MELATONIN LOADED CHITOSAN BASED HYDROGELS FOR CARTILAGE REGENERATION

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ABSTRACT

MELATONIN LOADED CHITOSAN BASED HYDROGELS FOR CARTILAGE REGENERATION

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Degeneration in the intervertebral discs (IVD) is a common worldwide health problem that is observed in most populations, especially in older age groups. It occurs as loss of disc height which causes severe pain and neurological disorders thus decreasing the quality of life. Tissue Engineering (TE) offers innovative and promising tools to restore, maintain, or improve the regeneration of intervertebral discs.

In this thesis, an injectable chitosan (CS) /oxidized-maltodextrin (ox-MD) hydrogel reinforced with oxidized microcrystalline cellulose (ox-MCCs) was developed with the ultimate aim of treatment of degenerated intervertebral discs. Melatonin (MEL) which helps to protect the structural integrity of IVD structure was also incorporated into the hydrogels. Different final concentrations of CS (1.5%, 2%, 2.5%), and ox. MD (0.5%, 0.25%, 0.125%) in the hydrogel compositions were investigated in terms of gelation time, degradation, injectability, and water uptake. Considering these properties 2%CS-0.5%ox.MD hydrogel was chosen for MCC incorporation and melatonin loading experiments. Different amounts of ox-MCC (0.5%, 0.25%, 0.125%) were incorporated into these hydrogels. MCC addition significantly decreased the gelling time (33 to 12 seconds) in proportion to the increase in MCC.

Besides that at the highest MCC-containing composition hydrogels could retain their weight up to 70-75% compared to 40% without MCC. Additionally, increasing ox-MCC content increased the Young Moduli of the hydrogels (the highest MCC content improved the Young Modulus by approximately 6 times). Melatonin release from the hydrogels with and without ox-MCC reached 35.29 % ± 2.3 and 26.65 % ±3.7, respectively at the end of 3 days. Cell culture studies conducted with nucleus pulpous (NP) cells isolated from bovine vertebral tissue showed that hydrogel groups had no toxic effects on cells and they supported proliferation at days 3 and 7 in melatonin-loaded groups. Therefore, melatonin-loaded (CS%2-ox.MD%0.5-ox.MCC%0.5) hydrogels may hold promise for use in the regeneration of IVD.

Keywords: Injectable Hydrogels, Cartilage Tissue Engineering, Intervertebral Disc (IVD) Degeneration, Melatonin, Chitosan

ÖZ

KIKIRDAK DOKU YENİLENMESİ İÇİN MELATONİN YÜKLÜ CHİTOSAN TEMELLİ HİDROJEL YAPIMI

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İntervertebral disklerde bozulmalar, dünya çapında özellikle ilerleyen yaşla yaygın görülen bir problemdir. Disk boyutunun azalmasıyla görülür, ciddi ağrılara ve sinirsel hastalıklara neden olarak hayat kalitesinin düşmesine sebebiyet verir. Doku mühendisliği uygulamaları; intervertebral disklerin fonksiyonlarının devam etmesini, yenilenmesini, veya yeniden kazanılmasını sağlayan yenilikçi ve ümit verici araçlar sunar.

Bu çalışmada, hasarlı intervertebral disklerin tedavisinde kullanma nihai amacıyla enjekte edilebilir chitosan (CS) ve oxide edilmiş maltodextrinden (ox-MD) oluşan, oxide microcrystalline parçacıkları (ox-MCC) ile güçlendirilmiş yeni bir hidrojel scaffoldu geliştirmek amaçlanmıştır. Ayrıca, IVD yapısal bütünlüğünü korumaya yardımcı olan Melatonin (MEL), hidrojel yapısına dahil edildi. Değişik son konsantrasyonlarda CS (%1.5, %2, %2.5), ve ox. MD (%0.5, %0.25, %0.125) hidrojellerde jelleşme süresi, degradasyon, enjekte edilebilirlik ve su tutma gibi yönlerden incelendi. Bu karakteristik özellikler göz önüne alınarak %2 CS-%0.5 ox. MD konsantrasyonu, MCC katılması ve melatonin yüklenmesi deneyleri için seçildi. Farklı oranlarda ox-MCC (%0.5, %0.25, %0.125) hidrojele eklendi. MCC eklenmesi, eklenen miktarla orantılı olarak jelleşme süresini önemli ölçüde düşürdü

(33-12 saniye). En yüksek MCC içeren hidrojel komposizyonunun, MCC içermeyen grubun yaklaşık %40'lık oranıyla karşılaştırıldığında, kuru ağırlığını %70-75'e kadar koruma imkanı oldu. Ayrıca, ox-MCC oranını arttırmak hidrojellerin Young Moduli değerlerini de yüksellti (en yüksek MCC konsantrasyonu, Young Moduli değerlerini yaklaşık olarak 6 kat artırarak iyileşme sağladı). Ox.MCC bulunan ve bulunmayan hidrojellerden 3 gün sonunda salınan MEL miktarı sırasıyla %35.29± 2.3 ve %26.65±3.7'a ulaştı. Sığır kuyruğu vertebral dokudan izole edilmiş nucleus pulpous (NP) hücreleriyle yürütülen hücre kültürü testleri, hidrojellerin toksik bir etkisi olmadığını ve melatonin yüklü gruplarda 3. ve 7. deney günlerinde hücre çoğalmasını destekliklerini gösterdi. Bulgular sonucunda melatonin yüklenmiş (CS%2-ox.MD%0.5-ox.MCC%0.5) hidrojellerin, IVD yenilenmesinde ümit vaad eden bir araç olarak kullanabileceği görüldü.

Anahtar Kelimeler: Enjekte Edilebilir Hidrojeller, Kıkırdak Doku Mühendisliği, İntervertebral Disk (İVD) Bozukluğu, Melatonin, Kitosan

To the greatest joy of my life, my dearest niece Almila, who has given me cherished moments and has always been a light in the darkest times. I hope it will also serve as a shining and exemplary guide in your life journey.

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INTRODUCTION

1.1. Cartilage Tissue

Cartilage tissue possesses a high level of organization of its special components that makes it structurally and functionally unique with specific biomechanical properties (Armiento et al., 2018). It is a highly hydrated, aneural, avascular tissue. This connective tissue is present in many parts of the body, including ribs, trachea, synovial joints (articular cartilage), nose, the external ear (auricular cartilage), and the intervertebral disc (annulus fibrosis and nucleus pulposus) (Figure 1.1). Specialized cells in this tissue are chondrocytes.

Cartilage tissue has a variety of functions in the human body depending on its type and place. These include structural support, form retention, and shock absorption (Chiu et al., 2016). It is classified into three forms (hyaline, elastic, and fibrocartilage) (Figure 1.2).

Hyaline cartilage is the most prevalent type of cartilage and is found in ribs, in the nasal septum, tracheal rings, and on the articular joint surfaces. It is predominantly made up of hydrated proteoglycan aggregates and Type II collagen fibers. The primary function of hyaline cartilage is mechanical resistance to bending, compression, and impact (Chiu et al., 2016).

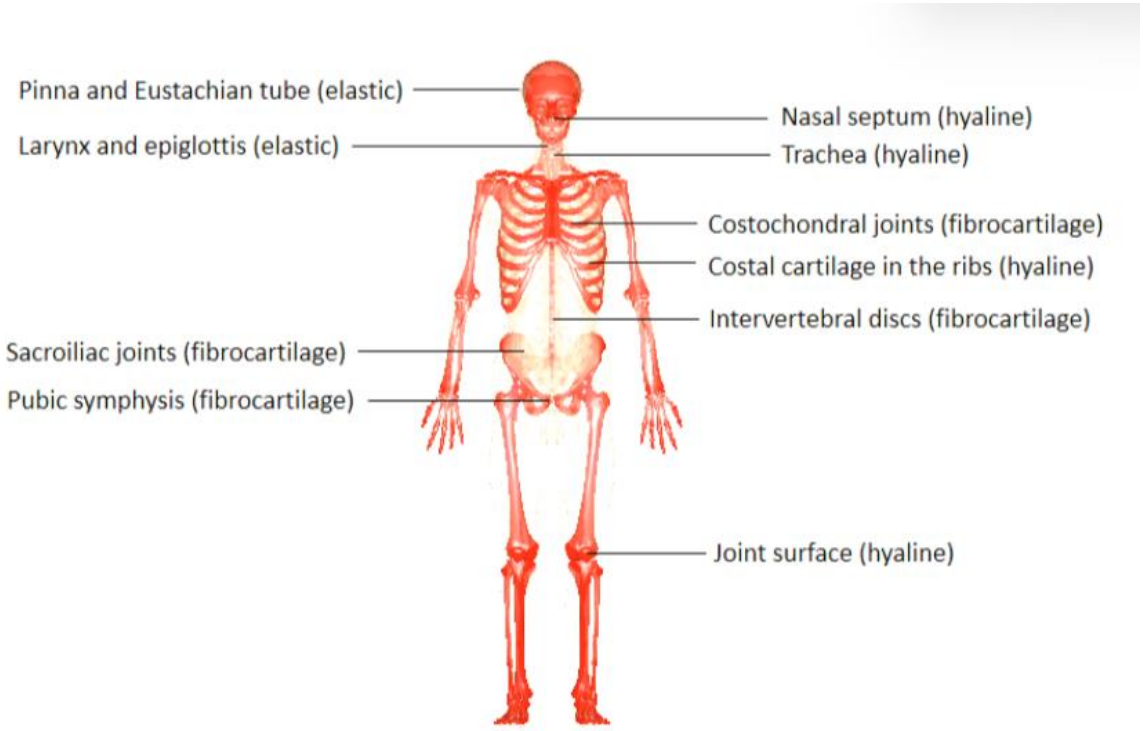


Figure 1.1. Cartilaginous regions in human body (Chiu et al., 2016*)*

The cartilage type which is found in the ear, as well as the larynx and epiglottis, is the elastic cartilage. It is mainly responsible for structural support, flexibility, and resilience in these body parts due to the elastic fibers and type II collagen fibers in it.

Intervertebral discs, costochondral joints, sacroiliac joints, and pubic symphysis, as well as specialized structures such as the meniscus, have fibrocartilage. This tissue is different than hyaline and elastic cartilage in terms of their composition. ıt contains a high concentration of collagen I and II fibers. These fibers may have various orientations depending on the specific fibrocartilage, such as in the annulus fibrous (of the intervertebral disc) and meniscus, which organize into longitudinal and circumferential fibers.

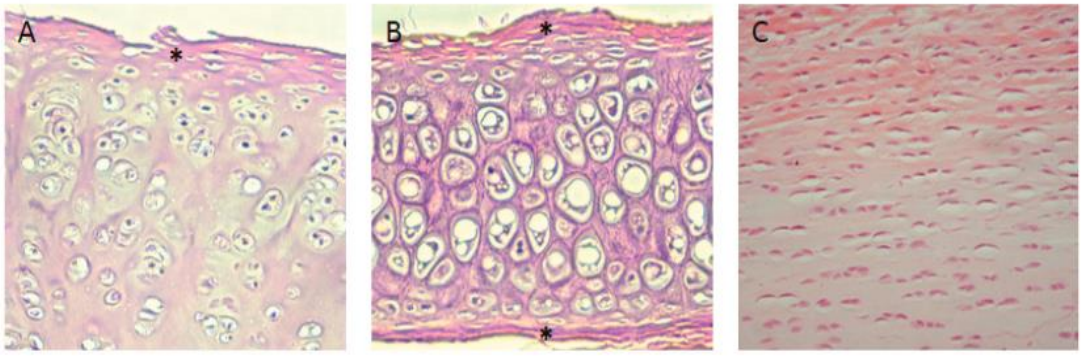


Figure 1.2. Three different types of cartilage. Hematoxylin and eosin staining of cross-sections of (A) hyaline cartilage in the human nasal septum, (B) elastic cartilage in the rabbit ear, and (C) fibrocartilage in the intervertebral disc (Chiu et al., 2016)

Collagen, non-collagenous proteins, and proteoglycans are the three main types of cartilage extracellular matrix (ECM) components. Each of these proteins gives the composite ECM material a particular function. A proteoglycan is a type of linear carbohydrate polymer that is created when one or more glycosaminoglycan (GAG) chains are added to a protein after translational modification. Repeating disaccharide units that make up the GAG chains, and the various GAG types employ distinct disaccharides are: hyaluronan, chondroitin/dermatan sulfate (CS/DS), heparan sulfate (HS)/heparin, and keratan sulfate (KS). The core protein is linked to the proteoglycan-forming GAGs, CS/DS, and HS. Keratan sulfate is either N-linked (KS type I) or O-linked (KS type II) to serine or threonine residues. In contrast to other GAGS, which are anchored to a protein core, transmembrane hyaluronan synthases extrude hyaluronan into the extracellular environment.

One of the proteoglycan's primary roles in cartilage is to apply swelling pressure, which enables the tissue to absorb and distribute mechanical load. The matrix of

aggrecan and hyaluronan makes this possible. Other cartilage proteoglycans serve as cell surface receptors or tissue reservoirs for soluble factors, both of which are essential functions in directing the assembly of the ECM. Proteoglycans also have the role of controlling the innate immune system (Aspberg A., 2016).

1.1.1. Intervertebral Tissue

The intervertebral disc (IVD) is a complex tissue that is located between the vertebrae and is mostly composed of cartilage tissue. By distributing mechanical load among the vertebrae, it contributes to the health of the spine. Inner soft nucleus pulposus part (NP) annulus fibrosus (AF), and cartilage endplates (CEP) are the three components of IVD (Figure 1.3). Because NP is hydrated and gelatinous, it plays an important role in resisting mechanical stimuli, and its main resident cells are the nucleus pulposus cells (NPC), which are in charge of extracellular matrix (ECM) synthesis and maintenance. The AF is divided into two sections: the inner and outer AF. This tissue, which surrounds the NP and connects the spinal vertebrae above and below the disc, is made up of ligament fibers rich in type 1 collagen and elongated fibro-chondrocytes. The cartilage endplate is the nutrition channel, and its thickness is typically less than 1 mm. It aids in nutrient transport in the endplates (Kirnaz et al., 2022).

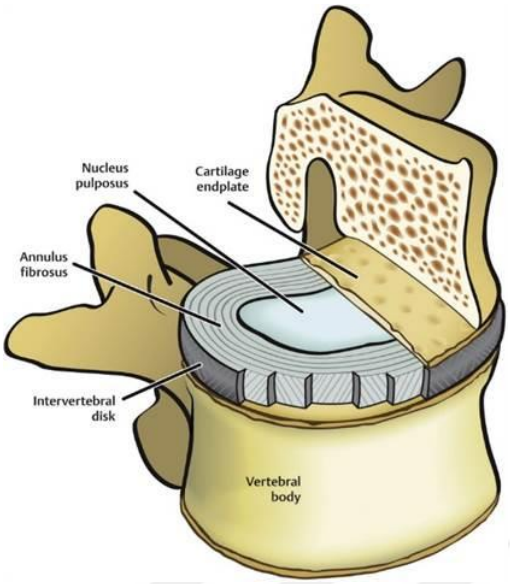


Figure 1.3. Anatomical organization of an intervertebral disc (Kirnaz et al., 2022).

Nowadays, it is very common that many cartilage-associated diseases (e.g. osteoarthritis (OA), trachea stenosis, chondrocalcinosis, cartilaginous tumors) and deformations owing to traumas affect the functioning of the tissue and thus, quality of life. Because of the lack of innervation and blood circulation in cartilage, once it is damaged, it is difficult to repair by itself (Agarwal et al., 2021).

Treatments are often invasive which increases procedure risk and recovery time with limited efficacy. Surgical methods are frequently used to correct cartilaginous abnormalities, either with or without the implantation of a prosthesis (Chiu et al., 2016). Most of the drug and supplementary-based treatments work well in decreasing the progress of the degenerations but have limited capacity towards tissue repair purposes.

Therefore, developing minimal invasive strategies is highly needed for effective cartilage repair as a new alternative approach to cartilage tissue engineering (CTE) offering innovative and promising tools for cartilage regeneration and repair.

1.2. Cartilage Tissue Engineering

Tissue Engineering (TE) offers innovative means to restore, maintain, or improve cartilage tissue functionality, by combining cells, growth factors, and biomaterials to fabricate biological substitutes. Producing bio-mimetic tissue with consistent biological and biomechanical performance is the goal in the field of cartilage tissue engineering (CTE) (Francis et al., 2018). For this purpose, the material that will be used in cartilage tissue engineering must be able to support the growth and expansion of cells (either chondrocytes or mesenchymal stem cells), facilitate their free diffusion and movement throughout the structure, and needs to be stiff enough to resist compression like forces.

Figure 1.4. shows the properties that an appropriate scaffold in cartilage tissue engineering should have (Jahani et al., 2020); biocompatible to prevent inflammatory reactions; three-dimensional shape to allow proliferation and cellular differentiation; porous to permit migration of cells and diffusion of molecules, nutrients, and oxygen; The matrix has to adhere to the host tissue to maintain its mechanical integrity; be degradable to integrate the physiological processes of tissue remodeling; must be applicable by mini-invasive surgery if possible via injection (Vinatier et al., 2009). It can also be bioactive and allow the homogeneous and controlled release of growth factors or agents.

Figure 1.4. Ideal Scaffold Properties in Cartilage Tissue Engineering (Jahani et al., 2020).

Design of the cartilage scaffolds starts with defining the most suitable materials and the most appropriate fabrication technique for creating constructs with the desired shape and functionality. A wide range of materials has been used for scaffold fabrication in CTE. Briefly, the mostly used materials are natural (e.g., alginate, collagen, chitosan, silk fibroin (SF)) and synthetic polymers (polylactide (PLA), polyethylene glycol (PEG), polyglycolide (PGA)) (Chiu et al., 2016). Natural polymers have superior biological properties, including the ability to promote cell

proliferation and differentiation, but are characterized by weak mechanical properties. Synthetic polymers are easily processable and have enhanced mechanical properties but are characterized by low or no biological properties like cell attachment sites and components for improving cell functionality for matrix synthesis [bioactive sites]. Soft polymeric materials such as hydrogels have similar biomimetic properties with cartilage tissue (Francis et al., 2018). Hydrogels are a three-dimensional network of hydrophilic polymers that absorb a large quantity of water. Their high water content allows rapid diffusion of nutrients and metabolites. Moreover, cells, growth factors, or bioactive components can be homogeneously incorporated. Such properties of hydrogels make them ideal candidates for CTE. For example, Hsu et. al., (2007) combined gold nanoparticles with porcine collagen II hydrogels. These nanoparticles improved the hydrogel's mechanical quality which raised its benefits. Researchers incorporate such nanoparticles into hydrogels to fabricate superior composites with desirable optical, electrical, and biological characteristics that are beneficial for cartilage repair. Hydrogels made up of materials like PEG are also favorable for the encapsulation of cells owing to their adjustable degradation properties (Chiu et al., 2016). Chondrocytes encapsulated in PEG hydrogels maintained their viability throughout the culture time (Nicodemus et al. 2007) demonstrating the hydrogels are useful for cells to be encapsulated. In another work, an alginate hydrogel system containing the growth factor BMP-7 was designed to stimulate chondrogenesis (Lim et al., 2010) which shows the utilization of hydrogels for delivering growth factors which is essential in the tissue engineering field.

1.3. Intervertebral Tissue Degenerations

Intervertebral disc (IVD) degenerations occur as a result of excessive mechanical loading, genetic or age-related diseases, and environmental factors (Widjaja et al, 2022). IVD degeneration is characterized by gradual tissue loss or degradation that reduces the height of the disc and eventually harms the vertebral body's mechanical stability. It is one of the most common causes of low back pain (LBP) and because

of risk factors such as aging, obesity, chronic stress, occupational exposure, and smoking, its prevalence is increasing (Oichi et al, 2020). Physical therapy, pain medication, and surgical intervention are currently available treatments for LBP (Urits et al, 2019). Discectomy and arthrodesis are the two most frequently performed surgical treatments for individuals with deteriorated intervertebral discs. Discectomy is the procedure of removing or excising the IVD's deteriorated section. Also known as spinal fusion, arthrodesis is the procedure of joining two neighboring vertebral bodies. Spinal fusion and discectomy are merely temporary fixes for degenerative IVDs or recurrent low back pain. Compared to fusion, an entire IVD replacement, or arthroplasty, offers advantages in that it reduces discomfort and increases patient mobility. Implants for disc replacement, by restoring disc height, eliminate damaged tissue and diminish discomfort (Whatley et al, 2012).

Surgical removal of the diseased tissue, implantation of a cage or prosthesis to restore the intervertebral space and vertebral bone fusion are included in current treatments for IVD degeneration. Although these might alleviate symptoms, it also changes the biomechanics of the spine and may accelerate the degenerative process in neighboring spinal segments. Biological regeneration of the deteriorating IVD would be ideal rather than surgical removal (Roughley et al, 2006).

As a result, since available treatments often do not cure the underlying disease and are inadequate for the healing process, tissue-engineering methods have gained attraction in recent years. This is because of the fact that it has the ability to cure the degenerated area by improving regeneration in the disc by biological treatments such as gene therapy, growth factors, stem cell injections, and due to their ability to restore the structural integrity and biomechanics of degenerated intervertebral discs (Huang et al, 2023). Hydrogels are excellent candidates for use as a biomimetic ECM due to their three-dimensional polymeric network structure with a high water content. Moreover, cells, growth factors or bioactive components can be homogeneously and safely incorporated to hydrogels due to no requirement of organic-harmful solvents. Bioactivity is important for the scaffold to promote cell proliferation and

differentiation and also for the hydrogel to be effective. Injectability of the hydrogel is also important because of the minimally invasive surgical procedure, in situ gelation, the filling of complex shape defects, and the easy incorporation of desired agents (Maisani et al, 2017).

1.4. Intervertebral Disc Degeneration Treatments

With modern lifestyles, IVD-related problems are increasing more and more and have become a public health concern. IVD degeneration is linked to low back pain, and it is one of the greatest reasons that decrease the quality of life. Current treatments are often invasive and increase procedure risk and recovery time with limited efficacy. There are also many side effects and often many surgery processes are needed. Tissue engineering offers innovative and promising tools by developing minimal invasive strategies and regenerating the tissue diminishing the symptoms of the disease significantly. The regeneration of the nucleus pulposus (NP) tissue is seen as a key target when developing strategies to restore disc function. The NP is very fluidic and abundant in glycosaminoglycans, proteoglycans, and type II collagen, so mechanical pressure that is passed through the spine is quickly absorbed by the cushioning ability it generates. Degeneration of the IVD is characterized by hydration loss and degradation of the NP's extracellular matrix. This, in turn, causes a progressive decrease in disc height, reducing the IVD's ability to cushion the space between vertebrae and eventually its degeneration (Frith et al., 2013). As a result, successful NP regeneration has the potential to significantly treat disc diseases. Figure 1.5 shows the approach of using injectable hydrogels in the treatment of IVD degenerations.

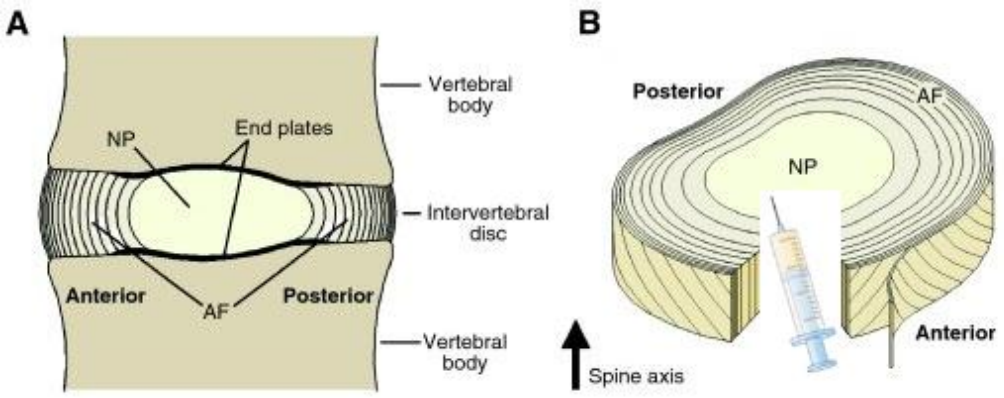


Figure 1.5. (A) Mid-sagittal cross-section of IVD showing anatomical regions. (B) Three-dimensional view illustrating the approach of an injectable hydrogel system for the treatment of IVD degenerations. (Smith et al., 2011).

1.4.1. Example Biomaterials Used in IVD Degeneration Treatments

Hydrogels have long been popular in the course of therapy of IVD degenerations since they share resembling mechanical and biological characteristics with NP. For IVD degenerations, many injectable hydrogel combinations are investigated using both natural and synthetic polymers (Table 1.1) (Peng et al., 2022).

Table 1.1. Overview of the natural and synthetic biomaterials used in hydrogel combinations for the treatment of intervertebral discs.

Biomaterial type	Example materials	Target tissue in IVD	References
Natural polymers	Elastin	AF	Boyd et al.,2006
	Hyaluronic Acid	NP	Revell et al.,2007, Ganey et al.,2009

	Alginate	NP	Leone et al.,2008, Guehring et al.,2008, Chou et al.,2009, Zhang et al.,2014
	Chitosan	NP	Richardson et al.,2008, Alinejad et al.,2019, Du et al.,2022
	Gelatin	NP	
	Fibrin/Fibrinogen	AF/NP	Wang et al., 2021
	Collagen	AF/NP	Stern et al.,2000, Gruber et al.,2004
Synthetic polymers	PCL	AF	Halloran et al.,2008, Tsaryk et al.,2015, Zhou et al.,2018, Friedmann et al.,2021
	PEG	NP	Hutmacher et al.,2001, Wan et al.,2008
	PLA/PLGA	AF/NP	Vernengo et al.,2008, Nguyan et al.,2019
			Sha'ban et al.,2008, Debusscher et al.,2009

The majority of hydrogel research for IVD treatment focuses on materials with a natural basis. While the features of synthetic polymers are simpler to manipulate, natural polymers offer a more accurate setting for simulating the NP of a disc transplantation (Whatley et al., 2012). Collagen, hyaluronic acid (HA), alginate, and chitosan are natural biomaterials that are mostly used in the field of IVD degeneration treatments.

1.4.1.1. Collagen:

The most prevalent polymer in the anatomy of a human is collagen. It makes up the majority of tendons and ligaments, along with the natural framework of bone and dentin, and it predominantly performs mechanical activities. It is also a crucial part of blood vessels, cartilage, skin, cornea, and the majority of other extracellular tissues (Wagermaier et al., 2015). Considering it has negligible immunogenicity and great biosafety, collagen has been employed extensively in several types of hydrogel materials, as it is a constituent of extracellular matrix (ECM). In a study, it has shown that after loading adipose stem cells, collagen hydrogel can acquire the NP phenotype and exhibit excellent biocompatibility (Mercury et al., 2014).

1.4.1.2. Hyaluronic Acid (HA):

Hyaluronic acid (HA) is a natural, linear, polysaccharide and is a crucial part of the extracellular matrix which is indispensable to several tissues (Cassano et al., 2023). Due to the fact that a hydrogel made solely of HA would rapidly break down, hydrogels are frequently created by cross-linking hyaluronic acid (HA) with collagen. Because HA has no immunogenic effects, it is the material of choice in the applications of tissue engineering. Also, it has been discovered that HA can promote stem cell differentiation and preserve the NP cell phenotype (Collin et al., 2011).

1.4.1.3. Alginate:

One biodegradable polysaccharide is alginate. Algae cell walls contain this anionic polysaccharide. Because of its features parallel to the extracellular matrix (ECM),

alginate is commonly utilized as a hydrogel material in tissue engineering. In a study, the hydrogel scaffold functioned like natural NP tissue and exhibited good swelling and hydrophilic qualities (Leone et al., 2008)

1.4.1.4. Chitosan:

Chitosan is a biomaterial that has advantages such as non-toxicity, biocompatibility, and biodegradability and is frequently used in areas such as tissue engineering and drug delivery. It is a hydrophilic natural polymer derived from chitin, has functional amino groups, and a net positive charge, and is also very similar to the ECM of the cartilage (Bhattarai et al., 2010).

Because of these positive traits, chitosan has been used extensively to make hydrogel in tissue engineering. Since chitosan has been demonstrated to increase the attachment and proliferation of chondrocytes and shares characteristics with natural cartilage's extracellular matrix (ECM), it is particularly appealing for IVD regeneration. Furthermore, chitosan hydrogels have the ability to restore NP at the initial phases of IVD degeneration and have been demonstrated to maintain significant levels of proteoglycans within NP cells (Roughley et al., 2006). However, like other natural polymers, chitosan has low mechanical strength and is fragile, but it can be modified with crosslinking and functionalized easily. For this reason, new combinations of polymers that can be used with chitosan are being investigated.

1.5. Reinforcement of Hydrogel Systems

Since injectable hydrogels could potentially be given to a designated area in a minimally invasive manner, they have attracted a great deal of scientific interest in tissue engineering applications. Yet, the practical usage of these materials is restricted by their comparatively weak mechanical characteristics especially when natural biomaterials have been used. However, there are several strategies to develop mechanically reinforced hydrogel systems. Incorporating nanoparticles as reinforcing agents into the gel is an advantageous way to reach the aim of enhancing

the properties of the hydrogel. As nanocomposite hydrogels are developed, the features of every single component are usually enhanced cooperatively. For example, as the mechanical integrity of the hydrogel system increases, the accumulation of the nanomaterial declines at the same time. Consequently, a variety of nanoparticles and nanofibers have been employed to improve the mechanical characteristics of hydrogel systems as well as to introduce functionality that is contingent on the reinforcing agent. (De France et al., 2020).

1.5.1. Cellulose Crystals as a Reinforcing Agent for Hydrogel Systems

The most prevalent replenishable polymer on earth, cellulose, is the source of cellulose nanocrystals (CNCs), which are biobased, rigid rod-shaped particles with a length of around 100–200 nm and a cross-section of approximately 5–20 nm. The most commonly used technique for creating CNCs is to hydrolyze cotton or wood pulp with sulfuric acid. Additionally, oxidative methods or different mineral and organic acids can be used to create CNCs.

By virtue of the substantial level of crystallization, CNCs also have robust intrinsic mechanical characteristics, adjustable surface chemistries, and considerable aspect ratios. Moreover, a number of investigations indicate that CNCs tend to be tolerated well in vivo and biologically inert, which makes them appealing for use in tissue engineering applications (Maren Roman, 2015). The effectiveness of CNCs as a reinforcement material can be further enhanced by utilizing the simplicity in which their surfaces can be functionalized to facilitate the cross-linking at the CNC–hydrogel interaction.

Briefly, CNCs have high strength, good biocompatibility, a low density, a large surface area, and can function both as a filler and a cross-linker (De France et al., 2020). With the recent commercial accessibility of high-grade CNCs, the utilization of CNCs as reinforcing agents for injectable hydrogel systems is a quickly expanding field of study.

1.5.2. Maltodextrin for Enhancing the Gelling Mechanisms

Maltodextrin, which can be derived from starch, is a polysaccharide. It can be functionalized easily and will be used to improve the properties of the hydrogel for the IVD tissue like injectability and friction properties during compression. It can also act as a depot energy source for cells in hydrogel after in vivo application (Velasco et al., 1997). The reason for using maltodextrin was its rapid bonding and gelling ability with chitosan via Schiff Base Reaction applying the aldehyde modification treatment on it.

1.6. Therapeutic Agents Used in IVD Tissue Engineering

Hydrogels, among a broad range of biomedical materials, have become increasingly popular in disc replacement, repair, and regeneration therapies thanks to their biocompatibility and similar structure with the nucleus pulposus tissues, also their allowance to high water uptake content and simplicity of usage due to injectability makes them ideal candidates in the field of IVD tissue engineering. They can be utilized to provide a localized and prolonged release of therapeutic agents, like growth factors or cells, drugs, and hormones to the spot of damage, which could boost healing results. There is a new increasing motivation for addressing a desired treatment outcome of IVDD thanks to these strategies, which combine biomaterials with cells, drugs, hormones, and growth factors (Han et al., 2023).

1.6.1. Growth Factors

Growth factors (GFs) are proteins that cells release to control ECM metabolism and promote cell migration, differentiation, and proliferation. GFs activate intracellular signaling processes by binding to certain receptors found on the surface of cells (Blanquer et al., 2015). According to recent research, IVDD is linked to growth factors in the intervertebral disc's extracellular matrix (ECM) that keep the ECM's homeostasis balanced by preserving the ratio of anabolism to catabolism in the disc cells. Growth factors can be combined or cross-linked with injectable

hydrogels to offer anabolic effects that persist longer (Gan et al., 2016, Gandhi et al., 2020). Inhibiting IVDD successfully requires more than just using growth factor treatment. To achieve the optimum treatment outcome, it is frequently required to combine transplanting cells to promote the cell number and secretory activity (Han et al., 2023).

1.6.2. Drugs

In the last few years, research on the therapeutic approach of hydrogel-carrying pharmaceuticals has gained a lot of attention. It has been demonstrated that several drugs alleviate IVDD (Pan et al., 2018, Bai et al., 2020). These drugs can assist restore the intervertebral disc's homeostasis, by interfering with the biological process that causes IVDD, and offer novel therapy options. Hence, drug-loaded hydrogels that are safe and controlled have significance in tissue engineering for applications like treating early IVDD (Han et al., 2023).

1.6.3. Hormones

Homeostasis in the extracellular matrix is essential to preserve the structure and function of the intervertebral disc. A class of endopeptidases known as matrix metalloproteinases (MMPs) is crucial for the homeostasis of the extracellular matrix (ECM). Numerous MMP subgroups exhibit markedly elevated expression and activity in degenerated IVD tissue, according to a number of recent studies. The discrepancy between ECM anabolism and catabolism caused by this overexpression of MMPs outcomes in the ECM to degrade and eventually IDD. Consequently, controlling MMP expression may be an effective avenue for the treatment of IDD since MMPs break down the ECM in IVD, and inhibiting the expression of them may have a huge potential in IDD treatment. Several hormones have demonstrated promise in the treatment of IDD since they can control MMP expression. Melatonin seems to have an important effect since it increases the formation of collagen type II and aggrecan in NP cells which prevents the breakdown of the extracellular matrix

while promoting the growth of NP cells. along with suppressing the expression of several MMP genes (Zou et al., 2023).

1.6.3.1. Melatonin:

N-acetyl-5-methoxytraptamine is the chemical name for melatonin. It is recognized for preserving circadian rhythm. Melatonin is an endocrine hormone that primarily the pineal gland produces and secretes in mammals. Melatonin production is regulated by the circadian cycle, which is activated at nighttime and inhibited during the day. Clinical research has demonstrated a favorable correlation between patients' serum melatonin levels and IDD (Turgut et al., 2006). Melatonin concentration is known to decrease with ageing and demonstrates a negative correlation with Intervertebral Disc Degeneration (IDD).

This hormone is also known to protect the structural integrity of IVD structure thus preventing the development of related diseases. Recent studies have shown that melatonin treatment promotes the recovery process of degenerated IVD tissue (Cheng et al., 2021). Melatonin supplementation may therefore be a promising therapeutic strategy for intervertebral disc diseases.

1.7. Aim of the Study

This research aimed to develop an injectable chitosan- oxidized-maltodextrin (ox-MD) based hydrogel strengthened with oxidized microcrystalline cellulose crystals (ox-MCCs) and loaded with melatonin for regenerative treatment of degenerated intervertebral discs. Chitosan, a natural polymer, was preferred to form the hydrogels due to its biocompatible, and hydrophilic properties that makes it preferable for cartilage tissue engineering applications. Ox-MD was used for its rapid bonding and gelling ability with chitosan via Schiff Base Reaction.

The hydrogel composition (Figure 1.6.) investigated in this thesis as a prospective biomaterial with the ultimate aim of treatment of intervertebral disc degeneration is studied for the first time in the literature.

The research questions of this thesis were:

- What is the optimum composition and condition for chitosan/ox-maltodextrin/ox-MCC hydrogels considering gelation, injectability, and stability requirements of the CTE system?

- Will new melatonin loaded chitosan/oxidized-maltodextrin/microcrystalline cellulose hydrogel provide suitable mechanical properties and support the proliferation of cartilage cells?

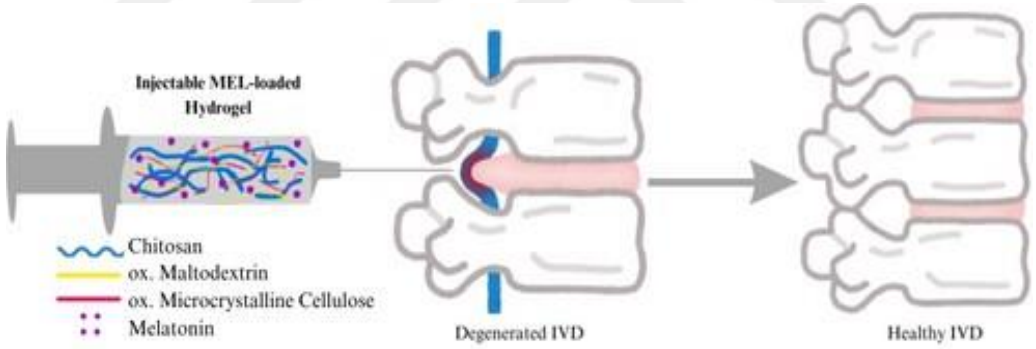


Figure 1.6. Illustration of the injectable MEL-loaded chitosan hydrogel approach of the study

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Microcrystalline cellulose powder (MCC) and cellulose dialysis tubing membrane (MWCO: 12000-14000, diameter 21 mm) were purchased from SERVA (Germany). Sulfuric acid 95-98% was from ISOLAB (Germany). Sodium (meta) periodate was from Sigma-Aldrich (USA). Maltodextrin was the product of TITO (Turkey). Hydroxylammonium chloride was obtained from Riedel-de Haen (Germany). Chitosan (medium molecular weight - 190,000-310,000 Da) was obtained from Sigma-Aldrich (USA). Hydrochloric acid (37%) was provided by Merck (Germany). Melatonin was obtained from Sigma-Aldrich (USA).

Dulbecco's Modified Eagle's Medium (DMEM) with stable glutamine and 15 mM HEPES, MEM non-Essential Amino Acids (NEAA 100x) was purchased from Biowest (France). Heat inactivated fetal bovine serum (FBS) was bought from Capricorn Scientific (Germany). Trypsin EDTA and penicillin/streptomycin were purchased from Biochrom (Germany). Dimethyl sulfoxide (DMSO) and Triton X-100 were supplied by AppliChem GmbH (Germany). Alamar Blue was obtained from Invitrogen by Thermo Fisher Scientific (Oregon, USA). All other chemicals were purchased from Sigma and Merck (New Jersey, U.S.A.).

2.2 Methods

2.2.1 Modification of Cellulose

2.2.1.1 Preparation of CNC

To create cellulose nanocrystals (CNCs) from microcrystalline cellulose powder (MCC), the typical sulfuric acid hydrolysis procedure (Bondeson et al., 2006) was followed with minor modifications for optimization purposes. MCC was mixed with sulfuric acid using different ratios (63.5%, 60% and 50% w/w) at 45 °C and stirred for 10h, 2 h, and 1h. Immediately after hydrolysis, the suspension was diluted 10-fold with distilled water to terminate the reaction. The suspension was then washed repeatedly with water and centrifuged 3–4 times (8000 RCF, 10 min for each cycle). The centrifugation step was finished when the wash water became clear. The resulting suspension was then collected and extensively dialyzed against distilled water using a cellulose dialysis membrane (MWCO: 3 kDa) at room temperature. After dialysis, the content was sonicated for 10 min with different amplitude ratios (20% and 40%) under ice cooling to prevent overheating and centrifuged again for 5 min at 10000 RCF. The supernatant which should include CNCs, was freeze-dried for 20h, and after obtaining freeze-dried powder the size and morphology of the obtained material were analyzed using SEM (TM-1000, Hitachi, Japan).

2.2.1.2 Preparation of Oxidized MCC

The microcrystalline cellulose was oxidized with sodium periodide ($NaIO_4$) according to a procedure in the literature (Liu et al., 2022). Microcrystalline cellulose powder (5 g) was mixed with 100 mL of distilled water and 10 g of $NaIO_4$ was added. Following this, the pH of the solution was adjusted to 6.8-7.2 using 0.1 M NaOH. Then, the solution was mixed in a water bath at 50 °C for 4 h. To stop the reaction, an excess amount of ethylene glycol was added. After this, the solution was transferred into the dialysis bag and dialyzed for 5 days against distilled water with changing the water twice a day at room temperature. The final product (ox. MCC) was obtained by freeze-drying.

2.2.1.2.1 Determination of Degree of Oxidation of MCC

Oxidation degree of ox. MCC was determined using the hydroxylamine hydrochloride titration method (Liu et al., 2017). The pH of the ox. MCC solution was adjusted to 3.5 with 1 M HCl solution. Then, 10 mL of hydroxylamine hydrochloride solution (5 % w/w) was added and mixed for 24 h at room temperature. After this, the solution was titrated using 1M NaOH until reaching pH 3.5. The oxidation degree was found by the consumption of NaOH using the formula below:

$$\mathrm{D.O.\%} = \frac{\mathrm{C}_{NaOH} * \mathrm{V}_{NaOH}}{\mathrm{m}/162} * 100$$

Where;

DO is the degree of oxidation of the samples,

C_{NaOH} is the concentration of aqueous NaOH solution,

V_{NaOH} is the volume of the NaOH solution used,

m is the mass of ox. MCC,

and 162 is the molecular weight of repeating unit in cellulose.

2.2.2 Preparation of Oxidized Maltodextrin (ox. MD)

2.2.2.1 Synthesis of Oxidized Maltodextrin (ox. MD)

Oxidized maltodextrin was synthesized following the similar procedure in literature with minor modifications (Chen et al., 2017). Maltodextrin solution was prepared by adding 1200 mg maltodextrin to 20 mL DI water. After obtaining a homogenous solution, maltodextrin was oxidized by adding 424 mg of $NaIO_4$ and mixing for 3 h in the dark at room temperature. The reaction was stopped by adding ethylene glycol. To remove the unreacted agents, the reaction solution was exhaustively

dialyzed against deionized water for 3 days. A cellulose dialysis tubing membrane with a Molecular Weight Cut Off: 12,000-14,000 Da was used for this purpose. The final product (ox. MD) was obtained by freeze-drying method for further use and long-term storage. In accordance with this objective, oxidized maltodextrin solution was freezed at -80 °C first and then placed into the freeze-dryer device (Labconco Corporation, Kansas City, USA).

2.2.2.2 Degree of Oxidation of Maltodextrin by Hydroxylamine Hydrochloride Method

Oxidation degree of ox. MD was determined using the similar hydroxylamine hydrochloride titration method and formula described above (Liu et al., 2017). Briefly, 0.1 g freeze-dried ox. MD was dissolved in 25 mL hydroxylamine hydrochloride solution (0.25 N) and stirred at room temperature for 24 h. The pH of the original hydroxylamine hydrochloride solution was recorded and NaOH solution (0.25 N) was used to titrate the solution until reaching this initial pH.

2.2.3 Preparation of the Composite Hydrogels: CS-ox.MD-ox. MCC

Chitosan (CS) was prepared in distilled water (2% w/w solution) adding hydrochloric acid (1 M) to make the pH approximately around 4 for the dissolution of chitosan. The solution was heated to 70 °C while stirring. Oxidized-MD (0.125-0.25-0.5% w/w) and oxidized-MCC (0.125-0.25-0.5% w/w) were added into the chitosan solution using different concentrations (Table 2.1) and the solution left to the incubator at 37 °C for 5 min to set the gelation.

Table 2.1. Final concentrations (w/v) in different hydrogel groups

ox. MD	CS	ox. MCC
0.125	*2*	0

0.125	*2*	0.125
0.125	*2*	0.25
0.125	*2*	0.5
0.25	*2*	0
0.25	*2*	0.125
0.25	*2*	0.25
0.25	*2*	0.5
0.5	*2*	0
0.5	*2*	0.125
0.5	*2*	0.25
0.5	*2*	0.5

2.2.4 Fourier Transform Infrared Spectrometry (FTIR) Analysis

The chemical structure of hydrogels was investigated by Fourier transform infrared spectrometer (FTIR, Shimadzu IRTracer-100, Japan). The FTIR spectra were obtained in the spectral range of 400–4000 cm^{-1} (METU Central Lab, and BIOMATEN, Türkiye).

2.2.5 Gelation Time

The gelation time and the injectability of the hydrogels were firstly determined with 3 different CS concentrations (1.5 w/v % CS, 2 w/v % CS, and 2.5 w/v % CS) at 37 °C. Afterwards, the experiment was repeated for 3 different ox. MD concentration groups in the hydrogels (final hydrogel compositions: 2 w/v % CS, 0.125 w/v % ox. MD, 2 w/v % CS, 0.25 w/v % ox. MD and 2 w/v % CS, 0.5 w/v % ox. MD). After that, the gelation time and injectability of hydrogels with ox. MCC addition (0.125, 0.25, 0.5 w/v %) were also tested.

The gelation time of the hydrogels was measured using the vial tilting method (Nguyen et al., 2011). Briefly, after the chitosan solution and oxidized maltodextrin solution were mixed in a narrow beaker (length 5 cm and inner diameter 4 cm) the

solution was transferred into an eppendorf tube and incubated at 37 °C in a water bath. The hydrogel was observed by inverting the tube and the gelation time was recorded when flowing ended (Figure 2.1).

Figure 2.1. Representative image of gel formed during the vial tilt assay

2.2.6 Swelling and Degradation Studies

In order to evaluate the stability of the hydrogels, swelling and degradation studies were done with (final hydrogel compositions: 2 w/v % CS, 0.125 w/v % ox. MD) (2 w/v % CS, 0.25 w/v % ox. MD) and (2 w/v % CS, 0.5 w/v % ox. MD). Furthermore, different amounts of ox. MCC (at a final concentration of 0, 0.125, 0.25, 0.5 w/v %) was added to the hydrogels composed of 2 w/v % CS, 0.5 w/v % ox. MD) to determine the effect of ox. MCC on the stability of the hydrogels. The hydrogels were prepared in a 48-well plate. After the gelation was set, they were freeze-dried for 20 hours. To assess the swelling ratio; the initial dry weight of the hydrogel was weighted (Wi), and then immersed in PBS in a shaking water bath (Nüve ST30, Türkiye), (set at 37 °C, 50 rpm). At predetermined time intervals, (1h, 2h, 4h, 24h-1d) excess water on the surfaces of the hydrogel was carefully removed

and the swollen hydrogel was immediately weighed (Ws). The swelling ratio of the hydrogel was determined using the following equation:

$$\text{Swelling Ratio } \% = \frac{\text{Ws} - \text{Wi}}{\text{Wi}} * 100$$

Ws; weight of swollen hydrogel (g) at time point, t

Wi; initial dry weight of the hydrogel (g)

To assess *in vitro* degradation; the initial dry weight of the hydrogel was weighted (Wi), and then immersed in PBS in a shaking water bath (at 37 °C, 50 rpm). At predetermined time intervals, (1d, 3d, 7d, 10d, 14d) the excess of water on the surfaces of gel was absorbed via rolling the samples very slowly on a filter paper and the hydrogel was freeze-dried again and then the last dry weights were weighed (Wt).

Weight loss percentages of the hydrogels were calculated using the following formula;

$$\text{Weight loss } \% = \frac{\text{Wi} - \text{Wt}}{\text{Wi}} * 100$$

Wt; dry weight (g) of hydrogel at time point

Wi; initial dry weight (g) of the hydrogel

2.2.7 Morphology of Hydrogel

The morphology of the hydrogel was perceived by scanning electron microscopy (SEM). For this purpose, the hydrogels were freeze-dried. Then, the freeze-dried samples were sputter-coated with gold, and their surface and cross-section morphology were monitored by SEM (TM-1000, Hitachi, Japan).

2.2.8 Mechanical Properties of the Composite Hydrogels

The hydrogels were poured into a mold with the dimensions (length: 1mm, width: 1mm, height: 4mm). Samples were tested with a 10N load of Univert Biomaterial Mechanical Tester (Cell scale, Canada). The preload value was adjusted to 0.02 N and the withdrawal speed was 1 mm/min. Mechanical tests were conducted for the

CS/ox. MD hydrogels (2 w/v % CS, 0.125-0.25-0.5 w/v % ox. MD) and also for the hydrogels composed of different amounts of ox. MCCs (0, 0.125, 0.25, and 0.5% (w/v).

2.2.9 Melatonin Release Studies from the Hydrogels

Melatonin-loaded hydrogels were prepared by dissolving melatonin in 0.5 % MD solution and adding MD-MEL solution to 2 % CS solution. The release of melatonin from the hydrogels with and without MCC content was investigated. Hydrogel solutions (1 mL) with and without MCC were put into the dialysis tube (Serva, Germany, MWCO:12000-14000) and incubated for 5 minutes to set the gelation. Then the gel-filled dialysis bags were put into the shaking incubator (at 37 °C, 50 rpm) in 50 mL falcons filled with 10 mL of PBS (0.1 M, pH7.4) at 37°C. Release media (1 mL) was taken from the falcons at predetermined intervals (15-30-45min, 1-2-4-6h, 1-3-7-10d) and the absorbance at 222 nm was measured with a spectrophotometer (U-2800A Hitachi, Japan). The amount of released drug was determined using the calibration curve constructed with different concentrations of melatonin (20, 10, 5, 2.5, 1.25, 0.625 μg/mL in PBS), (Appendix A, Figure A). Percent MEL release from the hydrogels was calculated using the following equation and the release profile from the hydrogels is represented as cumulative percent release

$$\text{Drug Release \%} = \frac{\text{Amount of released drug at a given time point}}{\text{Total amount of loaded drug}}$$

2.2.10 Cell Culture Studies

2.2.10.1 Nucleus Pulposus (NP) Cell Isolation from Bovine

Nucleus Pulposus (NP) cells which are present in intervertebral discs (IVD) cartilaginous tissue were isolated. The procedure for the isolation of nucleus pulposus cells was carried out according to the previous studies with minor modifications (Guus G. H. van den Akker et al., 2021; Basatvat et al., 2023). Briefly, bovine tail vertebral tissue (Figure 2-a) was obtained as fresh as possible from a local slaughterhouse (Nidanur Birlik, Sincan/Ankara) and was immediately placed in a sterile PBS solution with 100U Pen/Strep (Biochrom (Germany)). All steps were carried out in a laminar flow cabinet under sterile conditions and all equipment was sterilized before usage. The tissue was dissected using sterile scalpel blades (sizes 10 and 21). The dissected NP tissue piece (Figure 2-b) was put on a petri dish filled with DMEM containing 1% Pen/Strep to avoid drying. The NP tissue was minced (Figure 2-c) to as small pieces as possible and then put into the collagenase solution (collagenase type II 30U/mL), (Sigma-Aldrich, USA). For digestion, minced tissue pieces were incubated for 3 hours on an orbital shaker (Biosan OS-10, Lithuania), (set at 37 °C, 150 rpm). After digestion, the solution was filtered through a 70 μm cell strainer (Falcon, USA) (Figure 2-d) and washed with PBS twice. Then, the tube with cells was centrifuged for 8 minutes (320 RCF, Sigma, 3-30K, Germany). Pellet was very unstable and needed to be handled carefully. After removing the collagenase solution and floating debris by aspiration, cells were resuspended briefly with PBS by gentle pipetting and this centrifugation and washing step was repeated two more times. After removing the solution, cells were suspended in an appropriate volume of culture medium and put into the tissue culture T-25 flask (approximately 5 mL IVD expansion medium: DMEM/F12-HEPES with stable glutamine, 10% FBS heat inactivated, 1% pen/strep, 1% NEAA). NP chondrocytes were incubated (Panosonic Incusafe, Japan) until attachment which took place within 5-7 days. After the cell attachment, the media were refreshed regularly every two days.

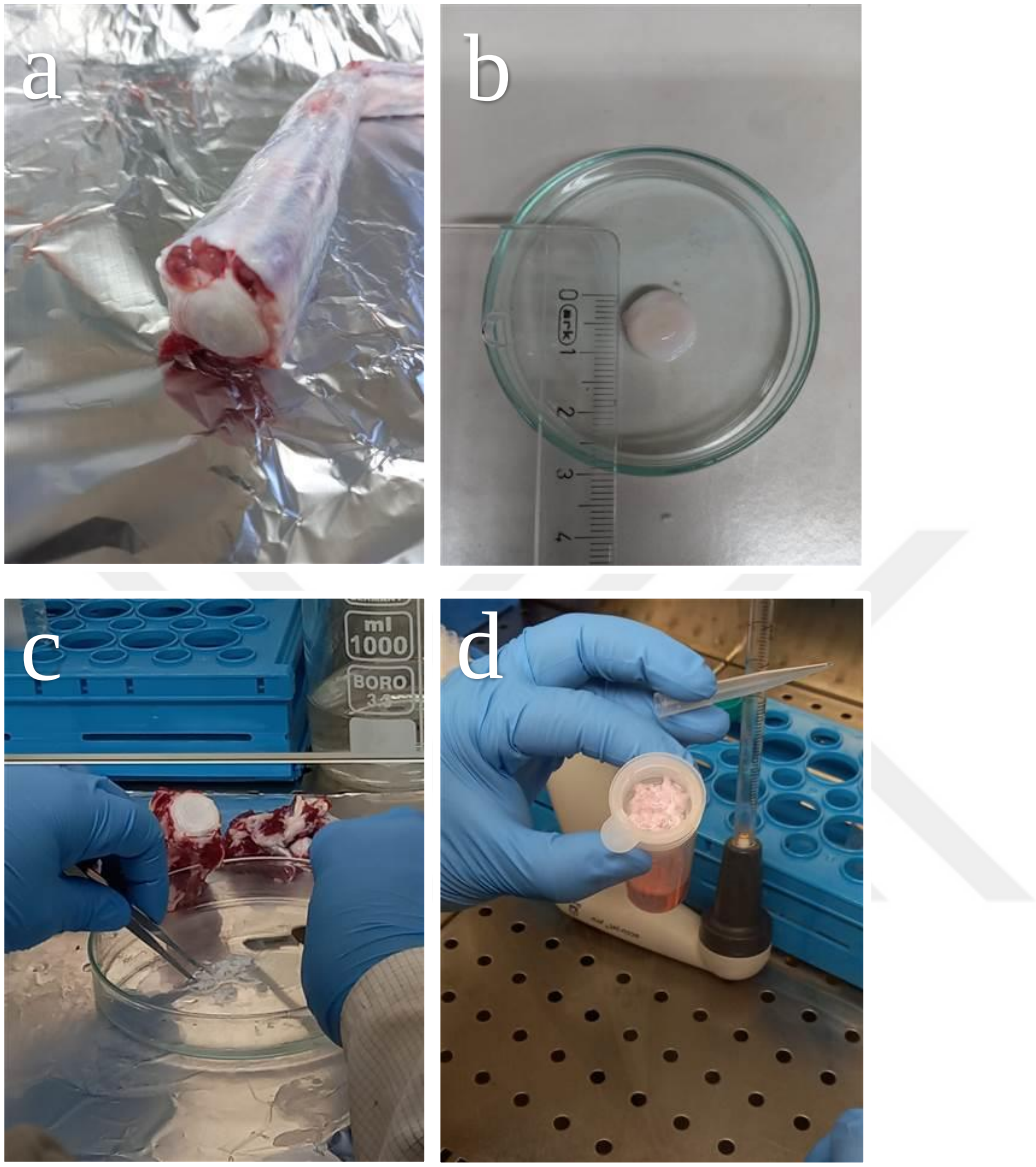


Figure 2.2. NP cell isolation steps (a- bovine tail vertebral tissue, b- dissected NP tissue piece, c- mincing the NP tissue piece, d- NP tissue pieces put into a cell strainer after digestion in a collagenase solution)

2.2.10.2 .Cell Viability and Proliferation

2.2.10.2.1 Dose-dependent Cytotoxicity of Melatonin

Cell viability analyses with Alamar blue assay were performed at the end of 24 h and 48 h of incubation of melatonin using different concentrations (0.05, 0.5, 5, 10, 50, 100 μg/mL). After seeding and incubating NP cells with cell culture media involving different concentrations of melatonin, the cell culture medium was removed, and the cells were washed with PBS. Alamar blue solution (10 % Alamar blue & 90 % DMEM without phenol red) was added to each well and incubated in the dark for 4 hours. Optical Densities of reduced solutions were obtained at 570 nm and 600 nm via a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, USA). The NP cells incubated in culture media without melatonin were applied as controls.

Percent relative cell viability of Alamar Blue can be calculated as indicated below formula;

$$\frac{(O_2 * A_1) - (O_1 * A_2)}{(R_1 * N_2) - (R_2 * N_1)} * 100$$

Where;

O1; molar extinction coefficient (E) of oxidized alamarBlue (Blue) at 570 nm

O2; E of oxidized alamarBlue (Blue) at 600 nm

R1; E of reduced alamarBlue (Red) at 570 nm

R2; E of reduced alamarBlue at 600 nm

A1; absorbance of test wells at 570 nm

A2; absorbance of test wells at 600 nm

N1; absorbance of negative control well (media plus alamarBlue but no cells) at 570 nm

N2; absorbance of negative control well (media plus alamarBlue but no cells) at 600 nm

2.2.10.2.2 Cell Viability and Proliferation Tests with Hydrogels

The cytotoxicity of hydrogels was investigated with the indirect elution test using isolated nucleus pulposus cells via Alamar Blue cell viability assay. Hydrogel groups (1: CS-MD, 2: CS-MD-MEL, 3: CS-MD-MCC, 4: CS-MD-MCC-MEL) were prepared and sterilized under UV light (30 minutes for both sides) before the experiment. Then, hydrogels were added into the 2 mL of sterile PBS in a 24-well plate and incubated at 37 °C in an incubator to collect the hydrogel extracts at predetermined intervals (1d, 3d, 7d). NP cells (5000/well) were seeded to the 96 well-plate and incubated at 37 °C for cells to adhere. After 1d of incubation of hydrogels in PBS, the extracts (50 µL) from hydrogels were taken and added onto the seeded cells in a 96-well plate diluting with cell media at a ¼ ratio. After incubation for 24h with the hydrogel extracts, the Alamar Blue Assay was performed as described above. Following the Alamar Blue Assay, the cells were washed with PBS and put into the incubator after adding media. After 3d, and 7d of incubation of hydrogels in PBS, the extracts (50 µL) from hydrogels were taken again, the same protocol, and Alamar Blue analysis was repeated for days 3 and 7. The wells without scaffolds were used as the control. The values were expressed as the mean ± standard deviation (n = 3).

2.2.11 Statistical Analysis

All experiments were conducted in triplicate, and the data were expressed as means with standard deviation. The SPSS software (SPSS Inc, USA) was used for the analysis. ANOVA or 2-way repeated-measures ANOVA statistical analyses and Tukey's test were applied to investigate specific differences. Statistical significance was defined at a p-value of < 0.05.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Modification of Microcrystalline Cellulose (MCC)

3.1.1 Disintegration of MCC

Injectable hydrogels have received significant attention from scientists for their potential in tissue engineering applications, however, their practical utilization is limited by their relatively poor mechanical properties, particularly when natural biomaterials are utilized. Yet, there are numerous strategies to overcome this problem like modifying the network and functionalization of the hydrogel or incorporating reinforcing agents such as nanoparticles or nanofibers like biominerals, silicate particles, and cellulose crystals (Ghandforoushan et al., 2023). In this study, injectable composite hydrogel which will gel once injected into intervertebral space is designed for the treatment of IVD degeneration. Incorporating micro/nanocrystalline cellulose (MCCs/CNCs) is one of the most effective ways that can improve the mechanical properties of the hydrogels. Cellulose, which is one of the most abundant biomaterials in nature involves a high molecular weight homopolysaccharide with β-1,4-anhydro-D-glucopyranose units. The fact that this polymer's monomer has three hydroxyl groups along with its hydrogen bonding ability has a crucial role on the reinforcement of the materials (Ramires et al., 2020).

Ramires et al. demonstrated the positive effects of both MCC and CNC on the mechanical properties of the polyurethane-based composites (an increase of approximately 520% and 670% respectively in the flexural modulus). CNCs usually have nanoscale dimensions of 2–50 nm in width and 100–2000 nm in length and because of their advantageous properties like reinforcement of the structure, and promotion of cell proliferation by mimicking the environment, they are widely investigated in the field of tissue engineering (Maturavongsadit et al., 2020). MCCs

are also a great point of interest due to having similar superior features, especially in tissue engineering with hydrogel-based scaffold studies (Liu et al., 2022). The morphology of MCCs used in this study is shown in Figure 3.1. A and Figure 3.1. B. It was observed that its crystalline fibril structure, within the size range of 10~30 μm, is similar to previous studies reported in the literature (Bao et al., 2021).

At first, experimental trials were applied to disintegrate this microcrystalline cellulose (MCC) powder to obtain cellulose nanocrystals (CNCs). Several attempts including the use of different sulphuric acid concentrations, various durations of incubation, and changing the amplitude of sonification were done. However, a homogenous nanocrystalline powder distribution could not be obtained. Probe sonification at 20% and 40% amplitudes for 10 minutes duration without changing other factors was tried and it was observed that using higher amplitudes caused formatişon of a film like structure (Figures 3.2 A and B) and also decreased the yield considerably. The effect of the sulphuric acid concentration was also investigated using 3 different concentrations and the results were presented in Figure 3.3 (A, B, and C). It can be observed that increasing acid concentration resulted in melted thin flakes (Figure 3.3 C). How the duration of reaction between cellulose and sulphuric acid affects the optimization was also questioned, and results given in Figure 3.4. (A, B, and C) was obtained. It was considered that excessive concentration of sulphuric acid and reaction time make the structure amorphous and less crystalline according to these results. The results of using excessive amount of acid concentration in making CNC was also investigated before and our results were in line with the previous reports (Ioelovich, 2012).

In the SEM micrographs results of CNCs, aggregates of microcrystalline cellulose crystals which are similar to CNC morphologies can be seen whose size was lower than the original size of the microcrystalline cellulose powder, but not in nanosize. Therefore, MCC powder was decided to be used in this study, since its reinforcement properties with hydrogels have also been proven in similar studies (Liu et al., 2022, Yin et al., 2022).

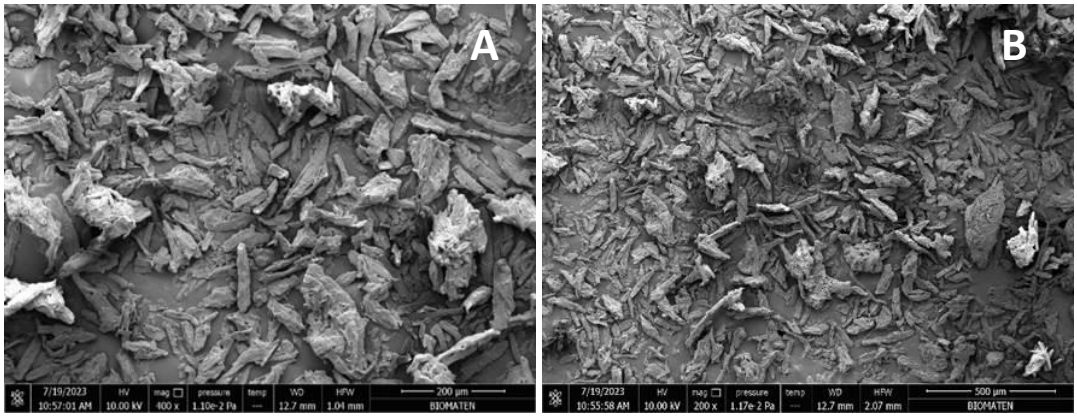


Figure 3.1. SEM images of A) MCCs (400X) and B) MCCs (200X) as obtained

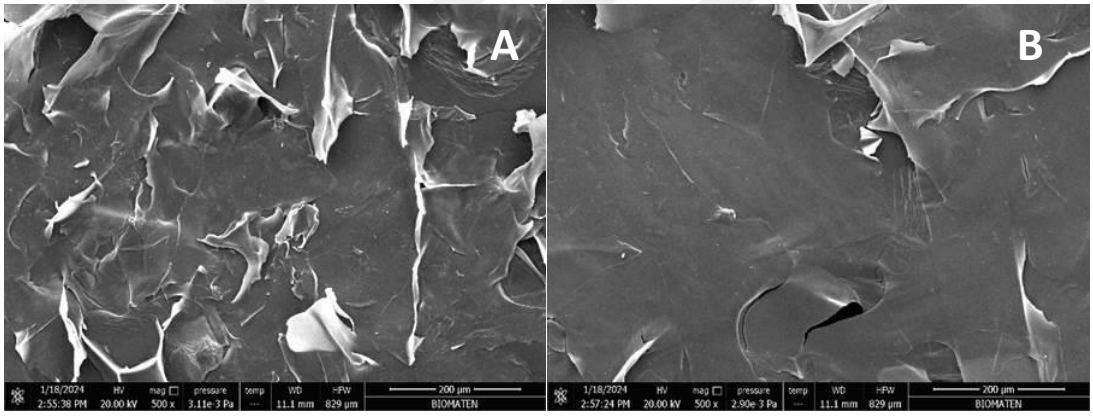


Figure 3.2. SEM images of CNCs treated with different amplitude ratios of sonication A) 20% A (500X) B) 40% A (500X)

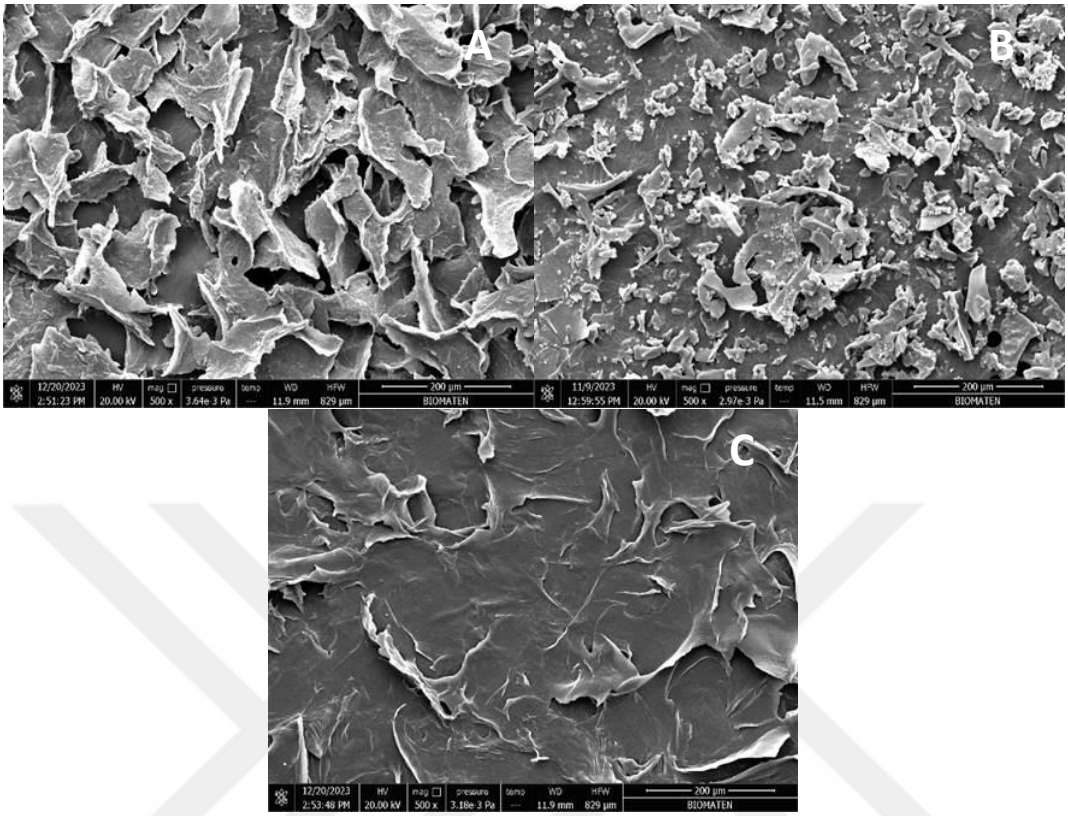


Figure 3.3. SEM images of CNCs treated with different concentrations of sulphuric acid A) 50 wt.% (500X) B) 60 wt. % (500X) C) 63.5 wt. % (500X)

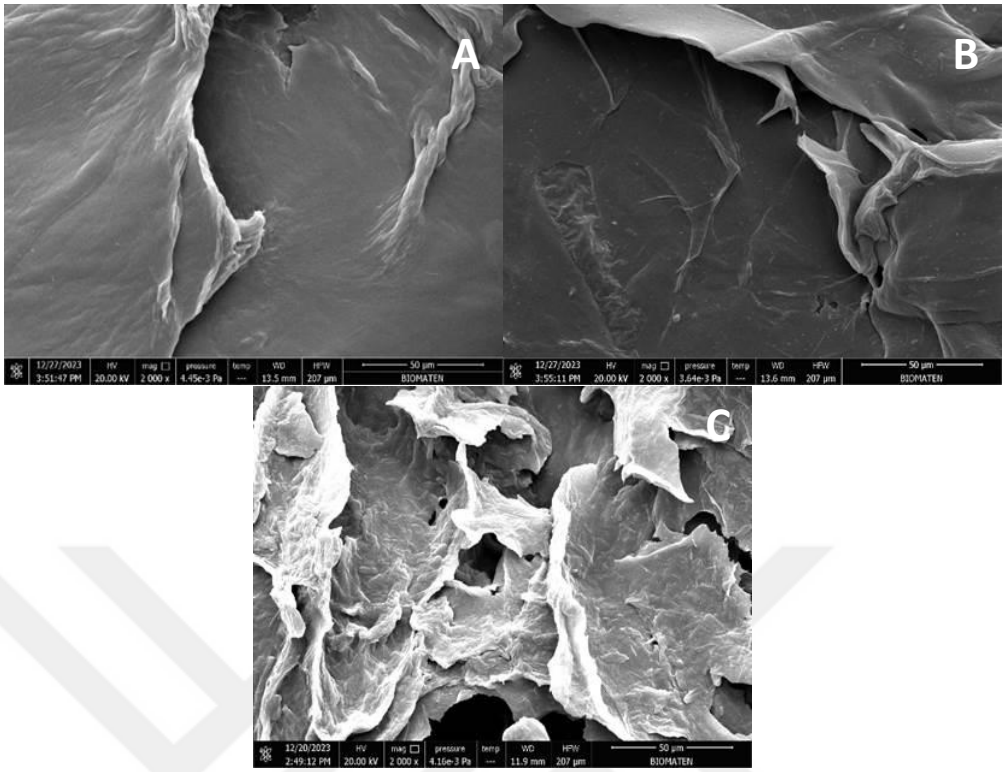


Figure 3.4. SEM images of CNCs treated with sulphuric acid at different durations A) 1h (2000X) B) 2h (2000X) C) 10h (2000X)

3.1.2 Oxidation of MCC

It was aimed to enforce the hydrogel structure using MCC to enhance the mechanical properties. MCC was oxidized to enable Schiff Base Reaction with chitosan for achieving this purpose. The oxidation degree was calculated with the titration method previously described and found to be 60.45±6.51% which is a close value to those reported in similar studies (Liu et al., 2022). Figure 3.5. shows the characteristic groups of MCC and ox. MCC evaluated with FT-IR analysis. The absorption in the spectrum of MCC at 3329 cm^{-1} and 2897 cm^{-1} was attributed to OH and CH_2 stretching respectively while the peaks at 1315 cm^{-1} and 1028 cm^{-1} were resulted from the existence of C-O-C and C-H stretching respectively. The oxidation of MCC was also proven with this analysis. A new absorption band at 1589 cm^{-1} in the spectrum of ox. MCC was observed. This demonstrates the oxidation reaction on

the hydroxyl group of MCC to prepare a basis for aldehyde group formation indicating close results with similar studies in the literature (Yin et al., 2022).

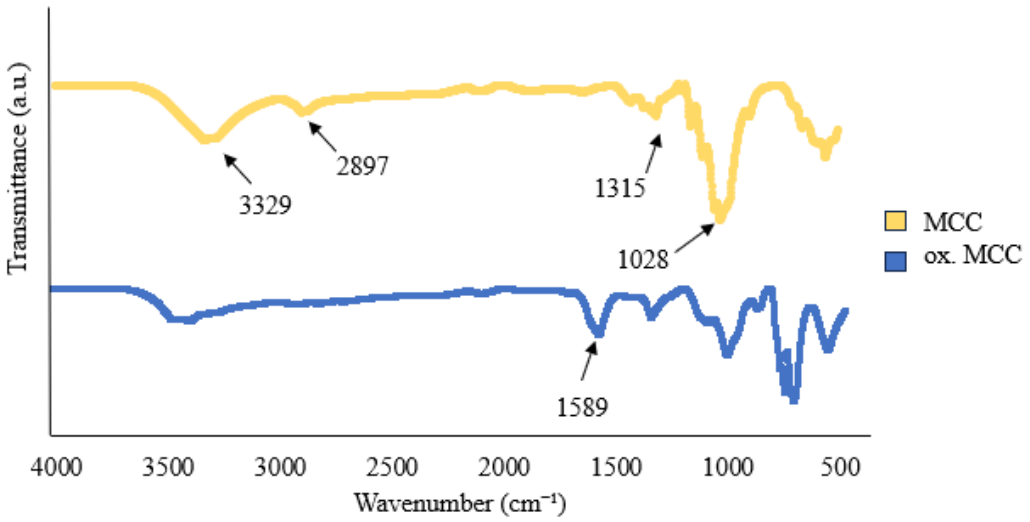


Figure 3.5. FTIR spectra of MCC and ox-MCC

3.2 Modification of Maltodextrin (MD)

Utilizing functionalized macromolecules derived from biocompatible polymers, namely oxidized polysaccharides is one of the effective ways to create functional chitosan hydrogels via Schiff Base Reaction (Serrero et al., 2010). Maltodextrin is a low molecular weight byproduct of starch hydrolysis which is more soluble, abundant, and was used for the preparation of injectable chitosan-based hydrogel in a recent research. It was expected that oxidized maltodextrin and chitosan would react via Schiff Base Reaction; forming an imine bond (C=N) between the -CHO group of ox. MD and -NH_2 group of CS (Lin et al., 2023).

The degree of oxidation of maltodextrin was found with the hydroxylamine hydrochloride titration method and calculated as 20.35±1.61% which was similar to those reported in the literature (Serrero et al., 2010). In the FT-IR spectra of ox.MD, a new absorption band at 1726 cm^{-1} with a small peak attributed to aldehyde groups (C=O stretching) was observed (Figure 3.6). The reason for the smallness of the peak

is relevant to the lower oxidation degree of MD respectively (20.35±1.61). In the FT-IR spectrum of maltodextrin, the characteristic peaks at 3298 cm^{-1} and 2394 cm^{-1} were attributed to the sugar ring's stretching vibrations of the -OH and -CH_2 groups, respectively. These results were consistent with the literature (Dai et al., 2023).

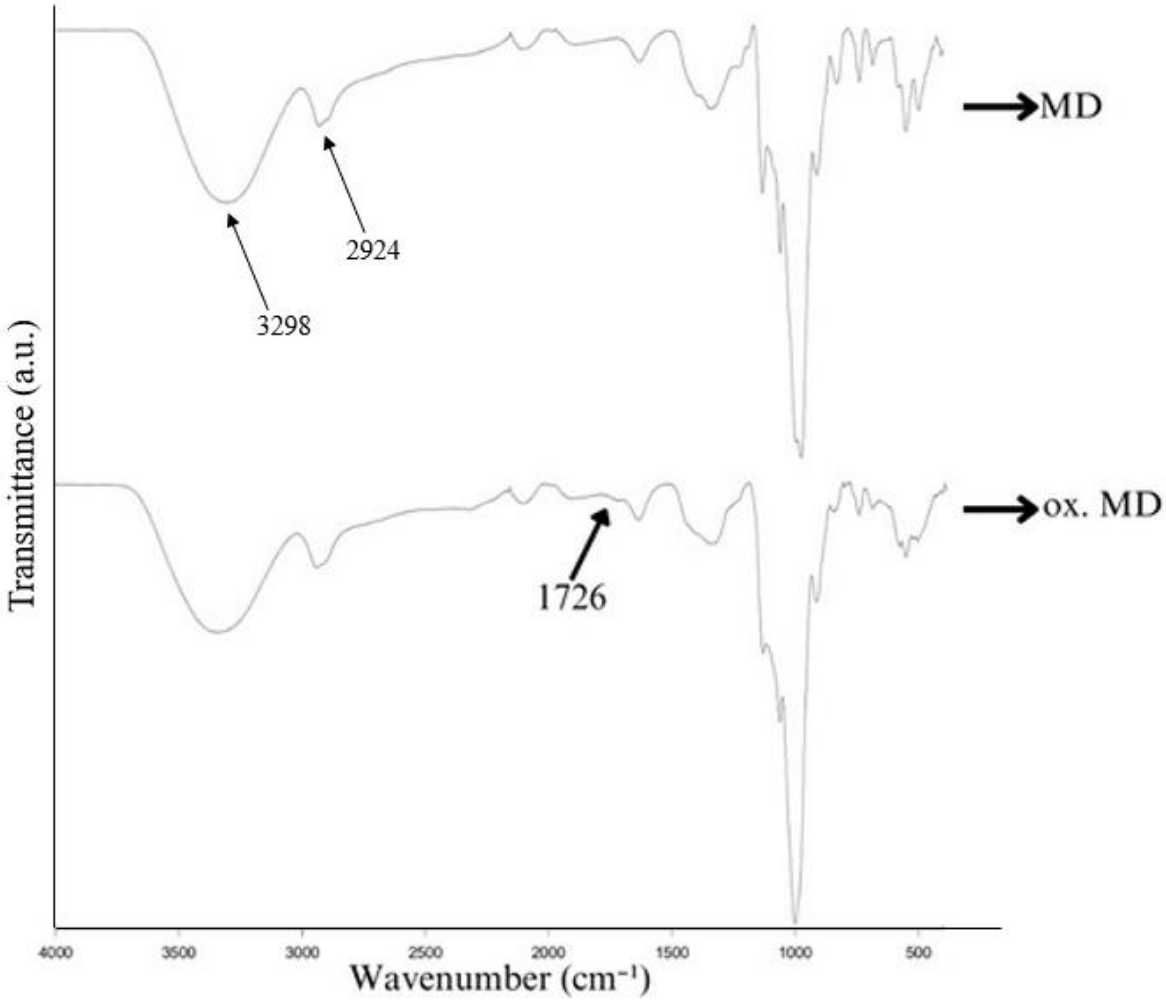


Figure 3.6. FTIR spectra of maltodextrin (MD) and ox-MD

3.3 Characterization of Hydrogels

Hydrogels are ideal biomaterial candidates in tissue engineering applications due to their favorable properties. The chemical and physical natures of native tissues especially NP region in the context of IVD tissue, can be imitated by chitosan hydrogels. Various methods have been established to produce chitosan hydrogels. In particular, employing a Schiff Base reaction between functional groups of chitosan with the functional groups of another polymer is widely preferred for preparing chitosan-based hydrogels (Serrero et al., 2010).

In order to obtain the best composition, different hydrogel concentrations using different ratios of chitosan, ox. maltodextrin and ox. microcrystalline cellulose were investigated (Table 2.1) according to their gelling time, injectability, swelling and degradation, as well as mechanical properties. To accomplish the Schiff Base Reaction for preparing chitosan-based hydrogels, firstly maltodextrin content was oxidized. Since there were some problems with gathering proper nano forms-CNCs, MCC was used instead after a similar oxidation process like in maltodextrin. This oxidation modification was thought to enable better incorporation into hydrogel via Schiff base reaction and also to strengthen the hydrogel matrix with increased bonding between molecules, (Figure 3.7) which will also improve the mechanic structure of the hydrogel via constructing imine bonds with chitosan with resultant carbonyl groups after oxidation treatment with sodium periodate ($NaIO_4$).

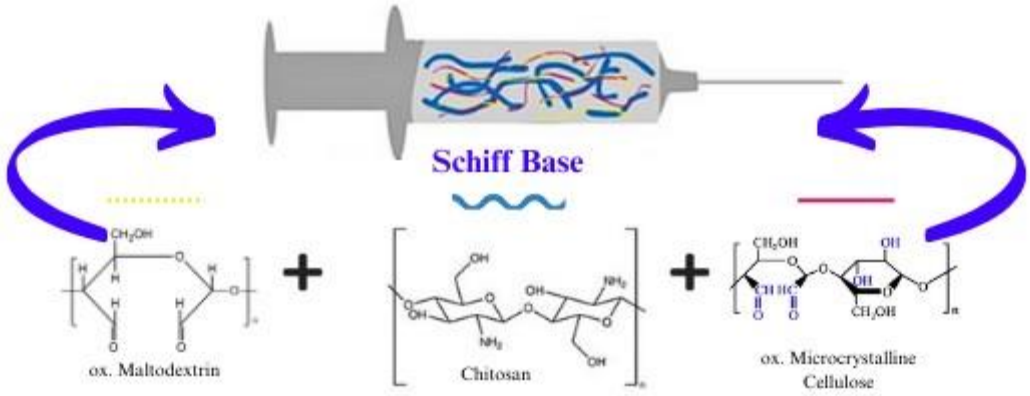


Figure 3.7. Illustration of the composite hydrogel components

3.3.1 Gelation Time and Injectability

Gelation time (Table 3.1) with tilting assay (Figure 2.1) and injectability of the hydrogels was studied. Results showed that the optimum final concentration of chitosan in the CS/ox. MD hydrogels was 2% (w/v). Below this concentration (1.5 % w/v), gelation did not occur and above (2.5 % w/v) gelation happened very fast that makes it difficult to inject the hydrogels from a syringe needle (21 Gauge). After that, the effect of adding different concentrations of oxidized maltodextrin to hydrogels was tested for gelling time and injectability by setting the final CS concentration to 2 w/v%. It was seen that the gelation time significantly decreased from 83.33±3.06 sec. to 39.67±1.53 and 38.33±3.51 sec. when ox. MD concentrations were 0.25 and 0.5 w/v% respectively. These results were in accordance with similar studies in the literature showing the fact that the gelation time - decreased as the content of oxidized substance (ox. MD) increased owing to more Schiff Base reaction during the gelation process (Yu et al., 2023, Wang et al., 2023). There were no significant changes between the 0.25 and 0.5 w/v% ox. MD groups in terms of gelling time. It was decided to continue the hydrogel experiments

with 2 w/v% CS-0.5 w/v% ox. MD composition since it showed the best results for the gelling time, injectability, and further characteristics (swelling and degradation experiments).

Furthermore, the effect of the addition of oxidized microcrystalline cellulose (ox. MCC) to the hydrogel was also investigated in terms of gelation time and injectability properties. Results showed that, incorporation of ox. MCC significantly decreased the gelling time to app. 21 s at0.25 w/v% concentration and to app 12 s at 0.5 w/v% concentration (Table 3..1) which again can be related with the incorporation of more aldehyde content and Schiff Base reaction. Also, after this point, the hydrogel showed instantaneous gelling properties which could be considered as a benefit while administration in a surgery in an operating room. At 0.125 w/v% concentration of ox. MCC, the data was not considered to be statistically significant -although it was still lower (33.33±4.73 s)- compared to non-ox. MCC groups.

Injectability observations of hydrogels (Figure 3.8) showed that all groups were injectable.

Table 3.1. Gelation time of the hydrogels prepared with different final concentrations of CS, ox. MD and ox. MCC (n=3)

CS (w/v%)	ox MD.(w/v%)	ox. MCC (w/v%)	Gelation Time (s)
2	0.125	0	83.33±3.06
2	0.25	0	39.67±1.53^{ns}
2	0.5	0	38.33±3.51^{ns}
2	0.5	0.125	33.33±4.73

2	0.5	0.25	21.67±3.51
2	0.5	0.5	12.67±2.08

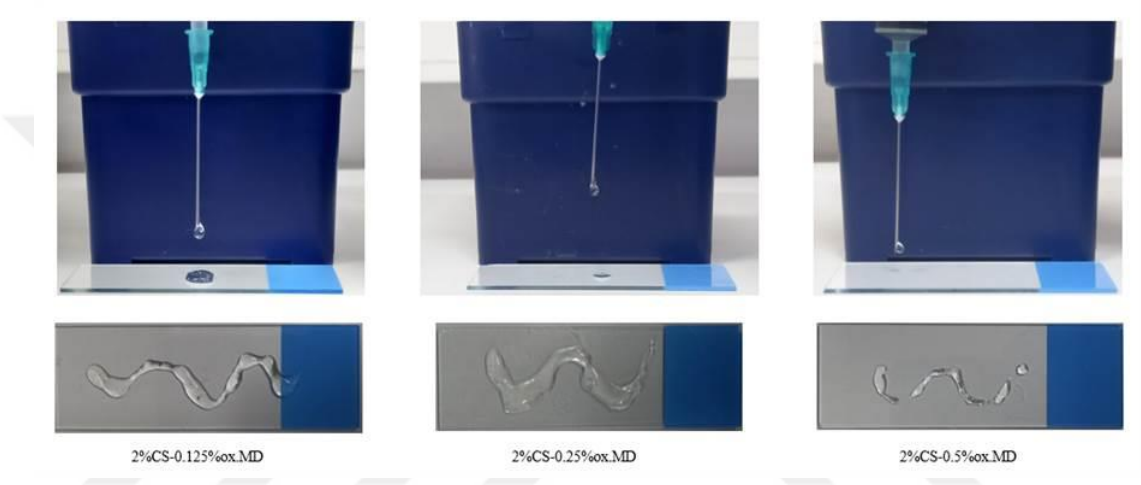


Figure 3.8. Injectability observation of the gels through syringe needle (21 G).

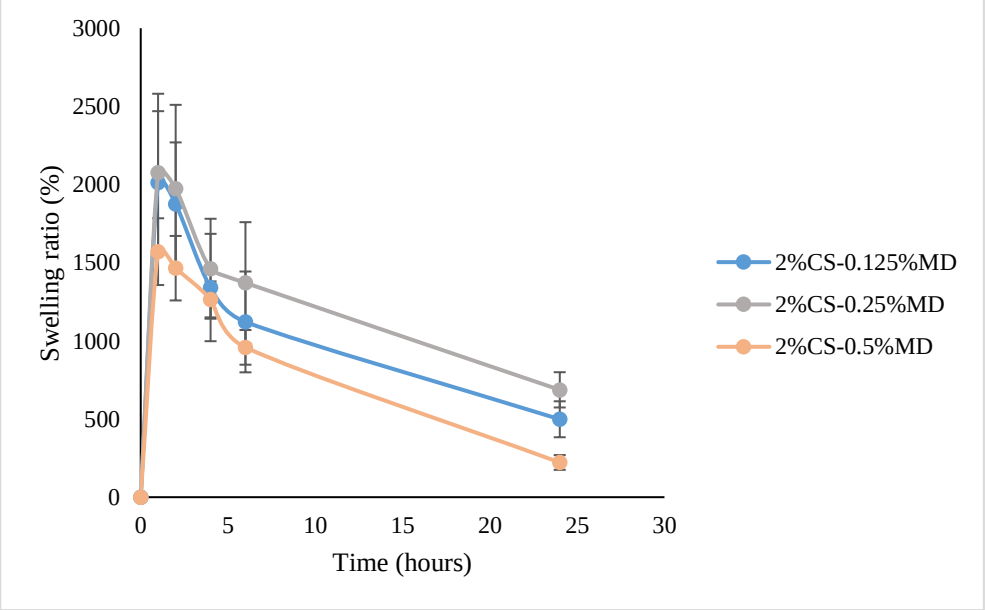
3.3.2 Swelling and Degradation Studies

Swelling and degradation experiments were conducted with 2% CS and different concentrations of ox. MD (0.5%, 0.25%, 0.125%) 2%CS-0.125% ox. MD and 2%CS-0.25% ox. MD hydrogel showed similar swelling ratios reaching a maximum swelling at approximately 2000%. (Figure 3.9-a) On the other hand 2% CS-0.5% ox. MD group had swollen slightly less than the other two and reached a maximum swelling ratio of about 1500% on the first day. The weight loss of hydrogels was also studied in PBS (0.1 M, pH 7.4) at 37°C. The hydrogel group with the highest ox. MD content (2%CS-0.5% ox. MD) showed the most stable hydrogel properties

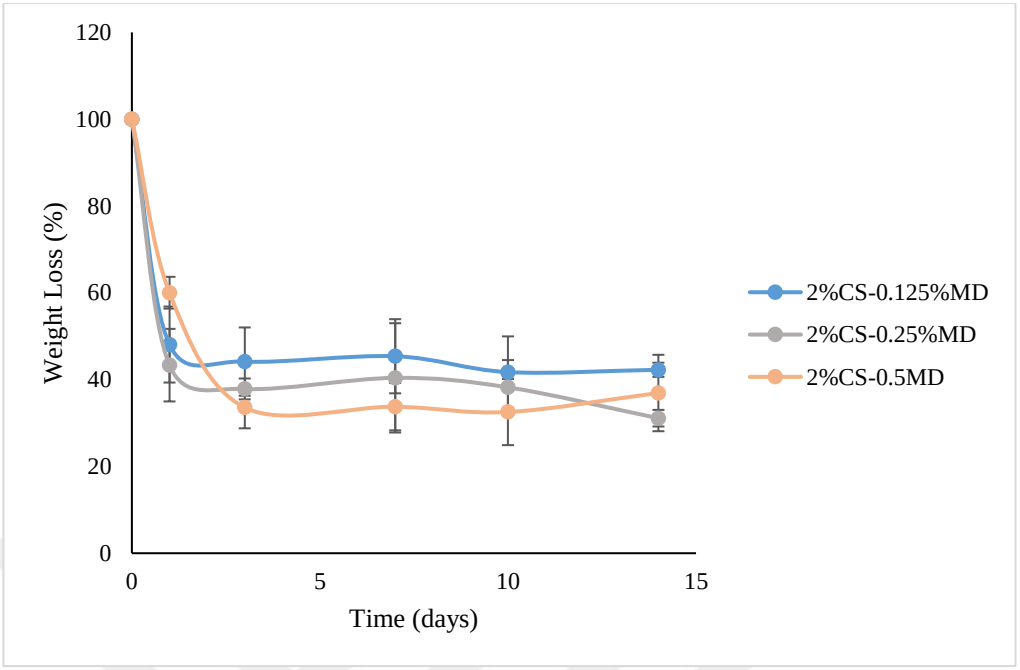
losing 40 % of its weight on the first day of the degradation experiment while the other two groups (0,125 and 0,25 MD groups) had more weight loss. This result can be explained by the fact that when more aldehyde content (ox. MD) is incorporated, the structure of hydrogel becomes more stable due to the existence of higher imine bonds between chitosan and ox. MD (Yu et al., 2023).

Also, to see the effect of MCC on the chitosan-based hydrogels, swelling, and degradation experiments were repeated. It can be said that hydrogels with ox. MCC content showed similar swelling properties compared to hydrogel groups not containing ox. MCC (Figure 3. 9-c). It was observed that incorporating ox. MCC made the structure more stable and enabled the hydrogels to retain their weight up to 70-75% of their dry weights (Figure 3. 9-d).

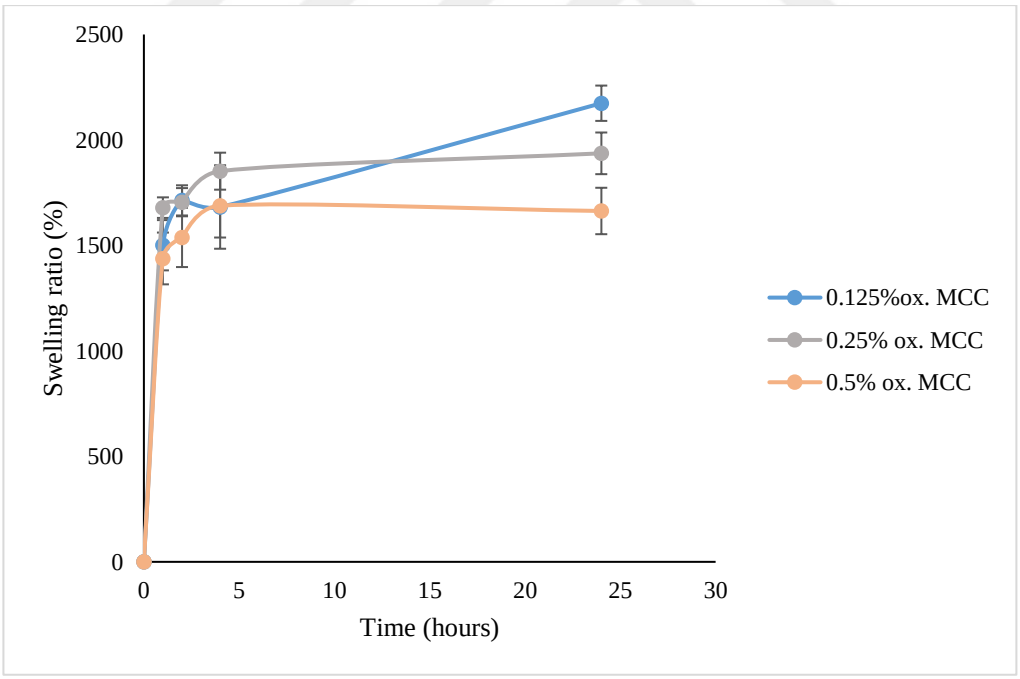
a)



b)


120
100
80
60
40
20
0
Weight Loss (%)
0
5
10
15
Time (days)
2%CS-0.125%MD
2%CS-0.25%MD
2%CS-0.5MD

c)


2500
2000
1500
1000
500
0
Swelling ratio (%)
0
5
10
15
20
25
30
Time (hours)
0.125%ox. MCC
0.25% ox. MCC
0.5% ox. MCC

d)

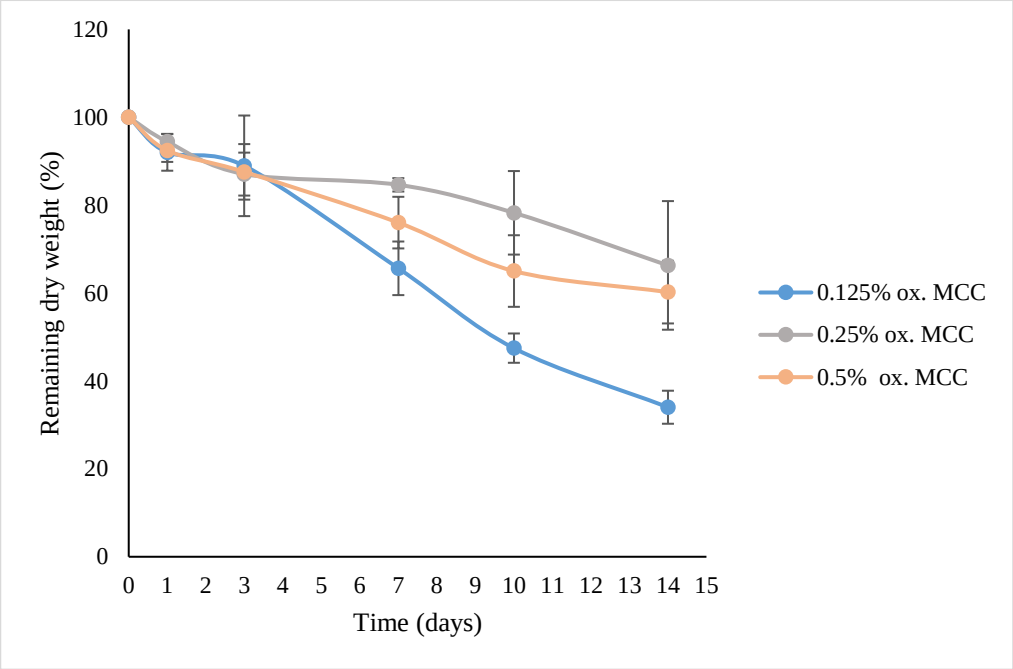
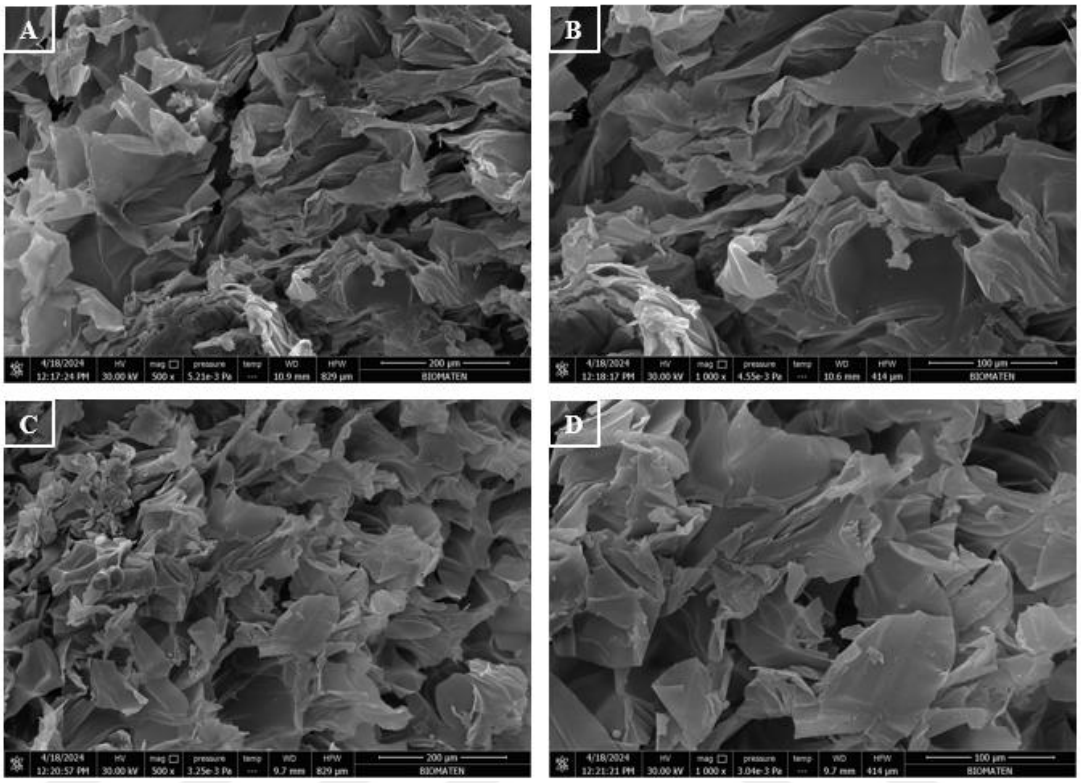


Figure 3. 9. a) Swelling graph of 2% CS hydrogels with 0.125%, 0.25% and 0.5% ox. MD concentrations b) Weight loss graph of 2% CS hydrogels with 0.125%, 0.25%, and 0.5% ox. MD concentrations c) Swelling graph of 2% CS-0.5% ox. MD hydrogels with 0.125%, 0.25%, and 0.5% ox. MCC concentrations, d) Remaining dry weight graph of 2% CS-0.5% ox. MD hydrogels with 0.125%, 0.25%, and 0.5% ox. MCC concentrations in PBS (0.1 M, pH: 7.4) at 37°C.

3.3.3 Morphology of the Hydrogels

Morphological analysis of different hydrogel groups was done with SEM analysis. It was observed that all 4 hydrogel groups (CS%2-ox.MD%0.5, CS%2-ox.MD%0.5-ox.MCC%0.125, CS%2-ox.MD%0.5-ox.MCC%0.25 and CS%2-ox.MD%0.5-ox.MCC%0.5) showed similar morphological properties and had relatively porous nature. It can be said that due to the escalating cross-linking density, increasing ox. MCC made the structure more compact and stabilized and led to relatively smaller pore sizes which could be observed in Figure 3.10-g, h which shows the SEM images of the highest ox. MCC content. These results are similar to the findings in the literature. For instance, in their work, Ghorbani et al. reported that they had more compact hydrogels with smaller pore sizes when the ox.

cellulose nanocrystal (CNC) content was increased in chitosan based hydrogels (Ghorbani et al., 2020).



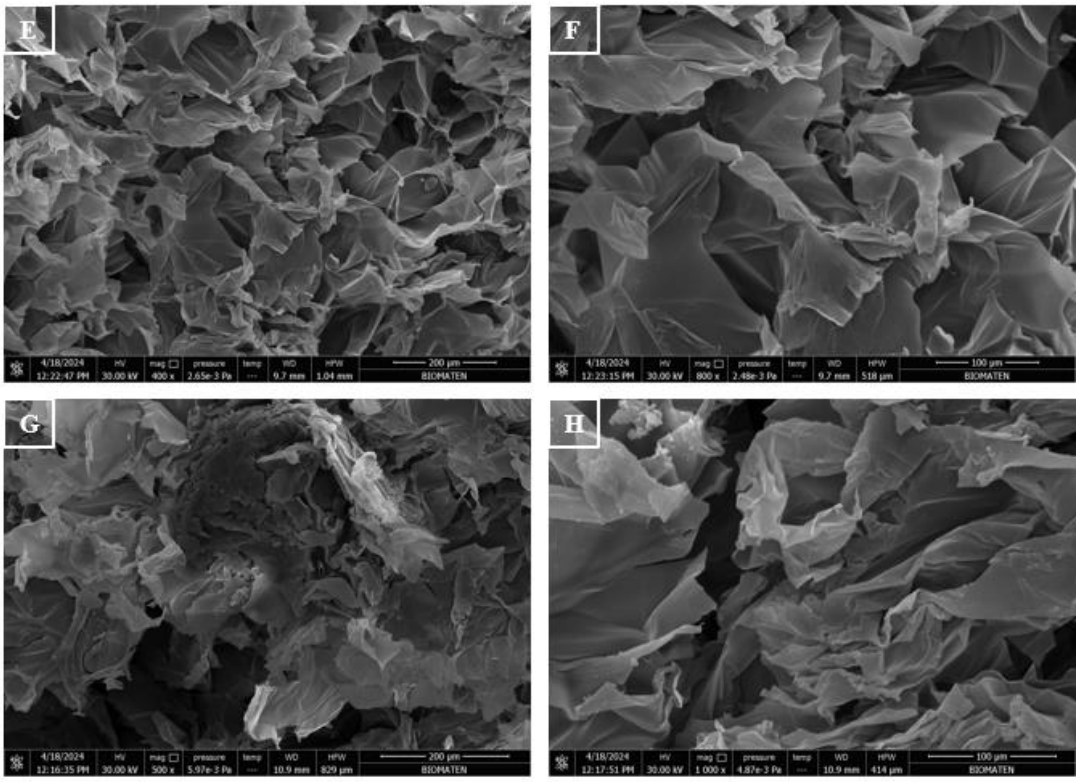


Figure 3.10. SEM images of the different hydrogel groups at different magnifications (500X and 1000X) a) CS%2-ox.MD%0.5 (500X) b) CS%2-ox.MD%0.5 (1000X) c) CS%2-ox.MD%0.5-ox.MCC%0.125 (500X) d) CS%2-ox.MD%0.5-ox.MCC%0.125 (1000X) e) CS%2-ox.MD%0.5-ox.MCC%0.25 (500X) f) CS%2-ox.MD%0.5-ox.MCC%0.25 (1000X) g) CS%2-ox.MD%0.5-ox.MCC%0.5 (500X) h) CS%2-ox.MD%0.5-ox.MCC%0.5 (1000X)

3.3.4 Mechanical Properties of the Hydrogels with MCC Reinforcement

After deciding the optimal chitosan and ox. MD concentrations (2%CS-0.5%ox.MD) in the hydrogel structure, it was tried to reinforce the hydrogel mechanically with the addition of ox. MCC to the structure. Mechanical test results (Figure 3.11) were obtained by using different concentrations of MCCs (0.125, 0.25, and 0.5% (w/w)).

Accordingly, there was a considerable improvement in Young's Modulus values with the increase in the concentrations of MCC in hydrogels (Figure 3.11). While the value was only 4.20±0.95 kPa for the hydrogel group not containing MCC, it

became 23.73±5.75 kPa in the hydrogel group that had the highest MCC, which resulted in the highest Young Moduli (an increment of approximately 6-fold in the Young Moduli value) compared to hydrogels without MCC. When the MCC concentration was doubled from 0.125% w/w to 0.25% w/w, Young Moduli values were nearly doubled (from 7.54±0.68 kPa to 14.84±3.88 kPa). These results were similar to those found previously in the literature that showing the effect of MCC on improvement of the mechanical properties of the hydrogel. (Yin et al., 2022, Ramires et al., 2020). In the literature, the compressive modulus value for the Nucleus Pulposus in IVD composition is reported between 3-31 kPa (Whatley et al., 2012). Looking at the mechanical test results it can be said that, MCC reinforced hydrogels could meet the requirements in terms of mechanical properties.

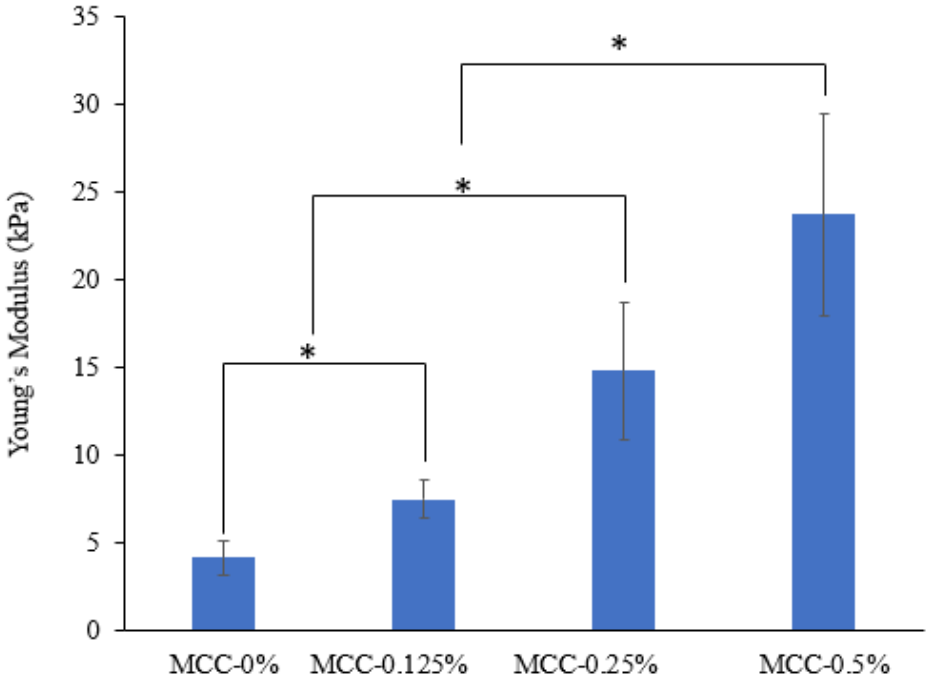


Figure 3.11. Young's Modulus comparison graph of chitosan/ox-MD hydrogels containing different concentrations of MCCs (0, 0.125, 0.25, and 0.5% (w/v)

3.3.5 Melatonin Release from Hydrogels

Melatonin release profiles of hydrogels with and without MCC content were investigated. Release profiles of hydrogels were constructed using the melatonin calibration curve in PBS (Appendix A, Figure A). Results (Figure 3.12) showed nearly similar MEL release profiles for the hydrogels with and without MCC added to the hydrogels. There was approximately a 10 percent increase in the release rate of MEL from the hydrogel involving MCC according to the experiment results. In both hydrogels, there was an initial burst release with approximately 31 % and 25 % being released from MCC involving and not involving hydrogels, respectively, within the initial 4 h. This was an expected outcome due to hydrogels' high water interaction capacity. While the hydrogel without MCC released 26.65 % ±3.7 MEL at the end of 3 days, the MEL release percentage reached up to 35.29 % ± 2.3 for the hydrogel with MCC. The release profiles of hydrogels showed similarity with the previous work (Zheng et al., Hou et al., 2023). In their work, Zheng et al. reported that melatonin was released from chitosan nanoparticles at the rate of approximately 35% at the end of 3 days (Zheng et al., 2023). After 24 h the release was very steady for both groups.

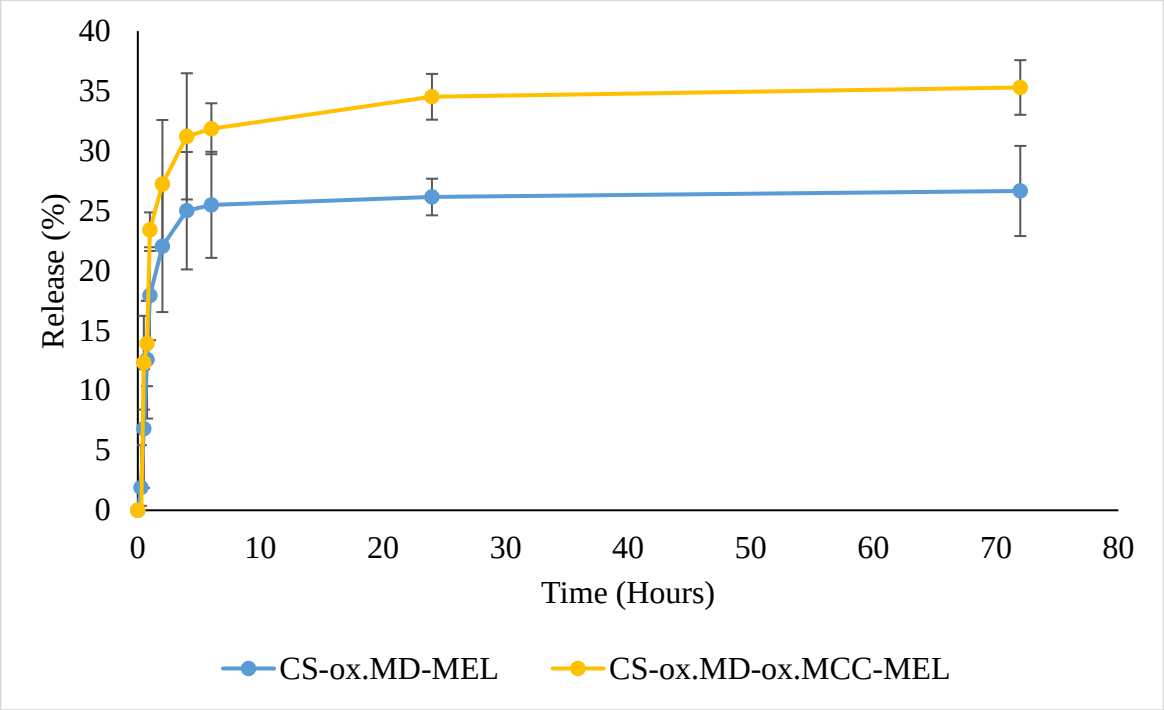


Figure 3.12. Melatonin Release profiles from hydrogels with and without MCC content in PBS media (0.1 M, pH: 7.4) at 37 ºC (n=3)

3.3.6 Cell Culture Studies

3.3.6.1 Isolation of Nucleus Pulposus (NP) Cells from Bovine Intervertebral Discs (IVD)

Nucleus Pulposus (NP) cells which are present in intervertebral discs (IVD) cartilaginous tissue were isolated from a bovine tail vertebral tissue (local slaughterhouse, Nidanur Birlik, Ankara). After the cell attachment, the media were refreshed regularly, and the phase contrast images of cells were taken at different periods of time (Figure 3.13). During the first week after isolation cells were round. They then became spread and gained fibroblastic morphology. The morphology of the NP cells was similar to that of previous studies (van den Akker, 2021). After the isolation, NP cells reached confluency nearly after 15 days. After the first passage, cells always reached confluency in 3 days and passaged when needed.

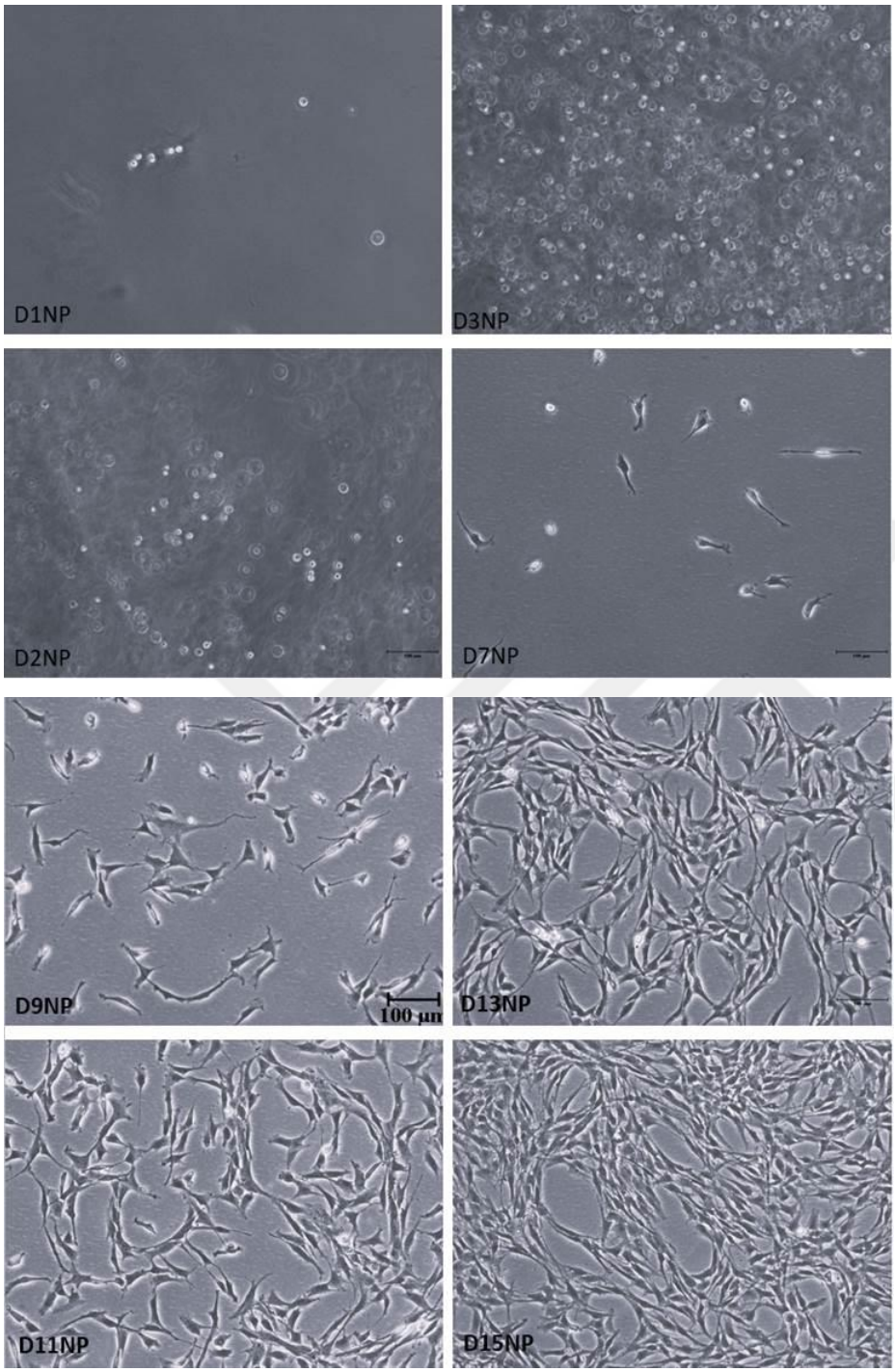


Figure 3.13. Phase contrast images of NP chondrocytes at different time periods (Scale bar: 100 µm)

3.3.6.2 Dose-dependent Cytotoxicity of Melatonin

Melatonin was reported to have important positive effects on the treatment of IVD degenerations (Ge et al., 2009). Dose-dependent cytotoxicity of melatonin on NP chondrocytes was investigated using the Alamar Blue assay (Figure 3.14). In similar studies in the literature, melatonin did not show obvious cytotoxicity below 1000 µM (Chen et al., 2019). In this thesis, dose-dependent cytotoxicity up to 100 µg/mL which is equivalent approximately to 430.51 µM was investigated. Similar to reports in the literature melatonin did not show any toxic effect on NP cells. No dose-dependent change in the morphology of NP cells was observed (Figure 3.15).

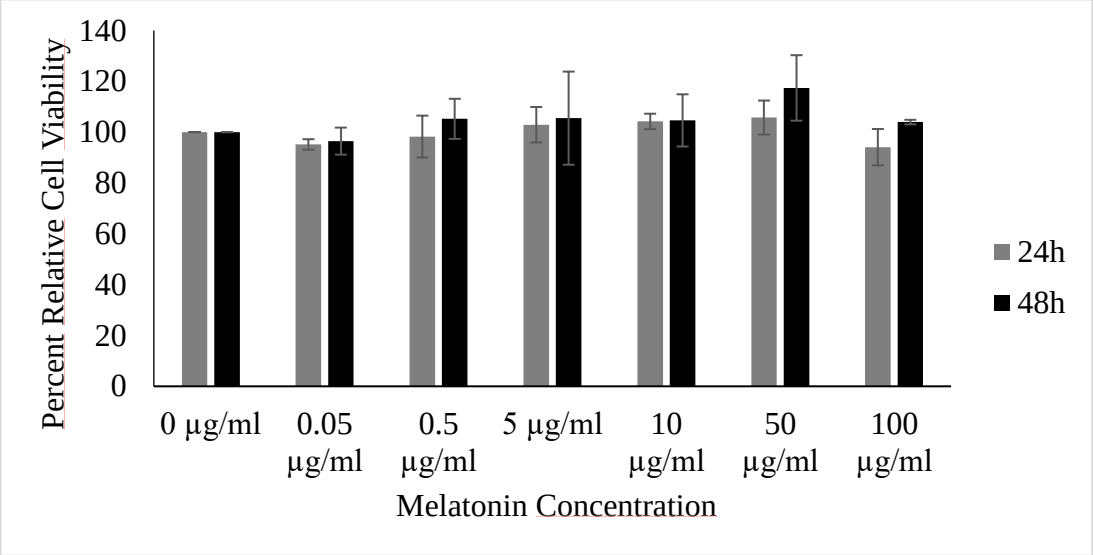
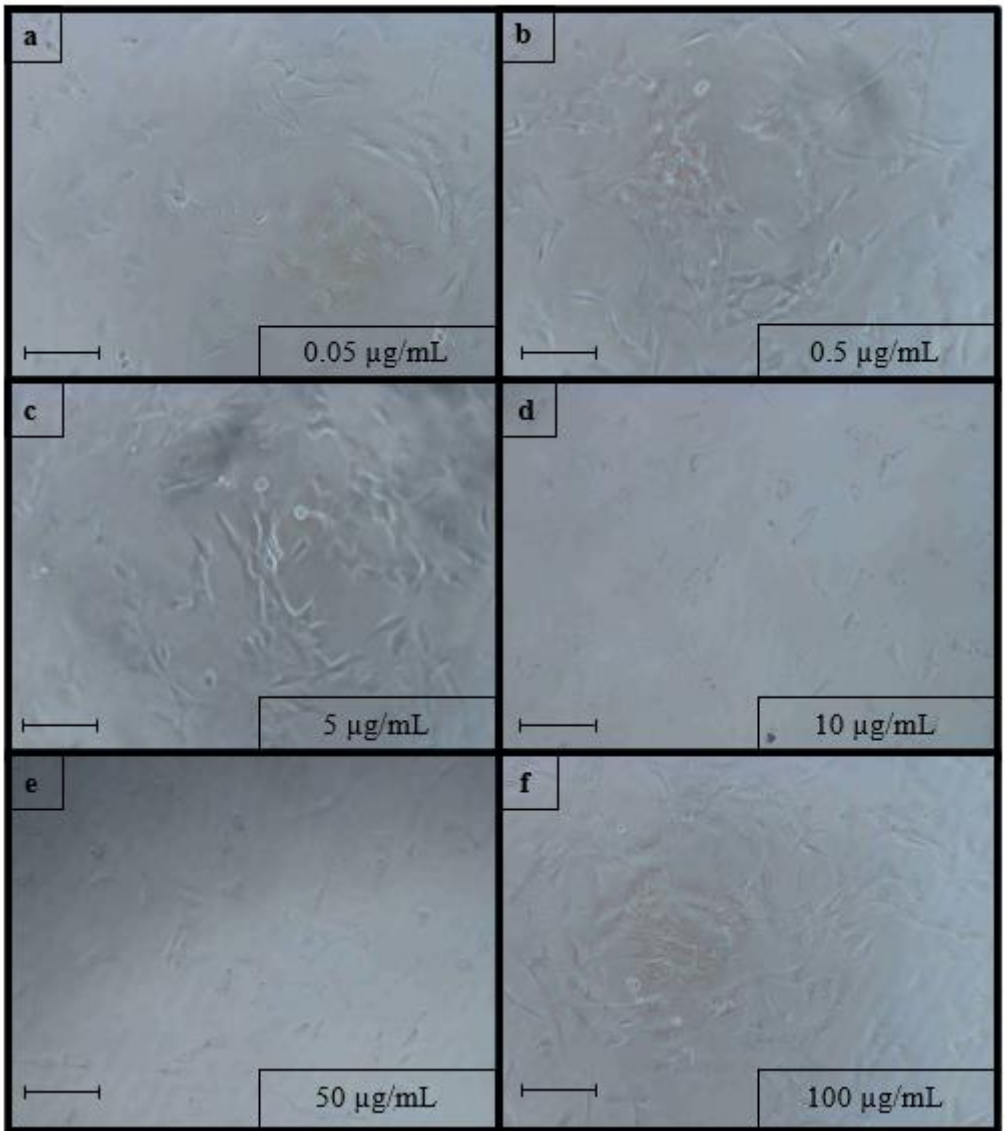


Figure 3.14. Percent relative cell viability of NP cells upon treatment with different melatonin concentrations (n=3)


a
0.05 µg/mL
b
0.5 µg/mL
c
5 µg/mL
d
10 µg/mL
e
50 µg/mL
f
100 µg/mL

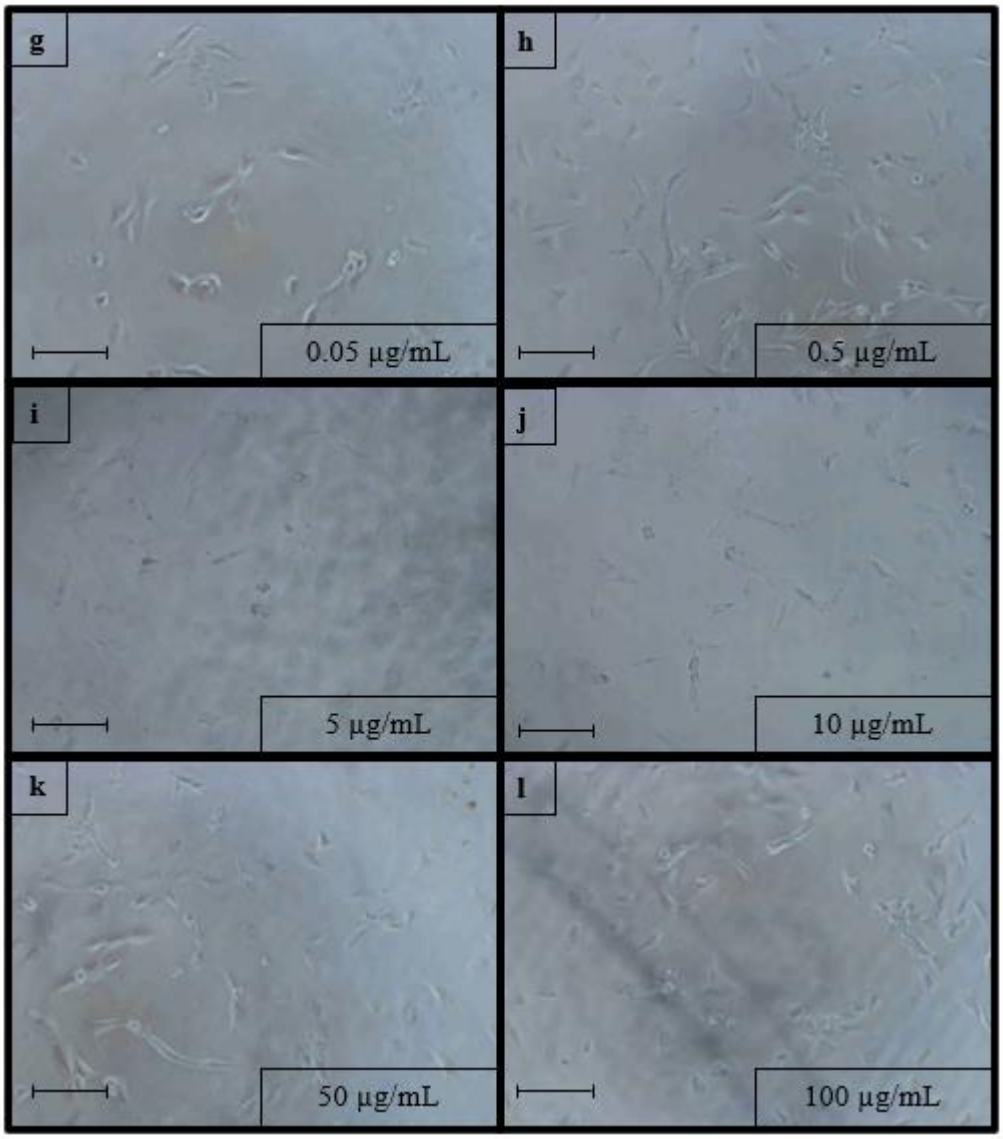


Figure 3.15. Phase contrast images of cells after 24 hours (a, b, c, d, e, f,) and 48 (g, h, i, j, k, l) hours of melatonin dose-dependent (0.05 µg/mL to 100 µg/mL respectively in the images) cytotoxicity evaluation using Alamar Blue assay (Scale bar: 100 µm).

3.3.6.3 Cell Viability and Proliferation Tests with Hydrogels

In order to evaluate the cytocompatibility of hydrogel groups (CS-ox. MD, CS-ox. MD+MEL, CS-ox. MD+ ox. MCC, CS-ox. MD+ ox. MCC+MEL), an indirect elution test was performed. The cells were incubated with the extracts obtained from the hydrogels and a 7-day viability study was performed using Alamar Blue Assay. After 24h duration, no toxic effect of hydrogels on the NP cells was observed (Figure 3.16). All four groups of hydrogels were cytocompatible and showed at least 95 % viability. The phase contrast images of cells treated with 4 different groups of hydrogel extracts were taken on days 1, 3, and 7 (Figure 3.17). There was no change in the morphology of NP cells. There were no significant differences between the cell viability of groups during the 7-day in vitro experiments. An increase in the viability of cells in the hydrogel groups was observed compared to their day 1 results. Additionally, on days 3 and 7 the extracts of hydrogels which included MEL resulted in a significant increase in the viability of the cells compared to the control group. This result could be correlated with the positive effect of MEL on the nucleus pulposus cells. The (CS-ox.MD+ox.MCC) hydrogel group also showed a significant viability increase on day 7 compared to the first day. Results were consistent with the previous work (Wu et al., 2023) in terms of the ameliorating effect of melatonin on NP cells in IVD degenerations.

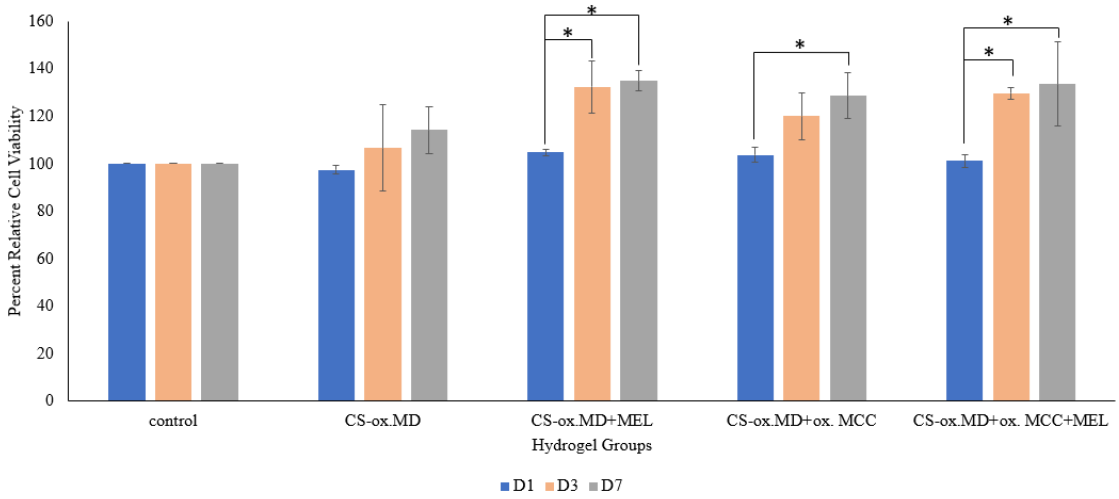
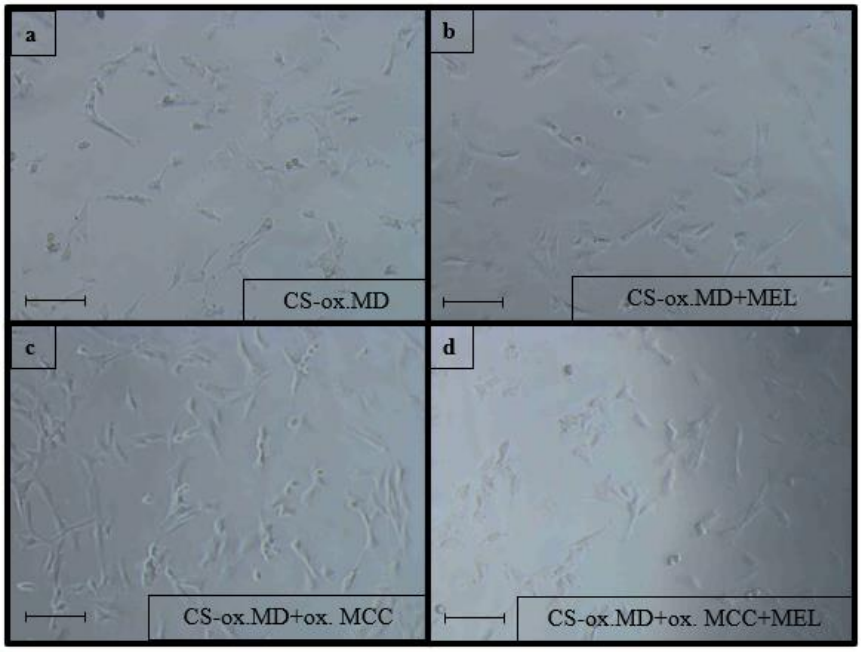
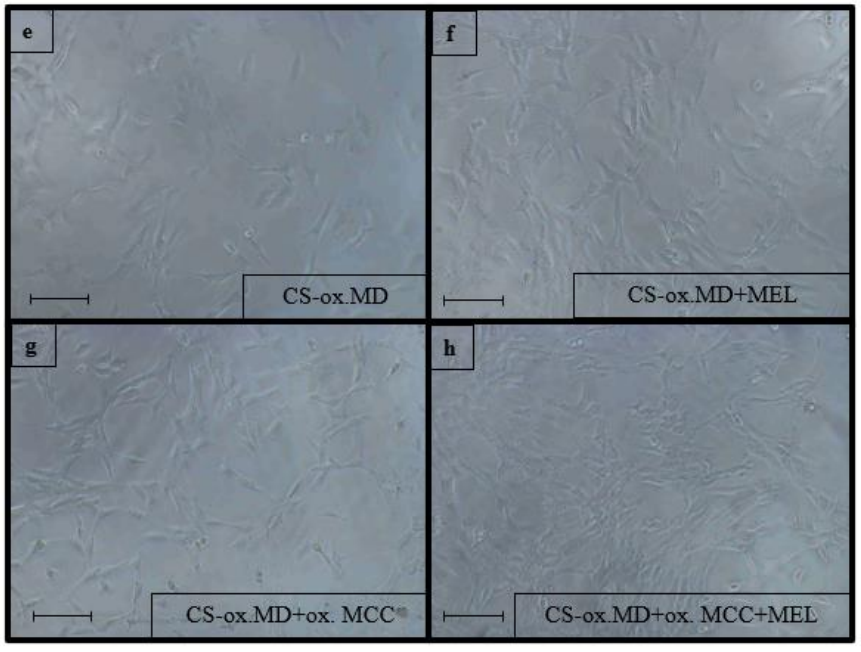


Figure 3.16. Percent relative cell viability of NP cells upon incubation with different hydrogel groups for 1 day, 3 days, and 7 days, NP cells without hydrogel extracts used as control groups (n=3)


a
CS-ox.MD
b
CS-ox.MD+MEL
c
CS-ox.MD+ox. MCC
d
CS-ox.MD+ox. MCC+MEL


e
CS-ox.MD
f
CS-ox.MD+MEL
g
CS-ox.MD+ox. MCC
h
CS-ox.MD+ox. MCC+MEL

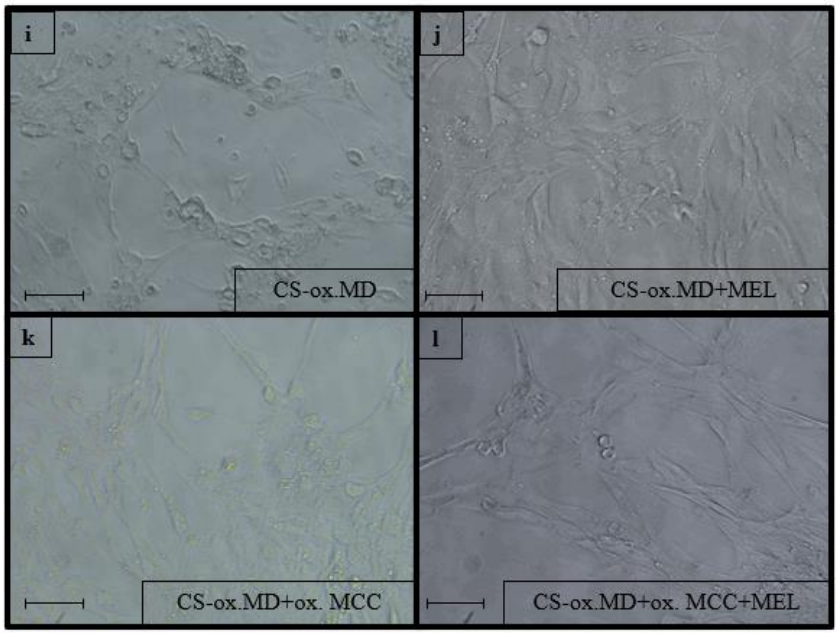


Figure 3.17. Phase contrast images of NP cells after 1 day (a, b, c, d), 3 days (e, f, g, h) and 7 days (i, j, k, l) treatment with different hydrogel extracts (CS-ox. MD, CS-ox. MD+MEL, CS-ox. MD+ ox. MCC, CS-ox. MD+ ox. MCC+MEL respectively in the images) cytotoxicity evaluation using Alamar Blue assay (Scale bar: 100 μm).

CHAPTER 4

CONCLUSION

Injectable hydrogels have gained attention in recent years due to their numerous benefits but most importantly because of their advantage of using minimally invasive strategies. In this thesis, injectable hydrogels were developed via Schiff Base reaction between amino groups of CS and aldehyde groups of oxidized MD and oxidized MCC for investigating their potential as a biomaterial for intervertebral disc applications. Best composition for CS and ox-MD was observed to be 2% and 0.5% respectively for hydrogel formation. A fast gelling and injectable formulation was achieved. The addition of MCC to this composition ameliorated the degradation profiles besides significantly improving the mechanical property (Young's Modulus) and further decreasing the gelation time of the hydrogels. Melatonin loaded hydrogels showed higher release amounts with MCC involving groups owing to the higher water holding capacity of this group which is also a desired characteristic for IVDs.

All of the hydrogel compositions were found cytocompatible for nucleus pulposus cells. In melatonin-loaded groups, better cell proliferation was also observed in 7-day cell culture experiments.

In conclusion, these findings reveal that melatonin-loaded injectable chitosan-based hydrogels reinforced with microcrystalline cellulose developed in this study hold great promise in cartilage tissue engineering, especially in applications of intervertebral disc degenerations.

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APPENDICES

A. Calibration Curve of Melatonin

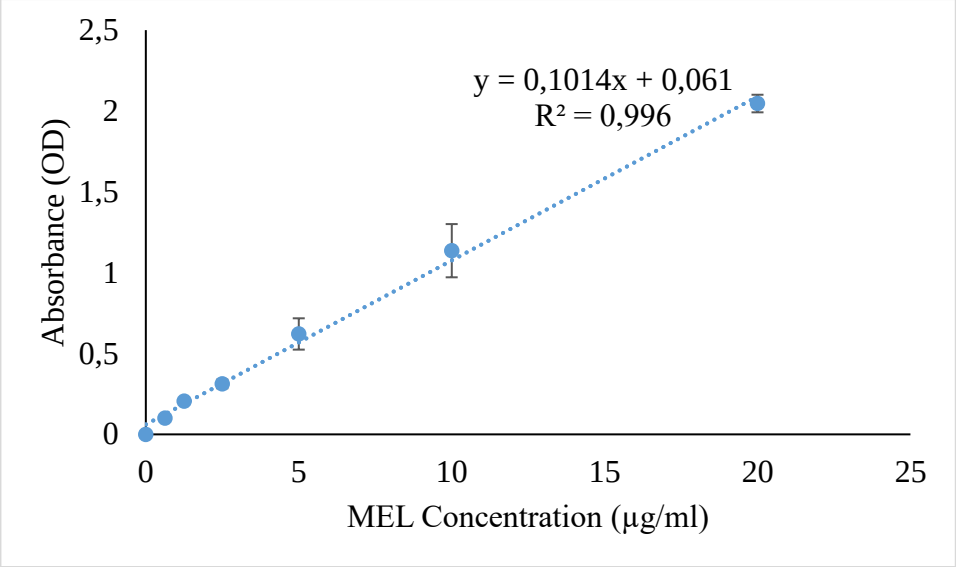

Figure A. Calibration curve of melatonin constructed with different concentrations in PBS at 222 nm (n=3)