very important for the evaluation of the biocompatibility and degradation of the material. It increases the interaction of the scaffold with cells and promotes cell attachment and proliferation which is very important for the scaffold in tissue regeneration [85]. Also, due to the presence of peptide bonds in gelatin helps to maintain its structural framework. These bonds also promote hydrogen bonding with surrounding groups, increasing the material's ability to withstand stress and deformation. The peaks may indicate changes in the structural conformation of gelatin or its interaction with methylcellulose due to the processes in the production or post-production of the scaffolds. Higher concentrations of methylcellulose may have disrupted some of the natural hydrogen bonds of gelatin, potentially altering the mechanical flexibility or stiffness of the scaffold.

The C-O-C stretching observed in the range 1050 - 1150 cm^{-1} represents the ether bonds in methylcellulose and indicates the structural integrity of the methylcellulose component. Ether bonds in methylcellulose can also interact with water, although not as strongly as hydroxyl groups. Ether bonds impart some flexibility to the polymer chains and may reduce overall stiffness compared to a fully hydrogen bonded network. However, these bonds help maintain flexibility in the scaffold. As the concentration of methylcellulose increases, the amount of ether bond increases, which can also affect the structural properties of the scaffolds and could also impact its degradation and water absorption behavior[86].

As methylcellulose concentration increases, the -OH groups that increase with the concentration, increase hydrogen bonding which is potentially making the scaffold stiffer and less elastic. However, more ether bonds provide flexibility that can counteract some stiffness and provide a stable, flexible scaffold with mod-

erate strength. In the case where methylcellulose concentration is low, it results in fewer ether linkages and hydroxyl groups and leads to a stiffer structure, probably due to the peptide network of gelatin. This may result in greater hardness but lower elasticity, as supported by the results of mechanical tests. In summary, hydroxyl (-OH) and amide groups contribute most to scaffold strength and stiffness through hydrogen bonding, while ether (C-O-C) linkages contribute to flexibility and elasticity. Varying the concentration of methylcellulose allows for the adjustment of the balance between these properties, enabling the scaffold to be optimized for specific mechanical requirements in tissue engineering applications.

5.3 Mechanical Evaluation - Compression Test

In tissue engineering for articular cartilage, aligning the mechanical properties of tissue scaffolds with natural cartilage is critical. As the stress-strain graph visualized in Figure 4.3 is examined, scaffolds exhibit a character similar to the native articular tissue. It can be inferred that all of the samples have properties such as low stiffness, high elongation and high elasticity [28].

Human articular cartilage has a compressive modulus ranging from 240 to 1000 kPa [30, 31]. However, one of the major limitations of using natural polymers in hydrogels is their reduced mechanical integrity [31]. Compared to real human articular cartilage, scaffolds have a compressive modulus range of 150 to 1300 times less. At this point, it can be concluded that the prepared tissue scaffolds are far from mimicking the native tissue mechanically. In terms of compressive modulus, it was observed that the 10MC group exhibited a higher value; closer to the native tissue. Accordingly, it can be concluded that scaffolds become less stiff (even more

than desired) as methylcellulose concentration increases.

As a result, it would be a better choice to prefer the group containing 10% MC when proceeding to the application phase. However, it may be preferable to add various ingredients to the scaffolds or change the geometric structure to mimic the native tissue more closely.

Quantitatively observed phenomena the color change of the scaffolds that are torn under compression, similar to a broken glass losing its transparency. According to Metelli, irregular reflection and scattering of light due to irregularities on their surfaces and distortions in their internal structures. This situation prevents the light from traveling homogeneously in the material and creates an opaque appearance [87]. Furthermore, the addition of EDC to scaffolds increases the covalent bonds, and hence the stiffness, while at the same time altering the surface roughness and water retention capacity [72]. In this context, the mechanical hardening and roughness increase observed in the polymers after crosslinking may lead to changes in light scattering during compression, which may cause the scaffolds to appear more opaque or white.

5.4 Contact Angle Measurement

Contact angle measurement gives an idea about the surface energy of a sample. Surfaces with high surface energy have a low contact angle and retain liquids better, in other words, as the contact angle value decreases, the hydrophilic character of the measured samples increases[87]. A low contact angle is of great importance in cell studies, for better adhesion of cells to the scaffold surface. It

is possible to improve biocompatibility by optimizing the contact angle of surface coatings [89].

Contact angle measurements were performed on cartilage samples to study how surface properties, particularly wettability, influence lubrication. It was found that the contact angle decreased significantly when the cartilage lost its phospholipid layer. This change in the contact angle is a strong indicator of the cartilage's ability to maintain lubrication [90, 91].

The surface of different materials is characterized by their wettability, which can be classified as non-wettable (highly hydrophobic ($\theta \sim$ 90-180°)) or wettable (highly hydrophilic ($\theta \sim$ 0-45°)). In healthy cartilage, the contact angle is typically around 103°. However, this angle can drop below 70°or even to 60°in degenerated cartilage, when cartilage tissue is in contact with dry air. In synovial fluid, it can possess superhydrophilic characteristic ($\theta \sim 0°$) [92]. Articular cartilage's ability to transition between hydrophilic and hydrophobic states is a critical adaptation for maintaining joint health. It allows the cartilage to function efficiently in wet environments while also responding to changes in hydration and environmental conditions [89, 91]. The degradation of this balance, such as in osteoarthritis, compromises its function, leading to increased friction and joint wear [6].

The results showed a correlation between methylcellulose concentration and contact angle. As long as MC concentration increases the contact angle also increases, so the surface energy gets decreased, and the wetting ratio as well, and so the material bears hydrophobic characteristics. This is similar to the characteristics of cartilage tissue in contact with air[92].

However, natural articular cartilage has a transitional character, and can be hydrophobic or hydrophilic depending on its condition, and the scaffolds that are produced have managed to mimic this tissue. On the other hand, tissue scaffolds main role is to induce cell proliferation in the tissue in which they are implanted and to perform a repair and healing activity. The hydrophilicity of the scaffold material plays a critical role in supporting chondrocyte attachment, proliferation, and overall functionality. For this reason, it would be more appropriate to choose the most hydrophilic one from the measured groups.

According to the results, the 15MC group has the highest contact angle value and it is closest to the native tissue in similar measurement conditions, its character closer to hydrophobic. Due to the excess of hydroxyl (-OH) groups in the FTIR results, the 15MC group is expected to be more hydrophilic. Likewise, in the Water Uptake experiment, the 15 MC group exhibited a higher water uptake in the first 24 hours, it also indicating a more hydrophilic character. This can be attributed to the amphiphilic characteristics of Methylcellulose. The increase in hydrophobic character is due to the higher concentration of methyl groups in the structure when the concentration of methylcellulose increases. In addition to this, it also has the highest variability. Therefore, it would not be a good choice for the application. The 10 MC concentration exhibited the lowest contact angle, indicating a hydrophilic surface with less variability. This property is advantageous for tissue engineering applications where nutrient diffusion and cell attachment have an importance.

5.5 Swelling & Degradation Study

PBS solution mimics the physiological conditions of the human body and therefore both swelling and degradation assays are studied under these conditions [93].

Swelling is a very important property for biomaterials; it helps in the absorption of nutrients and growth factors and promotes new tissue formation [94, 95]. Similar to natural cartilage tissue, scaffolds require high swelling behavior or water uptake. A low water uptake can inhibit nutrient diffusion, while a high-water uptake can affect the integrity of the biomaterial over time [95]. Scaffolds with swelling behavior resist compressive forces in cartilage as well as supporting nutrient transport. Therefore, designing scaffolds suitable for cartilage tissue requires a porous geometry containing hydrophilic groups in their structure.

Water uptake is higher in porous structures, in contrast, the bonds formed using crosslinkers make the structure denser and as the structure density increases, the swelling rate of the hydrogel decreases because the network becomes tighter and the water retention capacity decreases [95]. This bonding also increases hydrophobicity and according to Arıcı 's study showed that the most hydrophobic working group also had the lowest degree of swelling [96].

A hydrogel network placed in water absorbs water and swells, this causes the polymer chains within the structure to stretch and tend to dissolve by the effect of hydration or osmotic pressure driving force. This causes to polymer chains lose their elasticity, which initally gives them their swelling property.Therefore, the swelling capacity decreases over time.[97]. In case of excessive swelling, hydrogel

scaffolds may exhibit enlarged dimensions that do not fit the defect site. Therefore, they need to have appropriate swelling behaviors that match the defect site [95].

According to the swelling results,

It is possible to observe the effects of hydrophobicity caused by methyl groups when the methylcellulose concentration increases. Due to the hydrophobic character induced by these groups, it is seen that water uptake decreases as the concentration of methyl cellulose increases. Although there is no statistically significant difference between the groups. 10MC and 12.5MC groups would not be a good choice as they are too unstable for practical use. 15MC group may be more suitable for long-term applications as they are able to absorb water quickly and then retain it and showing a more balanced and controlled decrease. Working with this concentration of methylcellulose has the potential to both attract high amount of water initially, then retain it better over time.As spotted on the study, decreasing water uptake over time may indicate that the scaffold structure is beginning to deteriorate or dissolve. Also, scaffolds may gradually begin to dissolve or become structurally brittle as they absorb water. This deterioration can lead to a reduced water absorption capacity. It can be concluded that presence of more gelling agents such as MC causes more water retention. In long-term experiments, it is likely that some of the liquid into which the scaffold is immersed will be lost through evaporation or diffusion [98].

Degradation rate is crucial for the biomaterials as it regulates new tissue formation and breakdown of the scaffold. During tissue regeneration, degradation of the scaffold can trigger the environment to the reconstruction of tissue by changing ion concentrations [99].

Degradation of scaffolds induces by water absorption which leads to chain scission [95]. As water permeates the polymer structure, it induces swelling, which initiates the formation of oligomers and monomers. When these smaller molecules diffuse out of the scaffold, the weight decreases as well [100]. Degradation is also related with the functional groups surrounded by water molecules. Therefore the number of functional groups playing a significant role to determine the degradation rate [95].

Functional groups, such as carbonate and ester groups, are susceptible to attack by water molecules. In addition to these groups, various factors affect the degradation rate of polymers, including pH, copolymer composition, types of bonds within the biopolymer structure and water absorption. Environmental factors also have a major impact on the degradation process. Besides temperature and pressure, pH is one of the most important factors [100].

Chemical and physical changes accompany the degradation of biodegradable polymers, closely linked to the substrate concentration. Additionally, material composition is a crucial factor in determining both the hydrophilicity and degradation rate. Besides composition, A high surface area/volume ratio is also known as another value that increases the degradation rate. Also, with covalent bonds established by crosslinking, it is known that the degradation process can be modified [100].

The results showed a correlation between MC concentration and weight loss. It is known that as MC concentration increases, hydrophilicity increases depending on the amount of hydroxyl groups [15]. However, as the MC concentration increases, methyl groups become dominant, while the effect of hydroxyl groups

decreases. Therefore, as the MC concentration increases, the hydrophobicity of the scaffold becomes more evident. As the hydrophobic character increases, the water uptake of the scaffold decreases and this affects the degradation rate [95]. This can be explained by the amphiphilic nature of methylcellulose [15]. Initially, the hydrophilic groups in the structure attracted a high amount of water from the environment, which would typically lead to faster degradation. However, the hydrophobic character introduced by the effect of methyl groups may have slowed down the degradation by reducing water uptake, thus allowing the scaffolds to remain stable for a longer period of time, even though they lose more weight.

Besides, the 10MC group gave more consistent results with a lower variability value compared to the other groups. Therefore, it may be a better choice to prefer the 10MC group.

5.6 pH Study

pH is an extremely important parameter for biocompatibility [99]. pH control is critical to control the rate of degradation. By adjusting the pH, the biodegradation rate of the scaffold can be controlled by affecting ionic bonding and crosslinking, thus ensuring chemical stability. However, the importance of pH control is not limited to degradation. It is also an important parameter for tissue repair and structure formation [100]. It is possible to alter the surface charge by pH changes, which in turn can affect hydrophilicity and subsequent cell adhesion [29]. In general, neutral or slightly basic pH levels (7.2-7.4) provide an optimal environment for cell adhesion and proliferation [101].

Collagen II constitutes 90-95% of articular cartilage tissue [25]. Electrostatic and hydrophobic interactions drive collagen fibril formation. Ionization and electrostatic interactions between amino acids are regulated by factors such as pH [101]. The optimal pH value for forming organized collagen fibrils is 7; at a lower or higher pH, collagen usually transforms into globular or disordered structures. In addition to environmental factors such as pH and temperature, its concentration also has an effect on collagen formation [95].

During the study, the pH changes in the groups were observed between 7 - 7.60, and the averages were calculated between 7.35 - 7.47. This result is very close to mimicking the native tissue environment, as the pH was set at 7.4 in studies with real human articular cartilage environment [29, 92].

According to the results, as the methylcellulose concentration increased, the pH measured during the experiment also increased. This increase in pH can be attributed to the byproducts of the degraded scaffolds. This may also, explain the fluctiatons in Figure 4.10. 12.5MC group is the closest to the pH of native tissue. It can be concluded that all samples exhibit similar properties since the differences are not significant and very close.

6. CONCLUSION

In this study, Methylcellulose-Gelatin-based tissue scaffolds were produced using different Methylcellulose concentrations. As a result of the characterization studies performed with 10%, 12.5% and 15% methylcellulose concentrations, while keeping the gelatin ratio constant at 20%, the suitability of the produced scaffolds for the repair of cartilage tissue defects and which group would be a better candidate was evaluated.

Scaffolds that were produced demonstrate a similar property to real human articular cartilage. However, their mechanical properties are much lower than the native tissue. Adding new polymers, carbon nanotubes, graphene oxide, or changing the infill rate or scaffold architecture to increase the mechanical properties may be considerable, which can enhance the strength, stiffness, toughness, also swelling behavior, and water uptake [95]. After comparisons between groups, it can be concluded that the 10MC group demonstrates a better mechanical performance, making it more suitable for native cartilage tissue. According to CAM results, it has more hydrophilic character which enhances its potential for cell adhesion and growth. However, since swelling and degradation experiments may vary depending on the intended use of the scaffold, these studies alone do not provide sufficient insights without accompanying cell studies. Although the results of studies such as pH and swelling did not show a significant difference, here, as in most of the analyses, the lower variability of the results also favors the 10MC group.

Cell studies are planned as part of future research, which will ultimately determine the usability of the produced scaffolds as an alternative treatment method.

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