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Evaluación *in vitro* del efecto de estimulación eléctrica sobre condrocitos cultivados en un constructo tridimensional

Juan José Saiz Culma

Universidad Nacional de Colombia

Facultad de Medicina

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In vitro evaluation of the effect of electrical stimulation on cultured chondrocytes in a three-dimensional construct

Juan José Saiz Culma

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Thesis supervisor
Ph.D., Juan Jairo Vaca González
Thesis co-advisor
Ph.D., Diego Alexander Garzón Alvarado

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Biomimetics Laboratory: Tissues and Organs Mechanobiology Group

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I dedicate this goal to,

To my mother, a loving, charming and courageous woman, who taught me to be a kind person to others and improve myself every day.

To my father, you have always advised me on any subject. You are a role model.

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Abstract

Treatment of degenerative pathologies of articular cartilage remains a clinical challenge. Biophysical stimuli such as electric fields appear to be a promising non-invasive tool for cartilage tissue restoration. Previous studies have indicated that the electric field promotes the proliferation and molecular synthesis, such as glycosaminoglycans, in chondrocytes. However, in these studies there is no evidence of similarity in the results obtained, due to the different experimental setups used in the investigations. In this *in vitro* study, the effects of capacitively coupled electric fields were evaluated with an alternating voltage of 50 V (corresponds to 7.7 mV/cm) and 100 V (corresponds to 8.7 mV/cm) with a frequency of 60 kHz during 21 days, on cultured chondrocytes in gelatin hydrogels. Cell quantification and glycosaminoglycan detection were measured from the stimulated and control samples. The results on day 7 showed a reduction in cell proliferation by 24.7% and 39.2% ($p < 0.05$) in the samples stimulated with electric fields of 7.7 mV/cm and 8.7 mV/cm, respectively. However, on day 7, in the samples stimulated with an electric field of 8.7 mV/cm, an increase of 35.7% ($p < 0.05$) was obtained with respect to the control in the production of glycosaminoglycans. In conclusion, the results indicate that electrical stimulation has an effect on the synthesis of important molecules that make up the extracellular matrix of hyaline cartilage, such as glycosaminoglycans, which allows to conclude that electric stimulation is a very promising tool to enhance articular cartilage tissue engineering outcomes in therapies that use hydrogels.

Keywords: Hyaline cartilage, Electric fields, Chondrocytes, Cell proliferation, Glycosaminoglycans, Biophysical stimuli.

Resumen

El tratamiento de las patologías degenerativas del cartílago hialino sigue siendo un desafío clínico. Los estímulos biofísicos como los campos eléctricos parecen ser una herramienta prometedora no invasiva para la restauración del tejido cartilaginoso. Se han llevado a cabo investigaciones donde se evidencia que el campo eléctrico promueve la proliferación y síntesis molecular, como los glicosaminoglicanos, en condrocitos. Sin embargo, en estos estudios no se evidencia una similitud en los resultados obtenidos, debido a las diferentes configuraciones experimentales empleadas en las investigaciones. En este estudio *in vitro*, se evaluaron los efectos de los campos eléctricos acoplados capacitivamente con un voltaje en forma de onda sinusoidal de 50 V (corresponde a 7,7 mV/cm) y 100 V (corresponde a 8,7 mV/cm) con una frecuencia de 60 kHz durante 21 días, en condrocitos cultivados en hidrogeles de gelatina. A partir de las muestras estimuladas y controles, se llevaron a cabo mediciones cuantitativas de proliferación celular y detección de glicosaminoglicanos. Los resultados en el día 7, mostraron una reducción de la proliferación celular en un 24,7% y 39,2% ($p < 0,05$) en las muestras estimuladas con campos eléctricos de 7,7 mV/cm y 8,7 mV/cm, respectivamente. Sin embargo, en el día 7, en las muestras estimuladas con un campo eléctrico de 8,7 mV/cm, se evidenció un incremento de 35,7% ($p < 0,05$) con respecto al control en la producción de glicosaminoglicanos. En conclusión, los resultados indican que la estimulación eléctrica tiene un efecto en la síntesis de moléculas importantes que componen la matriz extracelular del cartílago hialino, tales como los glicosaminoglicanos, lo cual permite mejorar los tratamientos que se realizan en la actualidad en ingeniería de tejidos y medicina regenerativa con el fin de recuperar el tejido.

Palabras clave: Cartílago hialino, Campos eléctricos, Condrocitos, Proliferación celular, Glicosaminoglicanos, Estímulos biofísicos.

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1 Introduction

Articular cartilage is of high importance for the functionality of joints, it ensures a smooth and lubricated friction surface as well as the transfer of loads to the subchondral bone [1–3]. According to these, the viscoelastic characteristic of cartilage, which is mainly mediated by the Extracellular Matrix (ECM), is essential. Further characteristics of articular cartilage are the limited potential for self-repair and that nutrient and oxygen supply is ensured only through fluid movement by diffusion and mechanical loading [1,4].

In contrast to bone, mature articular cartilage cannot regenerate itself after traumatic lesions or tissue degeneration due to various reasons like lack of vascular supply and low cell turnover [5]. For this reason, the cartilage tissue is often irreversibly damaged by both trauma and subsequent degenerative processes [6]. Multiple surgical techniques for the treatment of cartilage defects are used depending mainly on the defect size: osteochondral autograft and allograft transplantation, microfracturing, matrix augmented bone marrow stimulation and autologous chondrocyte implantation (ACI), as well as matrix-assisted ACI (MACI). For ACI, autologous chondrocytes are isolated from articular cartilage tissue derived from non weight-bearing joint regions and expanded during *ex vivo* cultivation. After expansion, the cartilaginous cells are re-implanted in damaged regions of the articular cartilage. In MACI treatment, the expanded chondrocytes are seeded onto a three-dimensional (3D) scaffold before re-implantation to provide a more physiological environment, thus resulting in improved cell integration in the defect and regeneration of cartilage tissue [7].

However, there are still limitations and deficiencies that include tedious *ex vivo* cell manipulation, potential tumorigenesis, therapeutic translation risk and regulatory approval. Accordingly, it is of great clinical significance to develop and achieve a method of complete and permanent repair of damaged cartilage. This tissue, in turn, has a lower ability to withstand mechanical stresses during joint loading [12]. Fortunately, tissue engineering, consisting of scaffolds, cells, and favorable growth factors, has evolved into a most promising therapeutic strategy for cartilage tissue reconstruction [13]. To achieve the perfectible regeneration of damaged cartilage, it is essential to offer the biodegradable scaffolds, simulate local characteristics of specific tissues, transport tissue cells and growth factors, and provide supports to newly formed tissues [14]. Ideally, cartilage tissue-engineered scaffolds should be porous, nontoxic, biocompatible, and biodegradable, and they should enhance cell differentiation and tissue generation, which need to possess high performance, matched rate

between the degradation and new tissue formation, diffused nutrients and metabolites behaviors, adhesion to the surrounding native tissue fusion, and fulfilment of the damaged sites [15]. Hydrogels, composed of natural or synthetic hydrophilic polymer strands connected with each other at crosslinking points, possess a unique 3D crosslinked polymeric network encompassing a wide range of chemical compositions and bulk physical properties. The hydrophilic nature of constituting polymeric chains allows the hydrogels to absorb amounts of water (more than 1,000-fold compared to their dry weight) to be applied in a variety of technological biomaterials for drug delivery and tissue regeneration, among which the *in situ* hydrogels possess the advantage of simple drug formulation and the ability to deliver both hydrophilic and hydrophobic drugs. Based on the cross-linking properties, hydrogels are classified into “chemical” and “physical” network gels. Chemically crosslinked hydrogels are generally held together by molecular bonds of synthetic polymers and possess stable, homogeneous, and adjustable structures. While physically crosslinked hydrogels are generally aggregated by secondary interactions such as molecular entangling, hydrogen bonds, ionic bonds, or hydrophobic interactions force them to form a reversible structure and self-healing properties [16]. Natural polymers are employed extensively in tissue engineering to produce hydrogels due to biocompatibility, enzymatic degradation, and the ability to conjugate with various factors, such as growth factors [17]. A wide range of natural polymers, including collagen, gelatin, chitosan, alginate, agarose, hyaluronic acid (HA), and some other biological materials are used in cartilage tissue engineering [18]. It has been proven that gelatin hydrogels are biocompatible and biodegradable materials [19]. Hydrogel scaffolds, based on gelatin due to their properties have attracted much attention in regenerative medicine [20].

In order to develop new and improved treatment options in the field of cartilage tissue engineering, one approach is electrical stimulation (ES) of biological samples and tissue [21]. Relevant studies in which exposure of electrical stimulation was reported to stimulate proliferation and cause increases in the turnover of matrix molecules by chondrocytes [22]. These effects can probably be attributed to the unique structure, in addition to biomechanical and electromechanical properties of articular cartilage [16,21]. In the literature, different approaches have been described for applying electric fields to cells *in vitro*. These approaches can be divided into direct stimulation, inductive coupling, capacitive coupling, and semi capacitive coupling, where direct and capacitive applications have been mainly used *in vitro* [22]. To apply direct current, electrodes are placed directly in the cell culture medium, which serves as conductors. The disadvantage of direct coupling is the interaction of the electrode material with the cell culture environment and the possible resulting electrochemical reactions such as pH change, formation of hydrogen peroxide or reactive oxygen species, which can damage exposed cells. The capacitive coupling of the cell culture medium then takes place via the frequency adaptation of the generator in order to generate an electric field. The separation of charges leads to the creation of an electric field between the capacitor plates or electrodes [23]. In this study, a capacitive coupling system was implemented to generate

and homogeneously distribute electric fields over the 3D constructs based on natural materials such as gelatin. Cultured chondrocytes in the 3D constructs were stimulated using a scheme that consisted of applying electric fields four times a day for 21 days of culture. In this study, the combination of 3D constructs and electric fields will not only generate a novel methodology to stimulate viability, proliferation and molecular synthesis in articular cartilage cells, but it can also be used to improve therapeutic approaches focused on the treatment of pathologies that affect different tissues of the human body.

1.1 Hypothesis

In electrical stimulation on chondrocytes there are many discrepancies in the obtained results. Among various sources of discrepancies, a methodology to determine electric field strength is required to achieve the best rate of proliferation and molecular synthesis in chondrocytes cultured in a three-dimensional construct. Therefore, in this research an analysis *in vitro* is implemented to complement the experiments and evaluate the following hypotheses:

- Gelatin hydrogels can mimic the microenvironments of natural tissues and encapsulate cells homogeneously, which makes them attractive for cartilage tissue engineering. Accordingly, gelatin hybrids will provide an ideal extracellular environment to stimulate cell proliferation and glycosaminoglycan synthesis of chondrocytes encapsulated into the scaffolds.
- Although gelatin hydrogels provide an ideal environment for cartilage tissue engineering, it is necessary to use external biophysical stimuli to improve cell dynamics. In this context, it is hypothesized that electrical stimulation is useful to enhance chondrocyte proliferation and molecular synthesis. For this reason, the bioreactor used in this study will generate a homogeneous electric field distribution over chondrocytes cultured into gelatin hydrogels.
- There are many discrepancies in terms of proliferation rates and molecular expression of chondrocytes when are stimulated with electric fields. The main reason is because the electric fields strengths are unknown in several cases; therefore, the implementation of a computational approach using a finite element simulation method will allow predicting the electric fields intensities that are stimulating chondrocytes embedded into hydrogels.

1.2 Research objectives

1.2.1 General aim

To assess *in vitro* the effect of electric field strength in cultured chondrocytes in a 3D construct under a test setup with a capacitively coupling system and sinusoidal alternating voltages at 60 kHz during 21 days.

1.2.2 Specific aims

1. To design *in vitro* a protocol for the production of a 3D construct.
2. To implement *in vitro* a protocol to culture rat chondrocytes in a 3D construct.

3. To evaluate *in vitro* the effect of electric field strength in terms of viability, proliferation and molecular synthesis of cultured chondrocytes in a 3D construct.

1.3 Thesis outline

This thesis is divided into 5 chapters. **Chapter 1** corresponds to the introduction and objectives of the research project. In **Chapter 2**, the anatomy of articular cartilage is presented, highlighting its molecular composition: collagen fibers, proteoglycans, glycoproteins. On the other hand, a brief emphasis is made on the main pathologies that affect the articular cartilage and the possible treatments. In addition, the state of the art on the application of electric fields on chondrocytes cultured in three-dimensional constructs is developed, as a therapeutic alternative.

In **Chapter 3** a finite element computational simulation method is implemented to determine the electric field intensity in the capacitively coupled system. In this chapter, Method of Weighted Residuals is used to solve Poisson's Equation, which gives the magnitude of voltage in the system. Finally, the electric field strength in the system is determined using the electric field equation as a gradient.

After the intensity of the electric field applied in the three-dimensional constructs has been determined, in **Chapter 4** the effect of electric fields on chondrocytes cultured in a three-dimensional construct is analyzed. This chapter shows the production of gelatin hydrogels and their mechanical characterization. The isolation and encapsulation of chondrocytes in gelatin hydrogels is described. In addition, the stimulation schemes used are shown, which consisted of applying electric fields four times a day for 21 days through a capacitively coupled system. Finally, the effect of electric fields in terms of cell proliferation and production of glycosaminoglycans is evaluated.

Finally, in **Chapter 5** the main conclusions obtained by each of the experimental and computational analyzes carried out in this investigation are shown. In addition, the perspectives of this work are discussed and some recommendations for future work are made.

2 Anatomy, Molecular Structures, and Gelatin Injectable Hydrogels as a Therapeutic Alternative for Articular Cartilage Recovery

2.1 Articular Cartilage

Cartilage is a connective tissue that, depending on features of the extracellular matrix (ECM) and its location in human body, can be classified in three types: elastic, fibrillar and hyaline [1]. The former can be found in organs such as ear and epiglottis. The elastic tissue has low concentration of collagen type II (Col21-IIa), and it is mainly constituted by scattered elastic beams that form a threadlike network, which provides elasticity to cartilage. Similarly, intervertebral discs and meniscus are composed by fibrocartilage, which has a fibrous ECM rich in collagen type I. Finally, articular cartilage is rich in Col21-IIa and proteoglycans (PGs) and it can be found in two different parts of bones. On the one hand, articular cartilage (AC) is located at the end of bones [2–4], and its functional properties are reducing friction coefficient and supporting of mechanical loads between opposing joint surfaces [5]. On the other hand, a cartilaginous layer, known as growth plate (GP), is located between epiphysis and diaphysis, and it is responsible of longitudinal growth of long bones [6,7]. Given that articular cartilage fulfills a damping function, their cellular and molecular structures are crucial to support mechanical loading; therefore, its cellular component, the chondrocyte, is responsible for synthesizing and degrading main molecules of ECM such as collagen fibers and PGs [2,8]. These molecules provide dimensionality, elasticity and strength to articular cartilage to support mechanical loads [8,9].

2.2 Anatomy of cartilaginous structures in long bones

Since AC acts as a shock absorber, this tissue is exposed to different mechanical loads such as tension, compression, and shear stress (Figure 2-1A). Tension loading exerts an outward force increasing body elongation, while compression loading applies a thrust force that acts over the entire length of the tissue, making it flatter; finally, shear loading is tangential

to the body and deforms the tissue in several directions [2,3,8,10]. Cellular and molecular composition of ECM in AC are fundamental to support mechanical loading. Chondrocytes represent approximately 1 – 5 % of the total volume of the tissue [3], and their morphology varies according to their location inside the tissue (Figure 1B); thus, in the superficial zone (S-z) there are collagen fibers parallel to the articular surface and the chondrocytes are flattened. The intermediate zone (I-z) has a lower cell density of chondrocytes with a lightly oval morphology. Followed by this region, the chondrocytes become rounder and form column groups in the medium zone (M-z). In the deep zone (D-z), collagen is perpendicular to the joint and rather flattened chondrocytes are arranged into columns. Finally, it is in the calcified zone (C-z) where chondrocytes get hypertrophic and synthesize collagen type X [2,3]. The ECM represents more than 95% of the volume of the AC, where 60 – 80% is water, 15% is composed of collagens, including collagen type II, VI, IX, X, XI, and 9% is composed by PGs [1,3]. Nevertheless, collagen and PGs percentage vary depending on their location within AC. Thus, AC is organized in zones according to cell morphology and Col21-IIa orientation (Figure 2-1B) [1,9].

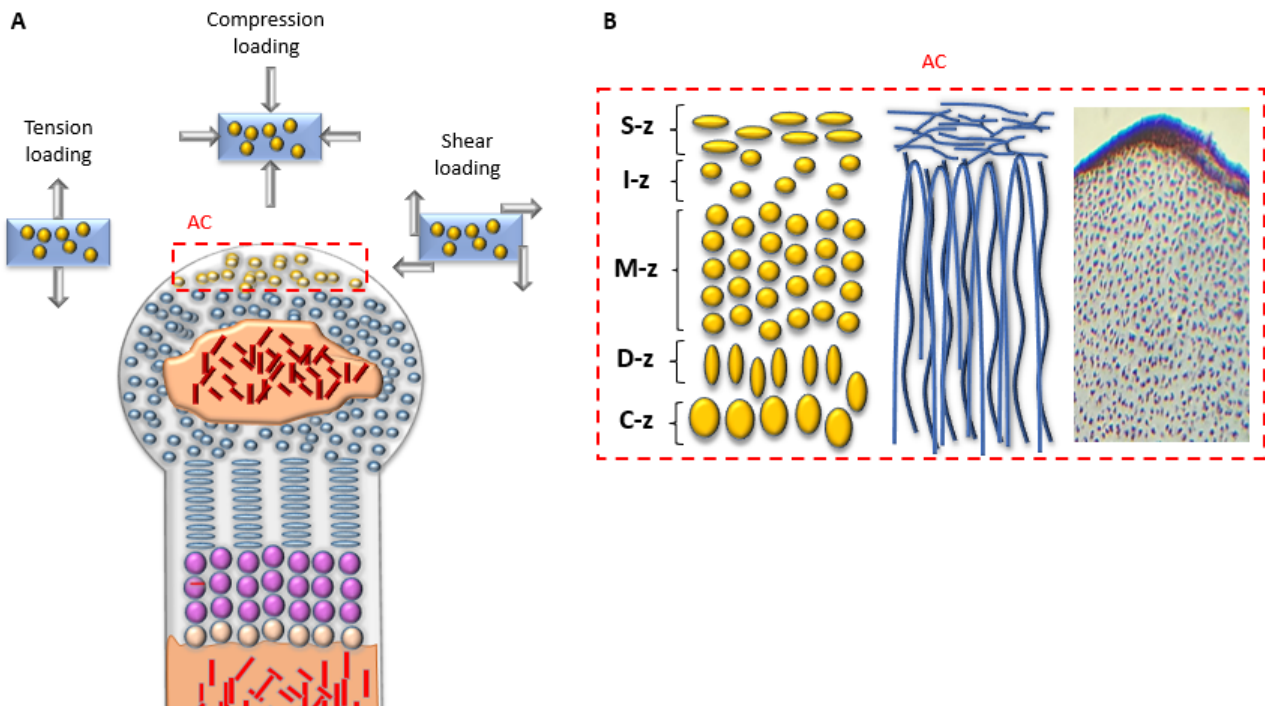


Figure 2-1: Cartilaginous structures in long bones. (A) Mechanical loads supported by AC. (B) Chondrocyte morphology in different AC zones; superficial zone (S-z), intermediate zone (I-z), medium zone (M-z), deep zone (D-z), and calcified zone (C-z). Collagen fiber organization within the ECM.

2.3 Molecular and biomechanical properties of articular cartilage

2.3.1 Molecular components of articular cartilage

Collagen fibers

The main component of ECM in AC is Col21-IIa (Figure 2-2). This is a fibrillar protein synthesized as a monomer, assembled as a trimer, and extracellularly processed to remove the amino and carboxy terminals [9]. Collagen fibers align to provide stiffness to cartilage depending on the direction of supported loads by the joint [14]. The AC has five types of collagens [8,9], where the most important is Col21-IIa, which constitutes the bulk of total collagen (about 80%). Col21-IIa is responsible for providing a high resistance to cyclic compression. Collagen type VI is concentrated around chondrocytes, helping them to adhere to ECM. Collagen type IX facilitates the interaction between collagen fibrils and PGs. Collagen type X organizes the collagen fibers in a tridimensional hexagonal lattice in the calcified zone of AC. Last, collagen type XI regulates collagen fiber size, forming a pericellular network around chondrocytes [15].

Proteoglycans (PGs)

PGs are very high molecular weight molecules, which provide support and dimensionality to cartilaginous tissues. PGs are constituted by a core protein known as hyaluronic acid with glycosaminoglycans (GAGs), that are perpendicularly attached through covalent bounds with a link protein (Figure 2-2) [9]. These long chains of linear carbohydrate polymers are negatively charged under physiological conditions due to their sulfate and uronic acid groups. Negatively charged sulfates attract positive ions, such as sodium, that in turn attract water. Thus, the AC is highly hydrated allowing cells to maintain a distance between them. PGs provide an osmotic resistance capacity and compressibility to the tissue [3]; additionally, they are joined to the chondrocyte cell membrane not only acting as signal modulators in communication processes between cells and their environment [2], but also promoting cell adhesion [3]. The main PG in the AC is Agc1, which is composed by a central protein of 210 to 250 kDa with three globular domains: two in the amino terminal (G1 and G2) and a third one in the carboxy terminal (G3). This third domain is separated by a region of two GAGs: the keratan sulfate (KS) and the chondroitin sulfate (CS) (Figure 2-2) [2,9]. The main functions of the globular domains are to provide aggregation, cell adhesion and chondrocyte apoptosis support, and supply hyaluronic acid binding sites [16]. Agc1 can interact with other proteins such as Col21-IIa, connecting the ECM with all constituents of the cellular surface [9]. Decorin and fibromodulin are other PGs with lower molecular weight, linked to Col21-IIa. Fibronectin is a glycoprotein that binds with cartilage membrane receptors, while cartilage oligomeric protein (COMP) mediates the interaction of chondrocytes with the

ECM through interaction with cell surface integrin receptors. Moreover, COMP maintain the structural integrity of cartilage interacting with other ECM proteins such as Col21-IIa [17,18]. These smaller PGs play important roles determining collagen fiber physical properties [1].

Transcription factors

The SOX9 is a transcription factor member of the SOX family. This molecule is essential for the differentiation of mesenchymal condensations into chondrocytes. Moreover, SOX9 is crucial for synthesis of cartilage characteristic molecules such as collagen type II, IX, XI and Agc1 [19]. During long bone development, SOX9 is synthesized by proliferative chondrocytes, and it is inhibited by hypertrophic chondrocytes [20]. Studies have demonstrated that SOX9, in presence of SOX-5 and SOX-6, regulates the endochondral ossification process [21].

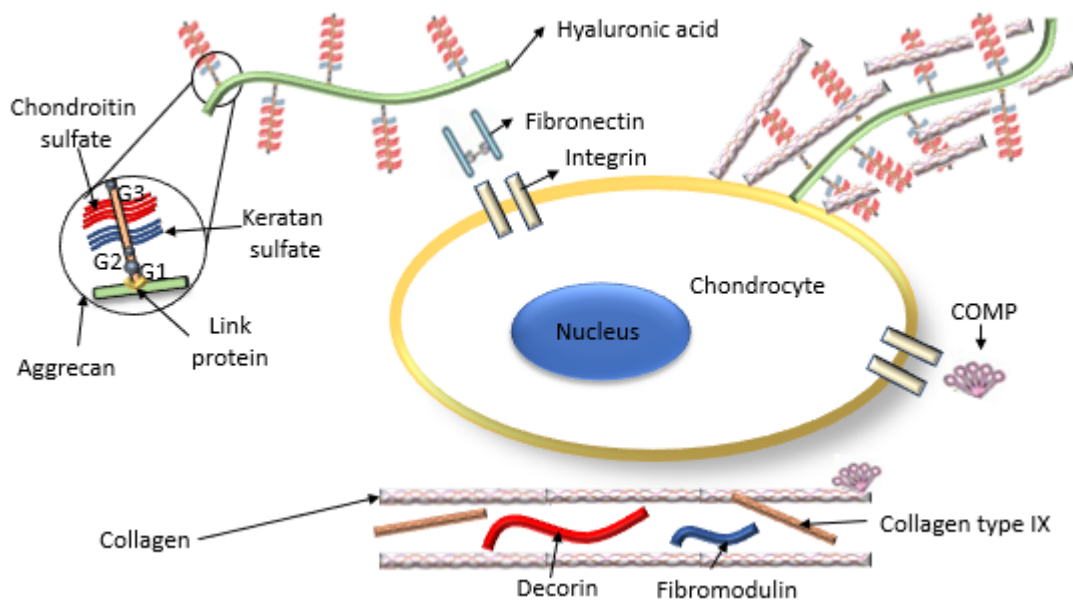


Figure 2-2: Schematic representations of articular cartilage and growth plate. Representative scheme of PGs, collagen fibers and GAGs secreted by chondrocytes in the articular cartilage

2.4 Mechanical loading supported by articular cartilage

Articular cartilage is a biphasic material which consists of an interstitial fluid phase and a porous-permeable solid phase [9]. On the one hand, the properties of the liquid phase

are given by the PGs, since they are negatively charged and allow the retention of water molecules within the tissue. On the other hand, the solid phase is provided by the distribution of collagen fibers, since they are strategically interlaced to give three-dimensionality to the cartilage [32]. The solid and fluid phases are crucial in distributing homogeneously external loads over the entire ECM when the cartilage is subjected to compression, shear and/or tension loads. This distribution is achieved by the permeable porous phase, which allows the flow of fluids that in turn creates a load transfer between both phases together with a pressurization of the interstitial fluid [33]. Accordingly, the cartilage intrinsic characteristics determine the main properties of the tissue such as compression resistance, tensile strength, shear stress resistance, hydrostatic pressure, swelling degree, permeability and viscoelasticity.

2.4.1 Compression

The compression loading is crucial for preservation of cartilage structure because this tissue is loaded primarily under compression. This load is supported by the solid phase of the cartilage, the fluid pressure and the nearly impermeable subchondral bone [34]. A volumetric change on cartilage structure occurs when the tissue is compressed. This internal morphologic change results in the flow of interstitial fluid, which generates a significant frictional resistance within the tissue [32].

2.4.2 Tensile strength

The cross-linked collagen in cartilage is the network responsible for supporting the tensile strength. When a tension loading is applied to the tissue, the collagen fibers tighten and align in order to absorb the tensile force [35].

2.4.3 Shear stress

This tangential load internally deforms the cartilage in an angular way affecting the inner zones of the cartilaginous tissue. The interaction between collagen fibers and PGs supports and distributes the shear stresses within the tissue. Collagen fibers, having a more elastic behavior than PGs, enable the absorption of shear loads [33].

2.4.4 Hydrostatic pressure

The fluid phase of the articular cartilage supports the hydrostatic pressures generated by compressive loads. These hydrostatic pressures are not uniform within the cartilage because the applied loads are heterogeneous across the tissue; therefore, a gradient in total stresses and pressures are formed, particularly near the joint surface. The hydrostatic pressures vary depending on the cartilage location; for example, the cartilage in the knee joints support

hydrostatic pressures between 3 and 10 MPa, while the cartilage in the hip joints support hydrostatic stresses of 18 MPa [36].

2.4.5 Swelling

Swelling is the mechanisms by which the residual stresses occur within the cartilage. Residual stresses and strains are physical magnitudes generated inside the cartilage after mechanical loading. These residual loads play a key role enhancing cartilage physiological behavior and reducing stresses when the tissue is loaded under physiological conditions [33]. Swelling is generated by the ionic constituents of the synovial fluid and the negatively charged GAGs (keratan sulfate and chondroitin sulfate) associated with the PGs molecules in the solid matrix of the cartilage [37].

2.4.6 Permeability

Permeability in articular cartilage is given by the PGs because they are the molecules that resist to fluid flow. It has been evidenced that a decrease in PGs concentration in the bone-cartilage interface lead to an increase of the permeability in the cartilage surface [38]. Moreover, collagen fibers orientation influences the permeability of the cartilage, due to the fact that permeability decreases in zones where the fibers are parallel to the surface [39]. Permeability in the knee cartilage of humans is between 1.14×10^{-15} to $2.17 \times 10^{-15} m^4/Ns$ [32].

2.4.7 Viscoelasticity

In cartilage, viscoelasticity is determined by the storage G' and the loss G'' moduli. The G' represents the capacity of the tissue to store energy for elastic recoil, while G'' characterizes the ability of a cartilage to dissipate energy [40]. Morphophysiologicaly, the collagen fibers and the fluid flow are responsible for determining the viscoelasticity of the cartilage. The cross-linking of the collagen fibers increases the viscoelastic shear modulus, while the fluid flow plays an important role as loading support in confined compression [41].

2.5 Pathologies affecting articular cartilage

When articular cartilage advances in age, it is conditioned to internal wear caused by the mechanical loads that are supported by the tissue. These mechanical loads generate morphological and functional alterations [42]. However, there are some injuries and diseases that may prematurely occur by reason of genetic, growth, metabolic, and/or traumatic factors [43].

There are three types of injuries that affect cartilage. First, there are lesions that disrupt and degrade the ECM of the tissue. However, the remaining viable chondrocytes increase their synthetic activity and repair the affected tissue. Second, there are changes in cartilage thickness that generate fissures in the structure of the cartilage. Last, there are injuries affecting the entire thickness of the cartilage that reach the subchondral bone. A possible recovery for this type of injury is carried out by progenitor cells migrating from the bone marrow to the injured tissue, generally replacing the tissue with fibrocartilage. This fibrillar tissue is mostly inefficient for load bearing and has a loss in friction properties [44].

According to the mentioned above and depending on the location of the articular cartilage, several pathologies may affect the cartilaginous tissue. On the one hand, osteoarthritis (OA) is the most frequent degenerative pathology of the articular cartilage. In this pathology both chondrocytes and ECM are affected [45–47]. Studies have revealed that OA affects both men and women usually over 60 years old. The OMS speculates that this disease will be the fourth cause of disability in the world by 2020 [48]. OA involves several factors that include mechanical damage of the joints, inflammation, and genetic predisposition [49]. It is manifested with high morphological, biochemical, molecular and biomechanical changes that result in an imbalance between synthesis and degradation of ECM components in articular cartilage. Thus, alterations of collagens and PGs decrease the tensile strength and the stiffness. The pathology leads to a decrease in thickness of the articular cartilage, subchondral bone remodeling and chronic inflammation of the synovial membrane [43,50,51]. Other pathologies such as neurogenic arthropathy, spondyloarthritis and osteochondritis affect the articular cartilage generating pain, inflammation, movement difficulties, fragmentation and joint fractures [52]. Overall, pathologies modifying morphology, composition and biomechanical properties of the articular cartilage may have an important impact on the quality of life of elder population. Therefore, there is a necessity to either develop appropriate treatments or find physiotherapeutic alternatives to restore cartilaginous tissue from lesions.

2.6 Treatments to restore articular cartilage

2.6.1 Conventional treatments to treat articular cartilage

Since different pathologies affect and degrade cartilage tissue, some therapeutic treatments have been implemented either to relieve the pain locally in the joint or to replace the affected articular surface with new cartilaginous tissue. For this reason, alternatives have focused on restoring articular cartilage through invasive and noninvasive treatments. The former focuses on physiotherapy, which makes use of noninvasive alternatives to treat injuries in the joints. This type of treatment is based on the application of different physical stimuli over the injured tissue. The most commonly used treatments are: ultrasound (stimulation with

frequency waves), magnetotherapy (application of static magnetic fields), electrotherapy (application of external electric fields), neuromuscular electrical stimulation and transcutaneous electrical nerve stimulation (TENS) [45]. According to the Osteoarthritis Research Society International (OARSI), therapies such as ultrasound, electrotherapy, neuromuscular electrical stimulation and TENS are treatments that need to be complemented with biomechanical interventions using knee braces, foot orthoses, strength training, weight management and physical exercise to relief pain in the knees with OA [56]. Although the non-invasive mechanisms used to treat cartilage have reduced the symptoms caused by the different diseases, a treatment that prolongs tissue healing has not yet been achieved. In fact, it has been demonstrated that several non-invasive therapies are of uncertain adequacy since they do not generate any effect in reducing local pain [56]. Accordingly, cartilage pathologies evolve to chronic states and in several cases the only solution is a surgical intervention.

On the other hand, the main purpose of invasive treatments is to replace osteochondral defects with a tissue that has the same characteristics of articular cartilage. One of the treatments is bone marrow stimulation, which consists in performing microfractures through the articular cartilage surface until the subchondral bone. These fractures allow the migration of mesenchymal cells to the affected site, and possibly their differentiation into chondrocytes to repair the lesion [1]. However, it is known that as the cells are not properly stimulated, the new formed tissue is generally fibrocartilage that will degenerate with time [44]. Another possible treatment uses autologous tissue transplantation, also known as mosaicoplasty. This treatment consists of taking small cylinders of healthy tissue from the edges of the articular cartilage, and inserting them into small holes previously drilled in the injured tissue [3]. Similar treatments are tissue grafting, which consists in taking small fragments of healthy articular cartilage from other joints of the body and inserting them into the affected cartilage. Osteotomy is another invasive technique, which consist in removing part of the bone-joint surface to reduce loads [2,8].

2.6.2 Tissue engineering to restore articular cartilage

Tissue engineering techniques have aimed to repair cartilage by autologous chondrocyte implantation (ACI), resulting either in regenerating or replacing the injured tissue [57]. ACI is a technique that consist in expanding the patient's chondrocytes through in vitro cultures. Once enough chondrocytes have been obtained, cells are re-implanted into the injured AC [58]. The use of scaffolds may enhance ACI, as these 3D structures play a pivotal role in regulation of mechanical loads that cells receive, affecting their proliferation, differentiation, and maintenance of the chondrocytic phenotype. Scaffolds act as temporal ECM until cells proliferate and start to synthesize characteristic molecules of the tissue [59]. Scaffolds in cartilage tissue engineering are a challenge, because finding a biomimetic material with similar

molecular properties to articular cartilage has proved to be difficult. Accordingly, several studies have focused on the development of biocompatible matrices mainly composed by collagen and GAGs, as they represent 95% of ECM [1]. Recently, injectable hydrogels have been used to mimic cartilage tissue, as these scaffolds are produced with biocompatible natural polymers that resemble natural soft tissues. These matrices are composed by a combination of polymeric networks insoluble in water with a well-established cross-linked structure [60]. Hydrogels are constituted by specialized materials with high permeability properties, which facilitate the fluid of oxygen, nutrients, and biomolecules through the scaffold. Moreover, these non-cytotoxic 3D structures are suitable for encapsulation of cells before cross-linking in situ, making hydrogels an attractive structure to be used in cartilage tissue engineering.

Novel therapeutic methods have been implemented to recover the cartilage such as autologous MSCs implantation and intra-articular injections of MSCs [1,44,61–64]. Considering that MSCs are multipotent cells capable to differentiate into several cells types such as cardiac muscle cells, neural cells, adipocytes, osteoblasts and chondrocytes [65], the aforementioned technique are based on obtaining patient MSCs, expand them through in vitro cultures, and re-implanted them into the injured tissue [63,66]. The autologous implantation has had mid-term effects in cartilage repair, and the treated zone are primarily filled with fibrocartilage tissue in several cases [67]. Regarding the intra-articular injections, it has been observed that MSCs encapsulated in hyaluronic acid solutions and injected in osteoarthritic knees have improved the treatment outcomes and the rate of cartilage regeneration [68]. MSCs have also shown their chondrogenic capacity to treat cartilage in the presence of growth factors and 3D environments [69]. In fact, 3D structures provide ideal environment to the MSCs to proliferate, migrate and differentiate into functional tissue-specific cells. Furthermore, 3D constructs enhance the diffusion of nutrients and bring a mechanical support for the cells in the first stages of regeneration [70]. Implement a biomimetic material with molecular properties similar to those of articular cartilage is challenge nowadays; in fact, different 3D constructs have been implemented to assess the cellular dynamics of MSCs and promote chondrogenesis such as alginate beads [71,72], agarose hydrogels [73–75], collagen fiber [76,77], micro-particles made of different polymers [78–80], and hyaluronic acid hydrogels [81–84]. Even though several hydrogels have been synthesized for tissue recovery, there is a need to characterize the biomaterial in terms of morphology, mechanical behavior and biocompatibility. For instance, the study conducted by 85 assessed the morphology, mechanical properties and biocompatibility of alginate, gelatin, and hyaluronic acid hybrid hydrogels. Results evidenced that both hydrogel mixture and precursor concentrations influence directly on pore size, young modulus, storage and loss modulus, swelling degree, and proliferation rate. Here, authors conclude that hybrid hydrogels at different concentration reveal a morphophysiological variation which make them attractive for tissue engineering applications. In fact, the raw material used to make the hydrogels has gained a lot of interest nowadays, since they are being used as bioinks in the 3D printing. Although this review

is not focused on bioprinting, it is noteworthy to mention that one of the most widely used biomaterials in bioprinting are the photo-cross-linkable hyaluronic acid hydrogels [86]. Here, authors highlight in a detailed way how hyaluronic acid hydrogels have been implemented in both in vitro and in vivo studies to treat cartilage pathologies, indicating that hyaluronic acid based bioinks are promising candidates for cartilage tissue engineering.

In general, hydrogels are used to mimic cartilage tissue because these scaffolds are produced with biocompatible natural polymers that resemble natural soft tissues. These matrices are composed by a combination of polymeric networks insoluble in water with a well-established cross-linked structure [87]. Hydrogels are constituted by specialized materials with high permeability properties, which facilitate the fluid of oxygen, nutrients and biomolecules through the scaffold. Moreover, these non-cytotoxic 3D structures are suitable for the encapsulation of cells before cross-linking in situ. These features make the hydrogels an attractive structure to be used in cartilage tissue engineering.

2.7 Hydrogels used for articular cartilage recovery

Hydrogels are composed by cross-linked hydrophilic polymeric chains. Morphologic organization and physicochemical composition of hydrogels favor retention of water molecules within the structure, while crossing points between polymeric chains avoid hydrogels dissolution in aqueous solutions [60]. Hydrogels provide a tridimensional structure that facilitate biophysical stimulation for cells to acquire an ideal morphology. Depending on polymeric materials used for hydrogel synthesis, different structures, with specific properties, can be obtained to be used in several applications [88]. For instance, cross-linking grade and biodegradability are factors that, depending on monomers employed and synthesis processes used, have direct influence in physicochemical properties such as water retention and mechanical properties [60]. Hydrogels can be fabricated using synthetic or natural polymers. On the one hand, synthetic polymers present advantages regarding mechanical properties; however, they represent disadvantages due to a lack of interaction between cell-scaffold and their grade of cytotoxicity after degradation. On the other hand, natural polymers mimic the ECM of soft tissues and increase adhesion, proliferation, and differentiation of cells without toxicity issues [88]. Most hydrogels are made either by proteins such as collagen, gelatin, elastin, and fibrin or by polysaccharides such as hyaluronic acid, alginate, agarose, cellulose, and chitosan [33].

Proteins and polysaccharides are present in the ECM of natural tissues; therefore, cartilage tissue engineering is focusing on the development of 3D structures that contain these types of materials to further mimic and regenerate the cartilaginous tissue. Some studies have shown that protein-polysaccharide hydrogels are suitable to maintain cell morphology and enhance protein synthesis. For instance, collagen type I hydrogels lead cells to undergo in distinct chondrogenic differentiation pathways [89]. In vivo experiments have evidenced that

collagen scaffolds may be used for ACI to recover AC defects [90]. Another protein used to make hydrogels is gelatin. This protein is derived by breaking the natural triple-helix structure of collagen into single-strand molecules by hydrolysis [60]. Gelatin is considered as a biomaterial, because it presents several advantages such as biodegradability, low immunogenicity, and low manufacturing cost. Gelatin is usually combined with polysaccharides to increase its biodegradability and biocompatibility properties. For example, gelatin-alginate hydrogels demonstrated influenced positively on bovine articular chondrocytes in terms of adhesion, proliferation, and glycosaminoglycan synthesis [91]. Gelatin and hyaluronic acid combination has been also studied and results have shown that this mixture constitutes a biocompatible and biodegradable substrate that increases synthesis of Col21-IIa and Agc1 [92].

2.7.1 Gelatin hydrogels

It is a natural polymer obtained from denaturation and partial hydrolysis of collagen, presenting the same peptide sequences as natural collagen [60]. Gelatin is formed by a combination of amino acids such as glycine, proline and hydroxyproline (Figure 2-3) [98]. These amino acids are linked by peptide bonds forming polymer chains of molecular weights between 10000 and 40000 g/mol [99]. Physical properties of gelatin vary according to its melting point; for instance, below its melting temperature (23°C) gelatin has helical chains, which act as cross-linking points. Nevertheless, these helical chains are unstable when gelatin is above melting temperature, which leads to a weakening of intramolecular bonds and to the dissolution of the polymer in water [100]. Gelatin is a non-toxic biocompatible polymer, which may be enzymatically degraded by collagenase; however, the low mechanical resistance limits its direct application, so it is usual to use gelatin in combination with other polymers [101]. Physiological environment and body temperature represent a problem in gelatin solubility; therefore, a chemical cross-linking is required to enhance gelatin physicochemical properties [102]. It has been reported that gelatin derivatives can be synthesized with phenolic hydroxyl groups using tyramine hydrochloride through condensation of the carboxyl groups of gelatin and the amino groups of tyramine [103]. However, gelatin can be synthesized by using hydroxyphenyl propionic acid (HPA) via carbodiimide active ester mediated coupling reaction and crosslinked with enzymatic oxidative reaction of HPA moieties using horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂) [104].

2.7.2 Recent advances on gelatin-based hydrogels to induce cell dynamics

The main advantage of gelatin-based hydrogels is that they are not cytotoxic and can be injected directly into injured tissues. It means that hydrogels can be inserted to fill the damaged zone before gelation and cover affected areas. This procedure can fill irregular shaped

defects, ensuring less invasive surgical interventions compared to surgeries to transplant preformed hydrogels [107]. Another advantage is that cells can be seeded within hydrogels before transplanting it. Once hydrogel-cells mixture is done, the dissolution is located into the wounded tissue and cross-linked in situ. This procedure allows the inclusion of growth factors, proteins and other substances that will be homogeneously distributed throughout the hydrogel once it is formed [87].

Regarding the gelatin, this protein contains arginine-glycine-aspartic acid adhesion sequences, which are recognized by integrins located in cell membrane to promote cell adhesion [110]. The formation of this complex adhesions, also known as focal adhesions, trigger the formation of actin cytoskeleton and expression of genes involved in proliferation, differentiation, and migration [111].

For instance, in vivo studies have demonstrated that hyaluronic acid – gelatin hydrogels crosslinked with UV provided an appropriate microenvironment for adipose derived stem cells proliferation [112]. In another study, gelatin and hyaluronic acid were crosslinked with different ratios of ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, the results showed that the hydrogels have no cytotoxicity and promote cell proliferation [113]. Similarly, human dermal fibroblast cultured in maleimide-modified hyaluronic acid and gelatin hydrogels crosslinked with polyethylene glycol have shown continuous proliferation [114]. Accordingly, a 3D structure composed by hyaluronic acid provides greater hydration and a better diffusion of nutrients. Moreover, this polysaccharide not only increases the mechanical properties of the construct such as greater support to compression, tension, and shear loads, but also enhances physicochemical properties such as swelling degree, viscoelasticity and permeability [115]. Additionally, if the 3D structure contains gelatin, it will generate an ideal environment in which cells can adhere to the ECM. In addition, gelatin promotes proliferation, differentiation, migration and synthesis of the characteristic molecules of a specific tissue. An in vitro study of hyaluronic acid – gelatin hydrogels with interpenetrating network crosslinked with transglutaminase and HRP, the hydrogels offered a high compressive strength and biocompatibility [116]. The use of photocrosslinked natural hydrogels based in hyaluronic acid and gelatin have been assessed to culture chondrocytes, the results showed a high mechanical strength and suitable elasticity, high biocompatibility and the hydrogels promoted ECM production [117]. Hyaluronic acid – gelatin hydrogels crosslinked with HRP enable differentiation of human stem cells to epithelial cells [118]. Furthermore, this type of injectable hydrogels has been studied to produce bioinks as materials for 3D printing biofabrication, it has been shown that these hydrogels crosslinked with different HRP concentration can provide a bioink with a high drug loading efficacy and homogeneous coverage due to wide range of gelation time, moreover, the bioink can obtain a printed structure with high biocompatibility and good stability [105]. Bioprinted hydrogels from gelatin derivatives and hyaluronic acid crosslinked with ruthenium (II) tris-bipyridyl dication and

sodium ammonium persulfate have been assessed to encapsulate human adipose stem cells, the results showed that the cells elongated and proliferated [119]. Overall, a combination of hyaluronic acid - gelatin is a 3D microenvironment rich in polysaccharides and proteins that provide water retention of molecules, resistance to compressive strengths and support to cyclic compression [120].

3 Computational simulation to estimate the electric field distribution in gelatin hydrogels

3.1 Introduction

Electric stimulation (ES) has garnered significant scientific interest as a physical modality employed in tissue engineering [121]. ES finds frequent application for both in vitro and in vivo cell stimulation, inducing a number of intracellular pathways involved in the regulation of cell metabolism, proliferation, migration and differentiation [122,123]. A meta-analysis of clinical trials has demonstrated the efficacy of neuromuscular electrical stimulation and interferential current in improving pain management and physical function among patients suffering from knee osteoarthritis [124,125].

ES has already been proven as a useful tool in cartilage tissue engineering. Cartilage primarily consists of non-excitabile chondrocytes [126,127]. Due to a lack of voltage-gated Na^+ and Ca^{2+} channels, these non-excitabile cells cannot generate action potential as a response to membrane depolarization [128]. Given the limited number of chondrocytes within cartilage and their feeble capability to repair extracellular matrix (ECM) damage, cartilage is susceptible to degenerative conditions such as osteoarthritis (OA) [129]. Various arthroscopic cartilage interventions, including chondroplasty, microfracture, or mosaicplasty [130,131,132], along with more modern approaches like autologous chondrocyte implantation [133], are currently in development. Nonetheless, their clinical efficacy remains to be definitively established. Stem cell-based tissue engineering strategies, particularly those harnessing adult tissue-derived mesenchymal stem cells (MSCs) capable of differentiating into chondrocytes, hold promise for cartilage damage repair. ES has emerged as a pivotal component of stem cell-based cartilage engineering, eliciting chondrogenic differentiation in MSCs even in the absence of growth factors [134].

The lack of standardized protocols for ES in tissue engineering introduces challenges in characterizing the exact mechanisms of its effect, since the electrical parameters (applied voltage, pulse or stimulus duration, frequency and field strength) can vary by several orders of magnitude [135]. Accordingly, different computational approaches have been implemented to

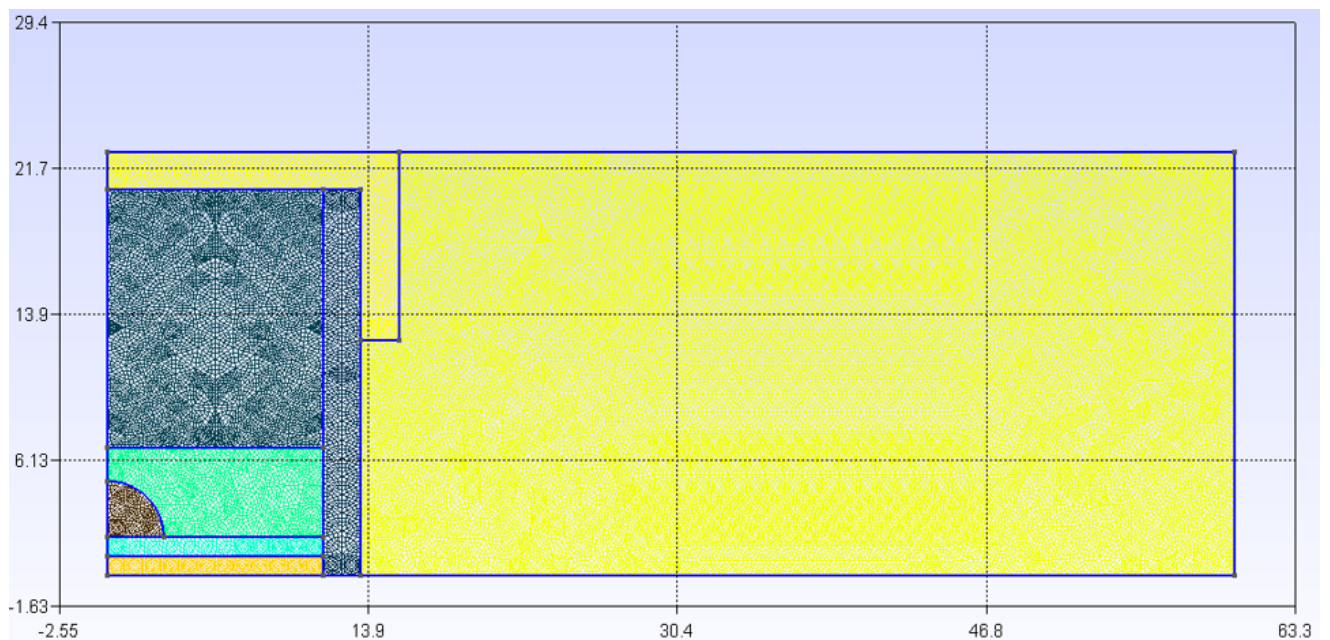
assess the effect generated by the application of electric fields (EFs) to cells. For instance, mathematical models were developed to observe the membrane potential in a spherical cell suspended in an electrolyte medium. Results evidenced that induced potential decreases due to surface conductance in small cells, while for bigger cells this potential decreases due to membrane conductance [24]. On the other hand, an electrolyte medium with low conductivity increases the charging time of the cell membrane; therefore, cells immersed in this kind of medium need to be stimulated with higher EFs to induce electroporation in the cell membrane [25]. In a study carried out by Krassowska et al., the polarization and physiological state of a single cell electrically stimulated was modeled [26]. A similar study analyzed the variation of the transmembrane potential on prolate and oblate spheroidal cells stimulated with EFs. Results evidenced that the transmembrane potential strongly depends on the cell orientation when a cell is being stimulated [27]. Similarly, computational models to simulate the effect of alternating current EFs on spheroidal cells were implemented. Results indicated that time-dependent geometry changes influence the induced membrane potential of the cell; additionally, the time-dependent charging and discharging of the membrane can be modeled when the frequency dependence is transformed into a time dependence [28, 29]. Even though the computational models evidenced that transmembrane potential vary according to cell shape, size and orientation, the models did not consider different frequencies and dielectric properties of the in vivo extracellular environment where the cell is located such as living tissues and three-dimensional structures. The computational model that has evaluated the effect of external EFs on a single cell cultured in monolayer was developed by Taghian et al., [30]. The results showed that the cell membrane behavior of the cell strongly depends on the frequency applied; however, the computational analysis was performed using a one-dimensional round cell morphology in one of the possible scenarios where a cell may grow. In this context, the role of the voltage, frequency and extracellular matrix in terms of distribution of EFs have effects on the cell culture. For this reason, a finite element computational method is implemented to simulate a gelatin hydrogel in a capacitively coupled system to determine the electric field intensity. The computational model was simulated to mimic the stimulation of a in vitro sample using two stainless-steel electrodes located at the bottom and upper surface of a culture well plate. The model could predict the field strength and electric field distribution within the biomaterial. The programming algorithm can be found in the Annex A.

3.2 Geometrical model and boundary conditions

A bi-dimensional axisymmetric domain was implemented to estimate the distribution of EF in the capacitively system coupling system and within the gelatin hydrogel. The coupled capacitive system is composed of two parallel stainless-steel electrodes located at the top and bottom of a 12 culture well-plates (Figure 3-1). The dimensions and dielectric properties of the different materials that compose the capacitive system in Table 3-1.

Table 3-1: Dielectric properties and measurements of the capacitive coupled system used in the simulation

Component	Parameter	Value	Reference
Stainless steel electrode	Separation [mm]	22	[156]
	Radius [mm]	60	
	Permittivity	1	
	Conductivity [MS/m]	1,73	
Well plate	Thickness [mm]	1,4	[156]
	Height [mm]	22	
	Radius [mm]	11,4	
	Permittivity	3,5	
	Conductivity [S/m]	6,60E-16	
Air	Permittivity	1	[156]
	Conductivity [S/m]	0	
Hydrogel	Permittivity @60kHz	8,00E+03	[157]
	Conductivity @60kHz [S/m]	7,10E-02	
Culture media	Permittivity @60kHz	2,70E+04	[157]
	Conductivity @60kHz [S/m]	7,20E-02	

**Figure 3-2:** Mesh of culture system used to stimulate a hydrogel

3.3 Model implementation

The procedures to determine the electrical field strength on a hydrogel are described in Figure 3-3, the model was implemented in MATLAB software. First, an axisymmetric model was selected to represent the domains of the electric field strength in a hydrogel. Then, the quadrangular mesh was generated in the Gmesh software. The region in between the electrodes is composed of various materials, each one with a constant permittivity. In the static case, first Maxwell's equation reduce to:

$$\nabla \times E = 0 \tag{3-1}$$

Where E is the electric field strength. Since equation 3-1 can be expressed in terms of the gradient of a scalar field, which is named as the electrostatic potential V as:

$$E = -\nabla V \tag{3-2}$$

The generalized 2-D Poisson's equation of materials with constant dielectric properties is expressed as follows:

$$-\frac{\delta}{\delta x} \left(\varepsilon \frac{\delta V}{\delta x} \right) - \frac{\delta}{\delta y} \left(\varepsilon \frac{\delta V}{\delta y} \right) = \rho \tag{3-3}$$

Where ε is the material permittivity, ρ is the electric charge density. Since free charges do not exist in a dielectric medium, $\rho = 0$ [121]. Therefore, the equation 3-3 can be expressed as follows:

$$-\frac{\delta}{\delta x} \left(\varepsilon \frac{\delta V}{\delta x} \right) - \frac{\delta}{\delta y} \left(\varepsilon \frac{\delta V}{\delta y} \right) = 0 \tag{3-4}$$

The approximation of equation 3-4 is constructed as a linear combination of a set of basis functions. Accordingly, instead of considering second-order partial differential equations, its weighted version used to obtain the approximate solution directly (Equation 3-5).

$$\int_{\Omega^e} \nabla w_l \cdot \nabla \hat{V}^e d\Omega^e = 0 \tag{3-5}$$

In the expression of equation 3-5, Ω is the domain, w is a suitably-chosen weight function and this result can be obtained by employing the Method of Weighted Residuals directly. Since the potential function is defined as a weighted sum of shape functions in each element, the electric field can be computed within each element by differentiating the weighted sum expression, Where the elementary approximation for a quadrangular element using Lagrange polynomials N_m , is constructed as follows:

$$\hat{V}^e = \sum_{m=1}^4 V_m N_m \tag{3-6}$$

A boundary value problem can be constructed by introducing suitable boundary conditions (BCs). The homogeneous Neumann BCs on the left and right boundaries are introduced under the assumption that these boundaries are sufficiently far away from the hydrogel, and the normal derivative of the potential is almost zero (i.e., the electric field is tangential to these surfaces). The Dirichlet BCs on the top and bottom are introduced under the assumption that these boundaries are stainless steel electrodes with a constant voltage and frequency (50 Vpp and 100 Vpp at 60 kHz). In summary, the following BCs must be imposed:

$$V = V_t \quad (\text{on top electrode}) \quad (3-7)$$

$$V = V_b \quad (\text{on bottom electrode}) \quad (3-8)$$

$$\frac{\delta V}{\delta n} = 0 \quad (\text{on the left and right outer boundaries}) \quad (3-9)$$

Once the domains were restricted with BCs (Equations 3-7, 3-8, 3-9), the voltage distribution in the domain (V) was determined by solving Equation 3-4 using the finite element method. Finally, the model was solved to determine the electric field strength (E) in each element with Equation 3-2. According to Equation 3-4, the model will use permittivity as the main parameter to determine the effect of the electric field on the hydrogel and the distribution of the electric field in the system.

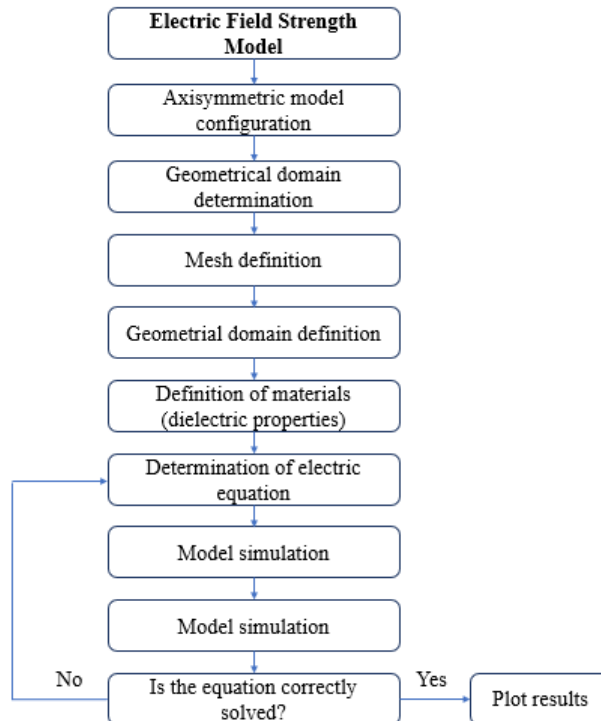


Figure 3-3: Flowchart of the computational model implementation

3.4 Results

An axisymmetric computational simulation was carried out in order to calculate the electric field strength in the capacitively coupled system.

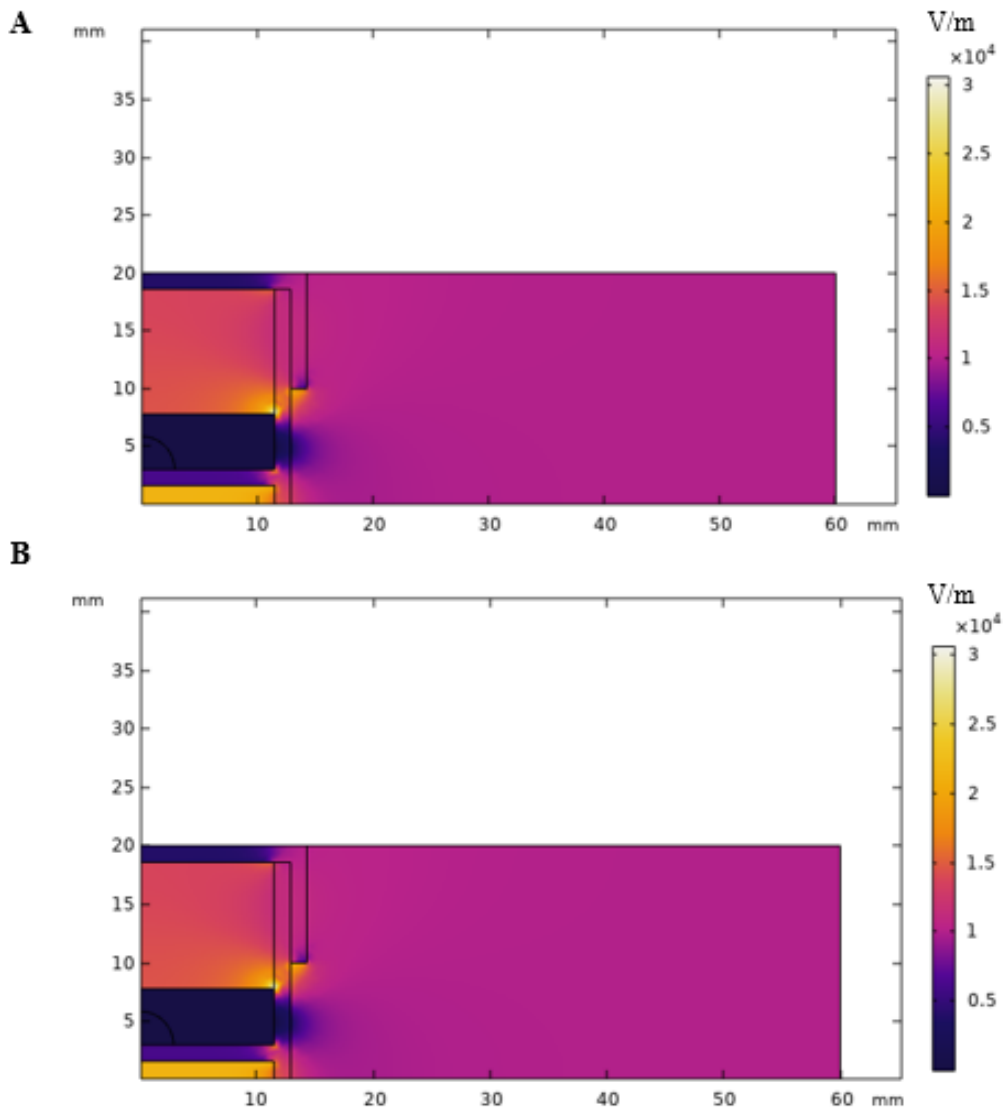


Figure 3-4: Distribution of the EFs in the capacitively coupled system. A) Simulation of electric field distribution with a scheme of 50 V at 60 kHz. B) Simulation of electric field distribution with a scheme of 100 V at 60 kHz.

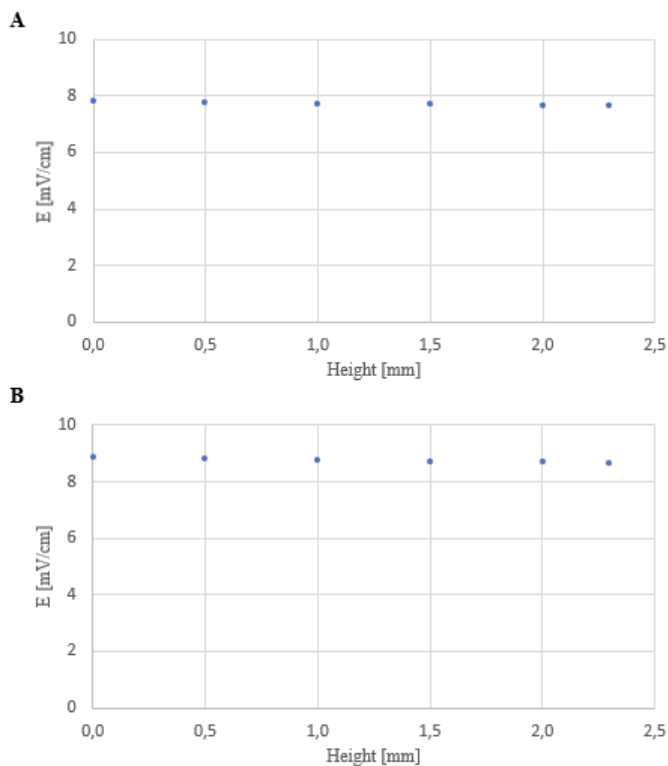


Figure 3-5: Values of the EFs within the hydrogel. A) Simulation of electric field distribution with a scheme of 50 V at 60 kHz. B) Simulation of electric field distribution with a scheme of 100 V at 60 kHz.

The EF distribution in both the capacitively coupled system is shown in Figure 3-5. Considering that the cells are immersed within the hydrogel, it is relevant to know the EF intensities within the whole surface of biomaterial. In Figure 3-5 it is possible to observe that the electric field strength within the hydrogel are 7.7 mV/cm and 8.8 mV/cm for 50 V and 100 V, correspondingly. The results were plotted in the Paraview software.

3.5 Discussion

This chapter presents a computational model that determine the electric field strength within a hydrogel generated by two stainless-steel electrodes located at the bottom and upper surface of a culture well plate. The findings demonstrated that the hydrogel is a biomaterial with dielectric properties and can have a homogeneous electric field strength with an externally generated field. The electric field strength depends on the frequency applied, due to the dielectric properties of the materials vary depending on the frequency [122]. The computational model was simulated to mimic the stimulation of a *in vitro* sample of encapsulated chondrocytes in a hydrogel, and the particular behavior that occurs in the membrane caused by electric fields has been investigated in the literature. For instance, it has been shown that

an increase in cytoplasm conductivity leads to lower EFs in the cytoplasm and higher EFs in the plasma membrane [123]. It is possible to mention that the plasma membrane acts as a capacitor, since the current that flows through a capacitor tends to increase as the frequency is higher.

Considering the cell membrane as a capacitor, the EFs in this computational model were applied in alternating current (AC). Furthermore, the model considered a variation of the frequencies, because a capacitor submitted to an AC with different frequencies is being constantly charged and discharged. In this sense, the plasma membrane is alternately charged and discharged at a rate determined by the frequency of the supply. This phenomenon is related with the activation of the plasma membrane ionic channels, as the application of external alternating EFs modifies the opening and closing of the Na⁺, K⁺ and Ca⁺ channels [124]. Even though the activation of ionic channels of chondrocytes was not modelled, there are reports that have demonstrated that the voltage-dependent calcium channels (VDCC) are responsible to trigger different signaling pathways, which are involved in cell dynamics and molecular synthesis [40]. According to our results, this computational model is a useful tool that can predict the electric field strength required to modify the influx and outflux of ions which may enhance the cell dynamics of chondrocytes. For this reason, the computational model presented here is a promising tool that can be used to estimate the required EFs to overcome the chondrocyte membrane potential and modify the ionic gradient concentrations responsible for activating the signaling pathways of the chondrocytes.

Depending on the EFs applied and the stimulation time, the *in vitro* procedures of chondrocytes cultured in monolayer can be modified in order to increase cell proliferation and molecular synthesis. In fact, some experimental studies have evidenced that EFs in chondrocytes cultured in monolayer increase cell population and enhance the molecular synthesis of molecules such as glycosaminoglycans, collagen type II and aggrecan [125]. Accordingly, this computational model could be improved if the molecular events are modeled when external EFs are applied to the cells. Combining these computational approaches, it is possible to establish the adequate parameters to stimulate cells, and accurately predict if the stimulation modifies the cell membrane potentials.

3.6 Concluding remarks

The purpose of this computational model was to carry out a novel framework to predict the electric field strength distribution within a hydrogel in a capacitively coupled system composed by two stainless-steel electrodes located at the bottom and upper surface of a culture well plate. Results showed that electric field strength distribution in the hydrogel is homogeneous and depends on material dielectric properties. The finite element computational simulation is a method to determine the electric field strength distribution in a biological

sample.

4 Electrical stimulation enhances cellular dynamics of chondrocytes cultured in gelatin hydrogels

4.1 Introduction

Articular cartilage is the connective tissue found at the surface of articular joints that permits friction less motion and absorbs/distributes loads [130]. Because of aging, repetitive joint use, and other factors, cartilage deteriorates and flakes or forming tiny crevices, eventually leading to osteoarthritis (OA) [131]. This chronic degenerative disease of articular joints is the most common joint disorder in the United States, affecting about 10% of American adults . It should be also noted that more than half of the affected adults are younger than 65 years of age [131], requiring long-term disease management. Several treatment methods have been clinically used to restore joint function, such as osteochondral transplantation and microfracture [132]. However, therapeutic options for cartilage repair are scarce and do not yield adequate results [133]. For example, osteochondral transplantation is a highly invasive procedure requiring large incisions and donor grafts that are not always readily available [135]. Microfracture, a less invasive procedure that involves microfenstration into the subchondral bone, has been shown to reduce pain and increase mobility in short-term follow up, but it has limited long-term success [136]. Total knee arthroplasty (TKA) represents an effective solution for end-stage OA because it can relieve pain, correct leg deformity, and help patients resume normal activities. However, TKA is an invasive surgery that replaces damaged biological joints with artificial prostheses. Infection, stiffness, and the limited lifespan of prostheses represent major risk factors for revision or repeat surgery [137].

More recent innovative treatment methods for OA, which have been studied in the field of regenerative medicine, involve the use of cells, scaffolds, and growth factors to biologically reproduce articular cartilage at the defect site. Regarding cells from different sources, human adult stem cells have drawn the most attention because of their demonstrated ability to differentiate into chondrocytes, as well as exhibit immunosuppressive and anti-inflammatory effects [137].

Biomaterial scaffolds, another critical component of regenerative medicine-based therapy,

have been used to provide a microenvironment conducive to cartilage regeneration, and serve as the carrier for cells and growth factors [138]. For years, different types of materials have been developed and tested to accelerate cartilage regeneration, with or without cells. In particular, hydrogel scaffolds are the most commonly used scaffold since they have many properties in common with articular cartilage. For example, they are highly hydrated, provide compressive strength, and permit load transfer from the environment to chondrocytes, similar to native cartilage [139]. To date, different types of natural and synthetic hydrogels have been tested in animal studies and clinical trials, including alginate/agarose, collagen, polyelectrolyte [140], and polyethylene glycol. For example, Dai et al. used scaffold free fibrin to induce the regeneration of full-thickness cartilage defects [141]. Silk fibroin [142] or bioprinted polycaprolactone [143] scaffolds have also been explored for cartilage repair. Unfortunately, no specific hydrogel material has accurately reproduced the tissue properties of cartilage. Therefore, further investigations are needed to optimize hydrogel materials and preparation methods [144].

Gelatin, a denatured form of collagen that has been widely used in the clinical setting, is recognized as an alternative for cartilage repair. Gelatin retains many of the native molecular epitopes for cell adhesion and signal transduction in collagen that are important for maintaining a chondrocyte phenotype [145]. It has been shown that a low-toxicity initiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate, to photocrosslink methacrylated gelatin (mGL), via gelation by visible light illumination allows direct cell encapsulation and is capable of rapid generation of cell-laden scaffolds with high cell viability, without the use of ultraviolet light or protective barriers. Scaffolds fabricated using this method are biodegradable, biocompatible, and resistant to swelling and contraction. The scaffolds also exhibit excellent in situ space-filling qualities in both air and aqueous solutions, without the use of protective barriers [146].

Cartilage tissue engineering has established technologies such as the application of biophysical stimuli in order to enhance flow of nutrients, cell attachment, molecular synthesis production and a prolonged chondrogenesis [147]. In this context, it has been reported that chondrocytes and Mesenchymal cells (MSCs) cultured either in monolayer or 3D constructs respond to a biophysical stimulus such as electric fields (EFs). For instance, in vitro studies have shown that EFs applied over MSCs cultured in monolayer change cell morphology and cell alignment [148], improve cell migration, and increase cell proliferation [149]. Regarding 3D cultures, it has evidenced that micromass MSCs experienced an increase in collagen type II, aggrecan and SOX-9 after an stimulation with EFs [150].

Recently, effects of electrical stimulation on cartilage tissue and chondrocytes have been studied and an increased proliferation and matrix synthesis, as well as a reduced matrix degradation, was revealed [151]. These effects can probably be attributed to the unique

structure as well as biomechanical and electromechanical properties of cartilage cartilage [150]. *In vitro*, it was found that EFs increase glycosaminoglycans and type II collagen synthesis by chondrocytes cultured on collagen scaffolds using a high-frequency capacitive system [152,153]. In contrast to other stimulation systems, higher frequencies up to 60 kHz are applied in capacitively coupled systems on cell cultures and cartilage explants enhancing the synthesis rates of collagen type II and aggrecan [154].

Taking the above mentioned, we hypothesize that electric field stimulation may improve the positive results observed with chondrocytes in gelatin hydrogels. To accomplish this, a capacitively coupled system was designed and implemented to generate and homogeneously distribute EFs of 7,7 mV/cm and 8,7 mV/cm at 60 kHz (sine wave-form). The hydrogels were stimulated using a stimulation scheme that consisted in applying EFs for 30 min four times per day during 21 days of culture. The mechanical properties of the hydrogels were measured by rheology. Cell proliferation was assessed by MTS assay, while gene expressions of GAGs were measured through a colorimetric test called 1-9-dimethylmethylene. In this study, the combination of 3D structures and EFs not only create a novel methodology to stimulate cell dynamics, but also may be used improve the therapeutic approaches focused on the treatment of chronic pathologies of articular cartilage.

4.2 Materials and methods

4.2.1 Materials

For the hydrogel synthesis the following reagents were employed: Hyaluronic acid sodium salt from *Streptococcus equi*, gelatin strength 300 type A, 2-(N morpholino) ethanesulfonic acid (MES), tyramine hydrochloride (HCl-Tyramine), N-Hydroxysuccinimide (NHS), dialysis tubing of 12.400 molecular weight cutoff (MWCO), sodium hydroxide (NaOH), hydrogen peroxide solution (H₂O₂), peroxidase from horseradish (HRP). All reagents were purchased from Sigma-Aldrich. Dialysis Tubing of 3.500 MWCO was acquired from Spectrum labs and carbodiimide hydrochloride (EDC) was obtained from Iris Biotech GMBH.

For the biological experiments DMEM (high glucose, GlutaMAX Supplement), Fetal Bovine Serum (FBS), phosphate buffered saline (PBS), antibiotics (penicillin/streptomycin) and trypan blue stain were obtained from Gibco. All the other reagents used in experiments were CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) from Promega.

The medium used to dissolve the polymers was the phosphate buffered saline (PBS), is a pH-adjusted blend of ultrapure-grade phosphate buffers and saline solutions which, when diluted to a 1X working concentration, contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄.

4.2.2 Synthesis of gelatin hydrogels

Gelatin is a natural polymer obtained from denaturation and partial hydrolysis of collagen, presenting the same peptide sequences as natural collagen [60]. Gelatin is formed by a combination of amino acids such as glycine, proline, and hydroxyproline (Figure 4-1) [98]. These amino acids are linked by peptide bonds forming polymer chains of molecular weights between 10,000 and 40,000 g/mol [99]. Physical properties of gelatin vary according to its melting point; for instance, below its melting temperature (23°C) gelatin has helical chains, which act as crosslinking points. Nevertheless, these helical chains are unstable when gelatin is above melting temperature, which leads to a weakening of intramolecular bonds and to the dissolution of the polymer in water [100]. Gelatin is a non-toxic biocompatible polymer, which may be enzymatically degraded by collagenase; however, the low mechanical resistance limits its direct application, so it is usual to use gelatin in combination with other polymers [101]. Physiological environment and body temperature represent a problem in gelatin solubility; therefore, a chemical cross-linking is required to enhance gelatin physicochemical properties [102]. It has been reported that gelatin derivatives can be synthesized with phenolic hydroxyl groups using tyramine hydrochloride through condensation of the carboxyl groups of gelatin and the amino groups of tyramine [103]. However, gelatin can be synthesized by using hydroxyphenyl propionic acid (HPA) via carbodiimide active ester mediated coupling reaction and crosslinked with enzymatic oxidative reaction of HPA moieties using horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂) [104].

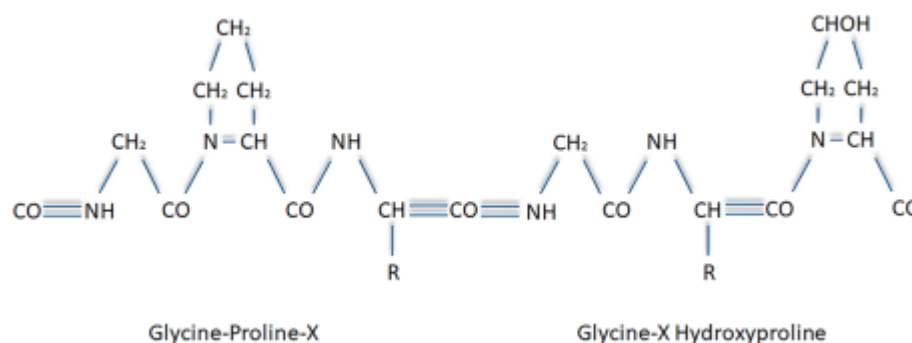


Figure 4-1: Chemical structure of gelatin. It is constituted by a repetition of units such as glycine, proline and hydroxyproline.

The cross-linking of gelatin is achieved by chemical modification adding phenol groups to carboxyl groups (COOH) of gelatin; thereafter, a cross-linking agent is added for hydrogel reticulation. Incorporation of phenol groups is carried out by adding tyramine, a monoamine that acts in the human body as vasoactive. Tyramine is bound to carboxyl groups of hyaluronic acid and gelatin through the activator N-(3-Dimethylaminopropyl)-N^o-ethylcarbodiimide (EDC) and the stabilizer N-Hydroxysuccinimide (NHS). The reaction between EDC-COOH generates

an O-acylisourea (Figure 4-2) [105]. The O-acylisourea reacts with both NHS and amine groups of tyramine to form an amide bond (Figure 4-2). This process induces the obtaining of gelatin chains modified with phenol groups for subsequent enzymatic gelation. Once tyramine is grafted onto polymeric chains, a cross-linking process is carried out by adding HRP, that catalyzes the cross-linking reaction and H₂O₂, which acts as oxidizing agent of tyramine molecules (Figure 4-3) [94].

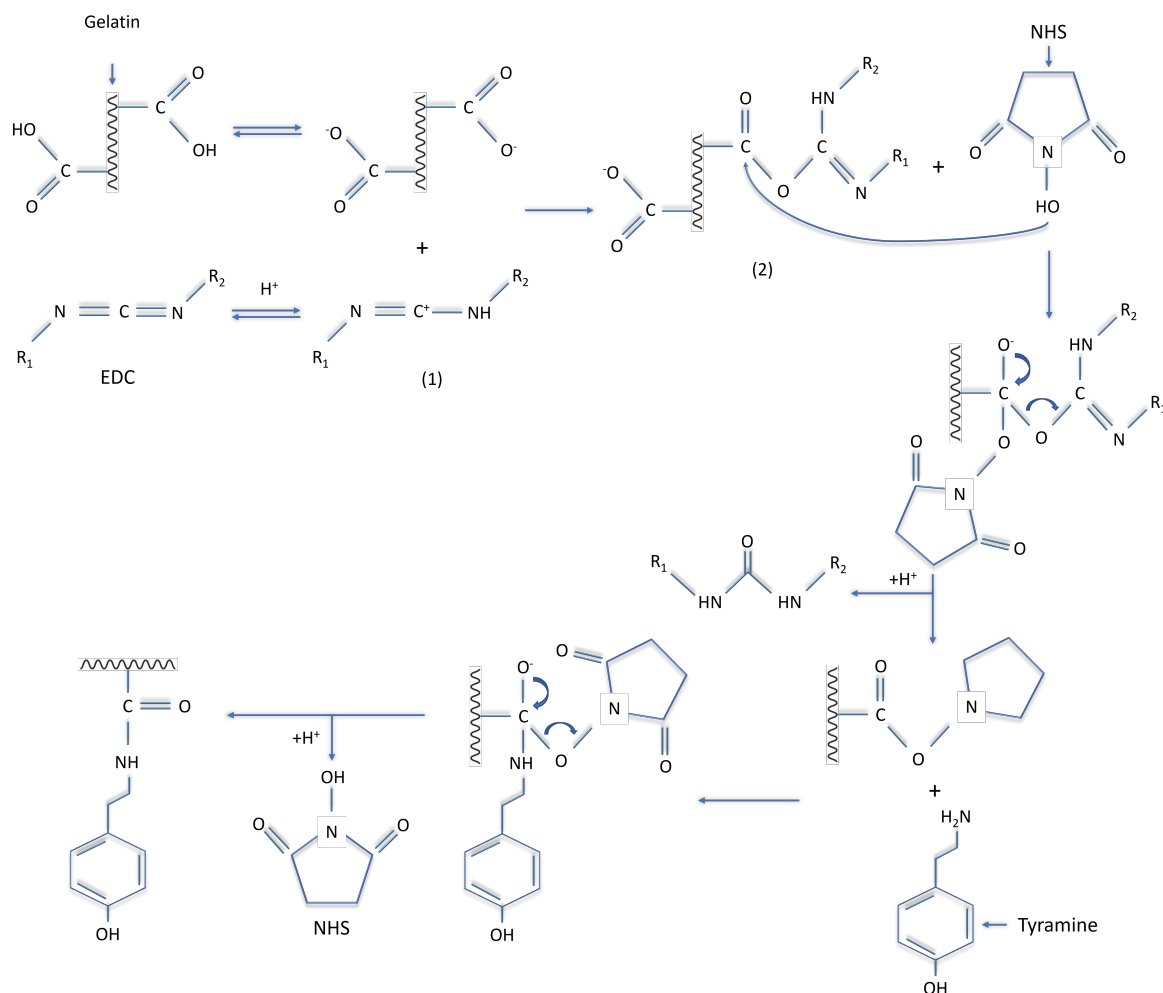


Figure 4-2: Gelatin hydrogels. (1) Reaction of hyaluronic acid or gelatin chains to form EDC carbocation. (2) O-acylisourea formation. (3) Gelatin chains reacting with the EDC forming an O-acylisourea. (4) The O-acylisourea then reacts with NHS and the amine group of tyramine forming an amide linkage. (5) Final chemical reaction for obtaining gelatin hydrogels grafted with tyramine using O-acylisourea. Yellow arrows indicate the chemical route of gelatin synthesis.

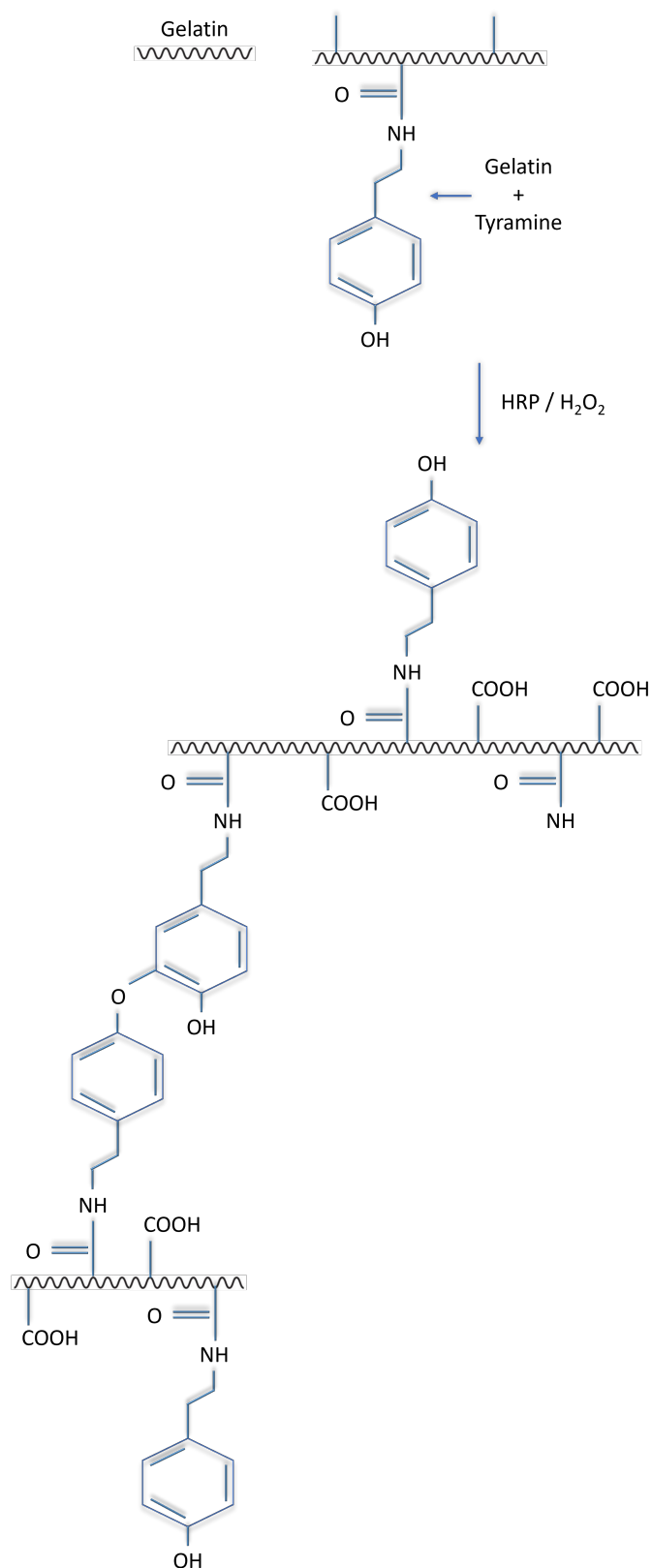


Figura 4-3: Diagram of the cross-link bonds formed in the gelation of gelatin hydrogels using HRP and H₂O₂.

4.2.3 Hydrogels preparation

Gelatin hydrogels (3% w/v) in a proportion of were prepared according to the protocol described by [85]. Gelatin (GEL) was dissolved for 30 min at 37 °C. A mixture containing 80% (v/v) of 2%w/v of gelatin and 10% (v/v) of HRP (12.5 U/mL) was prepared. Then, the GEL-HRP solution was cross-linked with 10% (v/v) of H₂O₂ (20 mM). The hydrogel was incubated at 37 °C and 5% CO₂ for 20 min to ensure hydrogel cross-linking.

4.2.4 Rheological measurements of gelatin hydrogel

Three different measurements were performed in order to mechanically characterize the HA–GEL hydrogel. First, an oscillatory time sweep for 20 min to register the gelation dynamics of the hydrogel was performed. The strain and frequency were selected at 1% and 1 Hz, respectively. Second, a dynamic strain sweep to calculate the range of strain amplitudes was carried out. Amplitudes ranging between 0.01% and 20% and a frequency of 1 Hz to measure the dynamic shear modulus as a function of strain were used. Finally, a dynamic frequency sweep test to determine the dependence of the dynamic shear modulus and loss factor on the frequency was done. Frequencies between 0.1 and 10 Hz and a strain of 1% were selected. The hydrogel temperature was controlled and maintained by a Peltier device at 37 °C. The hydrogel dynamic was measured using a gap around 1100 μm between the plates. Measurements were carried out by cross-linking gelatin (2 w/v %) either in PBS at 37 °C in order to determine which medium has similar mechanical properties to those of cartilage tissue. The protocol described for cross-linking process of hydrogels is showed in section 4.2.2. The rheological parameters were measured using a strain-controlled rheometer.

4.2.5 Chondrocytes isolation and cell culture

The distal and proximal extremities from humerus, scapulae and femurs of 2-day-old Wistar rats were isolated. The rats were obtained from the Pharmacy Faculty and sacrificed by decapitation at the Biomimetics laboratory of the Biotechnology Institute at Universidad Nacional of Colombia. Recommendations from the ethics committee were followed for this procedure. Taking into account the high cell density required for the experimental design, we decided to use the chondroepiphysis as it has been reported in literature [151]. This tissue is appropriate to obtain a high number of pure cells required for our study. Nevertheless, in this sample chondrocytes at different maturation stages are expected. First, the extremities were washed with PBS (Phosphate Buffered Saline) + 2% of antibiotics (streptomycin and penicillin, LONZA, Walkersville, MD USA). Next, the fibrous tissue and muscle were carefully removed from the epiphysis and metaphysis. After, the epiphysis was segmented in small pieces of approximately 4 mm and then washed with PBS + 2% of antibiotics. The fragments were submitted to a process of double digestion using 0.25% Trypsin/EDTA (LONZA, Walkersville, MD USA) during 1 h at 37 C, followed by digestion using 0.3%

of collagenase type I in HBSS (Hanks Buffered Saline Solution, LONZA, Walkersville, MD USA) during 4 h at 37 °C. The reaction was inactivated with 20% of fetal calf serum (FCS). The solution was homogenized by pipetting, then filtered with a sterile nylon membrane to eliminate remaining tissue, and finally, it was centrifuged. Cells were suspended in 1 mL of culture medium (DMEM-F12 with 50 µg/mL of ascorbic acid, 100 µg/mL of sodium pyruvate, 1% of streptomycin/ penicillin and 1% FCS) [156]. Then, the cell yield and viability were determined by counting in hemocytometer and trypan blue dye exclusion, respectively. Despite the isolation source, chondrocytes cultured in monolayer display a progressive loss of phenotype that increase with passages with the most striking phenotypic alterations becoming evident after passage four [157]. Therefore, for stimulation assays, 1.000.000 cells were seeded at day zero into a T-75 culture well-plate; moreover, chondrocytes expanded in culture at passage one were used for all experiments. Cell cultures remained in atmospheric conditions of 37 °C and 5% CO₂ and the cell culture media was changed every other day. This procedure was implemented for cultures under electrical stimulation and controls.

4.2.6 Electrical stimulation assay

Electrical stimulation was performed using a coupled system that deliver homogeneous EFs [158]. The electrodes were placed in teflon supports to eliminate any contact with the incubator surface. The positive and negative terminals of the electrodes were connected to an electronic circuit (oscillator) that generated the voltage and frequency required to create the EF (Figure 4-4). The oscillator was energized with a dual source (Lendher – HY3003D-3, Shenzhen, China) and signal verification before and after electrical stimulation was monitored using an oscilloscope (Keysight – DSO1052B, Santa Rosa, CA, USA). Stimulated cultures were placed between electrodes, while control cultures were incubated in the same way, except that electrodes were not connected to the oscillator. Hydrogels were exposed to an EF of 7,7 mV/cm and 8,7 mV/cm at 60 kHz sine wave-form during 21 days of culture. According to a previous report the EF was delivered at first day of culture using an exposure time of 30 min delivered four times per day according to following the scheme: 30 min of stimulation corresponds to 5.5 h without stimulation.

4.2.7 Cell proliferation assay

Proliferation was assayed on days 7, 14 and 21 using MTS assay. First, culture media was removed and the hydrogels (five replicates) were washed with PBS. Then, samples were transferred into new 48 culture well-plate, and a solution containing culture media without phenol red and MTS reagent (ratio 5:1) was added. Cell-cultured samples were incubated at 37 °C and 5% CO₂ for 2 h in dark. Thereafter, the absorbance of 100 µL of supernatant was measured at 450 nm with a Multiskan FC Microplate Photometer (Thermo Scientific).

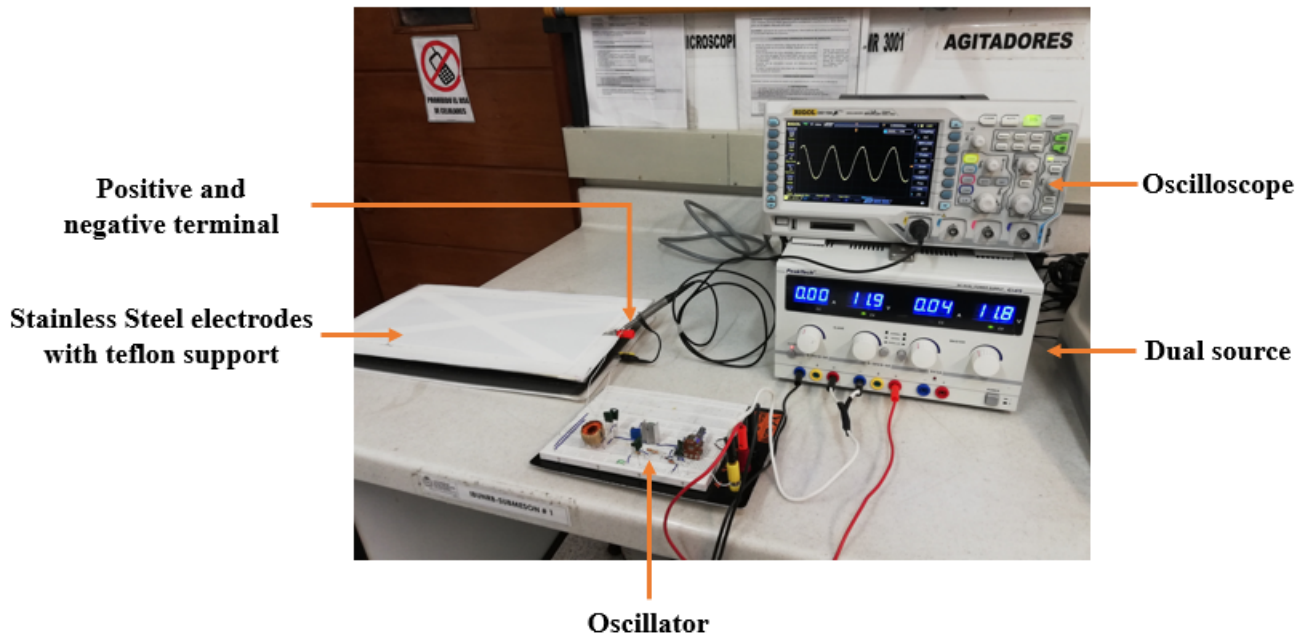


Figura 4-4: Capacitively coupling electrical stimulation in vitro. Connection of the dual source, oscillator and oscilloscope to the parallel electrodes.

4.2.8 Glycosaminoglycan quantification

The quantity of Glycosaminoglycans (GAGs) produced by each cell, in stimulated cell cultures and controls, was estimated in the cell culture media by a colorimetric assay using 1- 9-dimethyl methylene blue, 31 μM at pH 8.0. GAGs was quantificated on days 7, 14, after electrical stimulation, and stored at -20°C . All samples were analyzed with a spectrophotometer (BioSpec 1601, Shimadzu) to a 520 nm wavelength. The quantification was performed using a calibration curve of chondroitin sulfata B, ranging from 0 to 35 $\mu\text{g}/\text{mL}$ against reactive blank.

4.2.9 Statistical analysis

All analyses were performed as mean \pm SD ($n = 3$). Cell population growth and GAGs synthesis were analyzed throughout an Shapiro-Wilks test to verify normality of data. Then, homoscedasticity was analyzed by Levene's test and significance was obtained by analysis of variance with a t-studen test. The statistical analysis was carried out in IBM SPSS Statistics software.

4.3 Results

4.3.1 Hydrogels preparation

Gelatin chains were modified by grafting their carboxylic groups with the tyramine amine group using EDC and NHS as reaction catalyzers (Figure 4-2). Gelatin crosslinked hydrogels were obtained by covalently bonding the tyramine groups of the grafted polymers using HRP and H₂O₂ (Figure 4-3). H₂O₂ acts as the oxidant of the tyramine molecule, and the HRP catalyzes the crosslinking reaction, oxidizing two tyramine molecules every crosslinking cycle.

Figure 4-5 shows the gelatin hydrogel used for cell dynamics studies in a 12 culture well-plate. The pictures correspond to 50 μ L hydrogels with encapsulated cells after their crosslinking. In Figure 4-5A the hydrogels are not swollen in equilibrium and that is why they appear to be similar in size compared to hydrogels immersed in culture medium (Figure 4-5B).

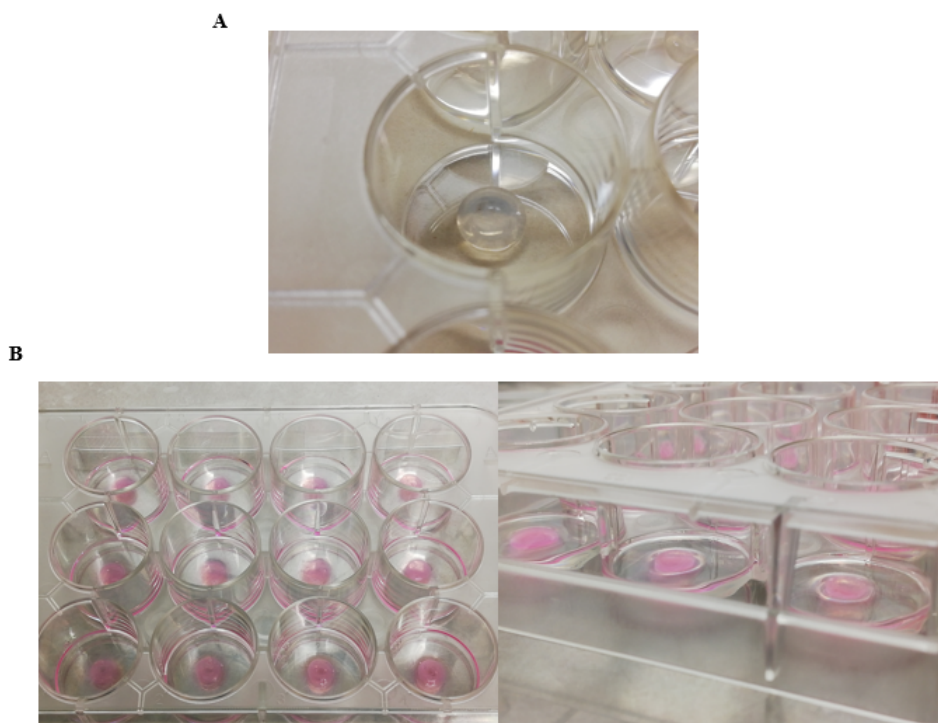


Figura 4-5: Macroscopic image of gelatine hydrogels used in cell culture experiments. A) Hydrogels prior to add culture medium. B) Hydrogels after to add culture medium.

4.3.2 Mechanical properties of the hydrogels

The rheological properties were measured over polymers that were dissolved in PBS. The variation of the storage modulus (G') and loss modulus (G'') as a function of reaction time are

shown in Figure 4-6. The storage modulus gives information about the amount of structure present in a material. It represents the energy stored in the elastic structure of the sample. If it is higher than the loss modulus the material can be regarded as mainly elastic. The loss modulus represents the viscous part or the amount of energy dissipated in the sample. The variation of the storage modulus (G') and loss modulus (G'') as a function of strain and frequency are shown in Figure 4-6 and Figure 4-8, respectively. Results indicate that hydrogels are mainly elastic.

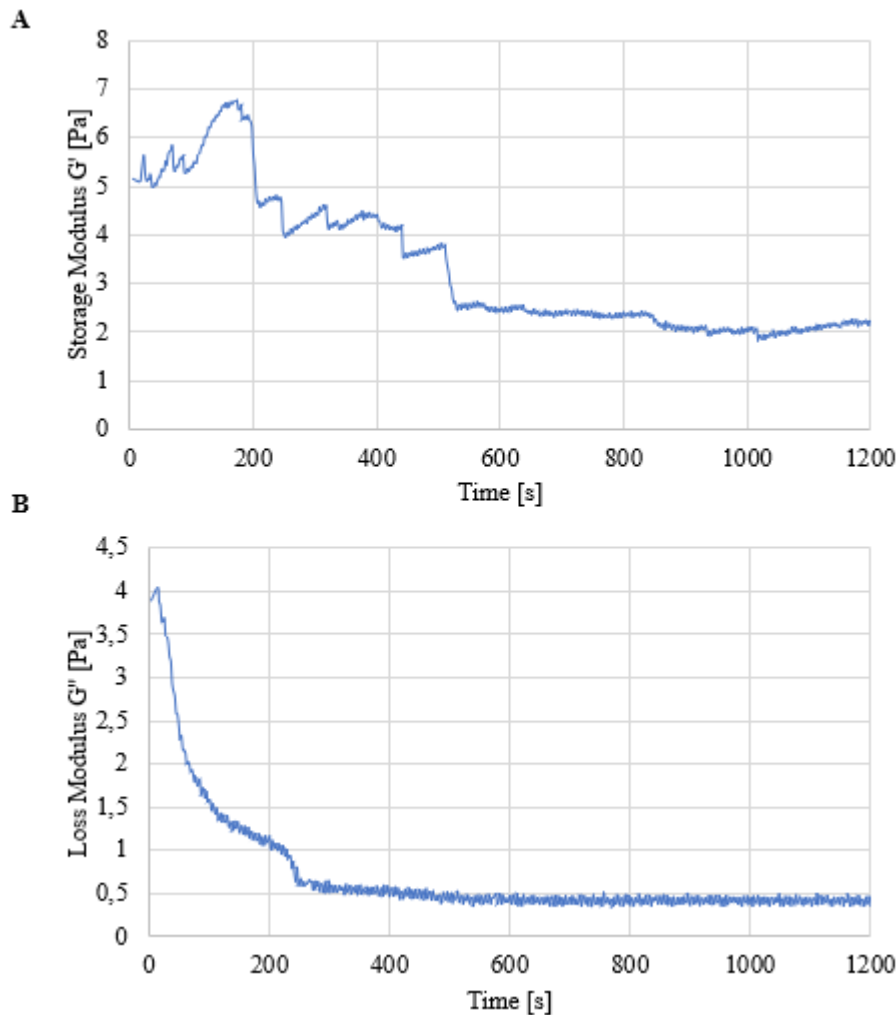


Figure 4-6: Gelation dynamics of the hydrogel in oscillatory time sweep. A) Storage modulus (G'). B) Loss modulus (G''). Each curve corresponds to the average of three different samples.

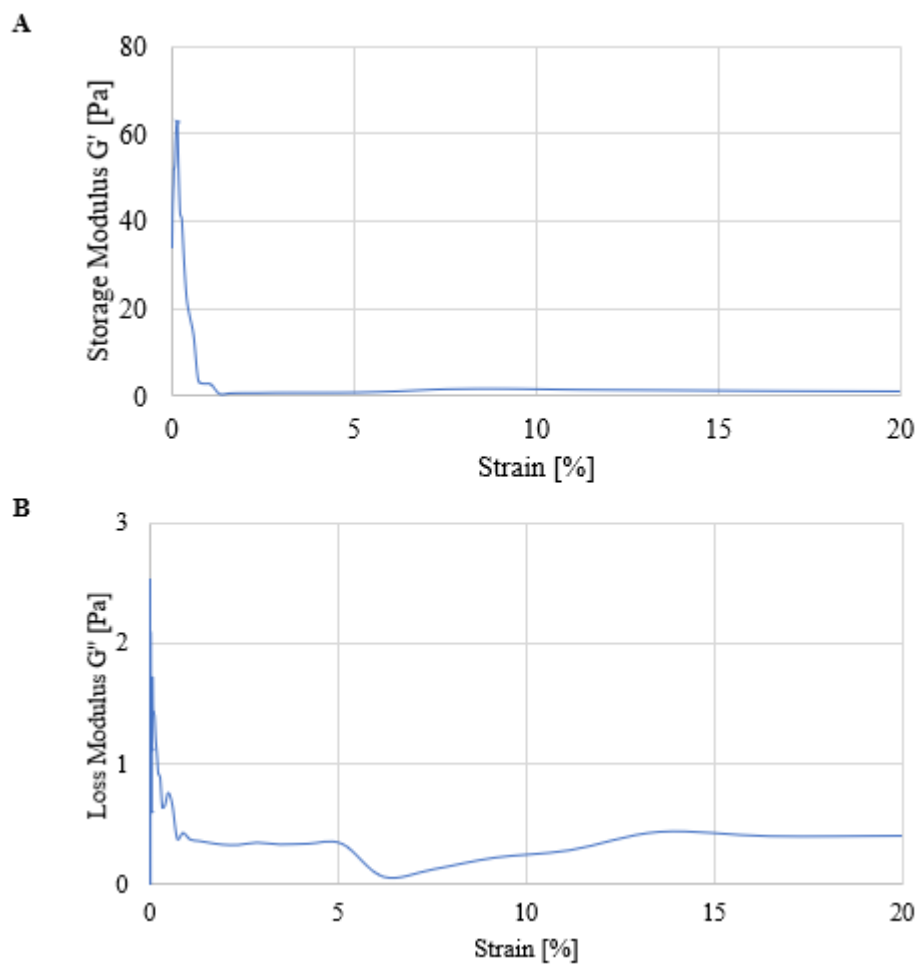


Figure 4-7: Dynamic strain sweep to calculate the range of strain amplitudes. A) Storage modulus (G'). B) Loss modulus (G''). Each curve corresponds to the average of three different samples.

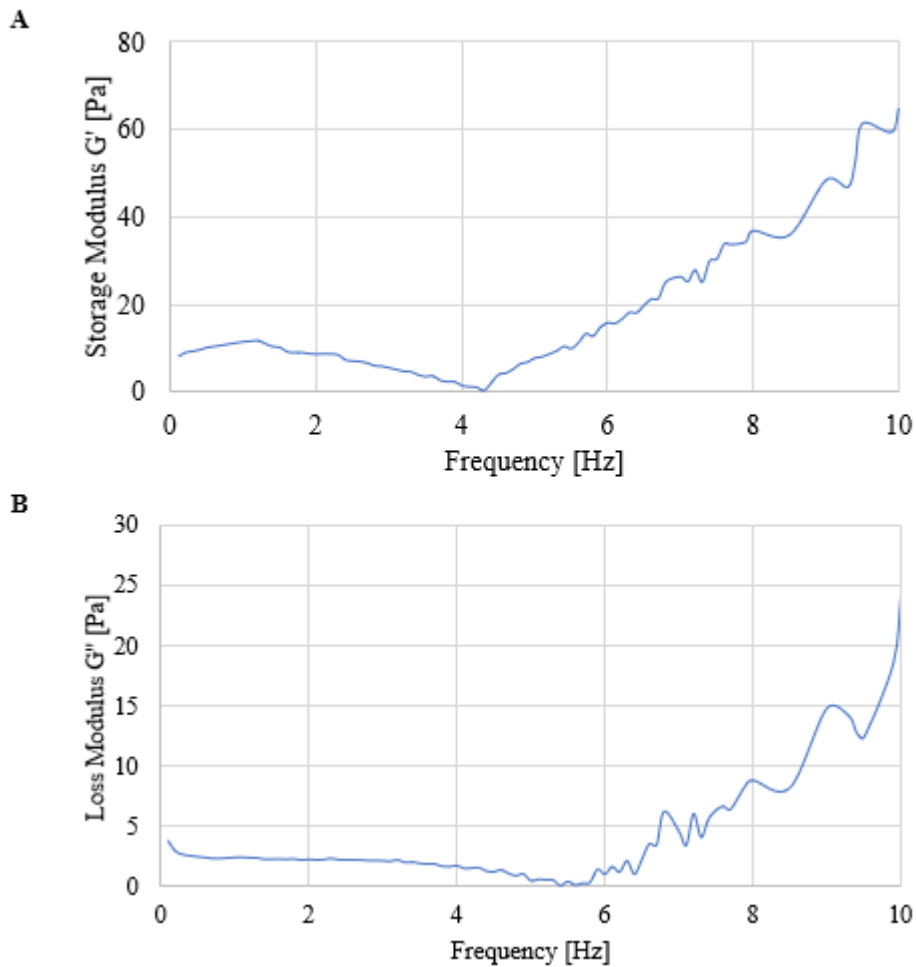


Figure 4-8: Dynamic frequency sweep test to determine the dependence of the dynamic shear modulus and loss factor on the frequency. A) Storage modulus (G'). B) Loss modulus (G''). Each curve corresponds to the average of three different samples.

4.3.3 Cell proliferation

Cell proliferation in electrically stimulated cell cultures, compared to that of controls, showed differences depending on the intensity of the applied EF. At days 7 and 14 of culture the controls evidenced a higher proliferation rate in comparison with the stimulated hydrogels with a electric field strength of 7,7 mV/cm (Figure 4-9A), no significant differences were found between controls and stimulated hydrogels at day 21 ($n=3$, $p > 0.05^*$). Similarly, at day 7 of culture the controls evidenced a higher proliferation rate in comparison with the stimulated hydrogels with a electric field strength of 8,7 mV/cm (Figure 4-9B), no significant differences were found between controls and stimulated hydrogels at days 14 and 21 ($n=3$, $p > 0.05^*$).

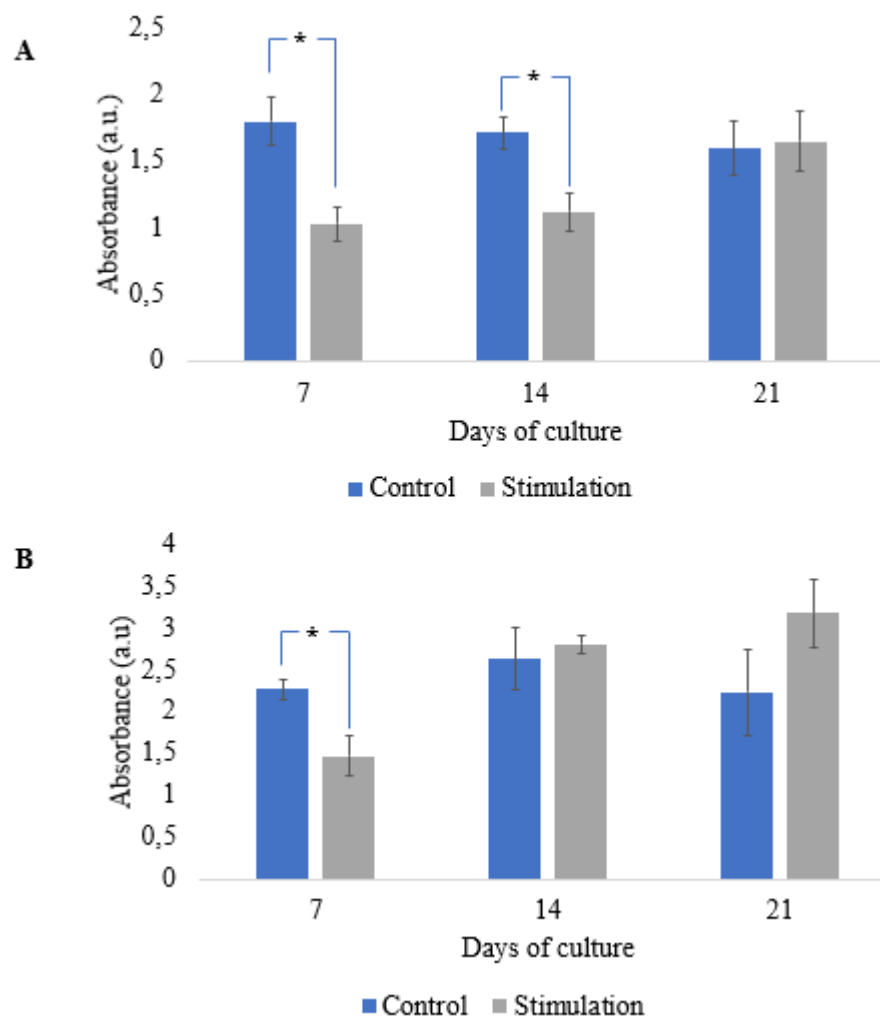


Figure 4-9: Cell proliferation of controls and stimulated hydrogels. A) Electric field of 7,7 mV/cm. B) Electric field of 8,7 mV/cm

4.3.4 Glycosaminoglycan quantification

The amount of GAGs produced by stimulated cell cultures and controls are shown in Figure 4-10. GAGs produced by encapsulated chondrocytes in hydrogels tend to stay the same with time. This behavior was observed in cell cultures stimulated with EFs of 7,7 and 8,7 mV/cm. However, encapsulated chondrocytes in hydrogels that were stimulated with an EF of 8,7 mV/cm demonstrated an increase in GAGs at day 7 (Figure 4-10B).

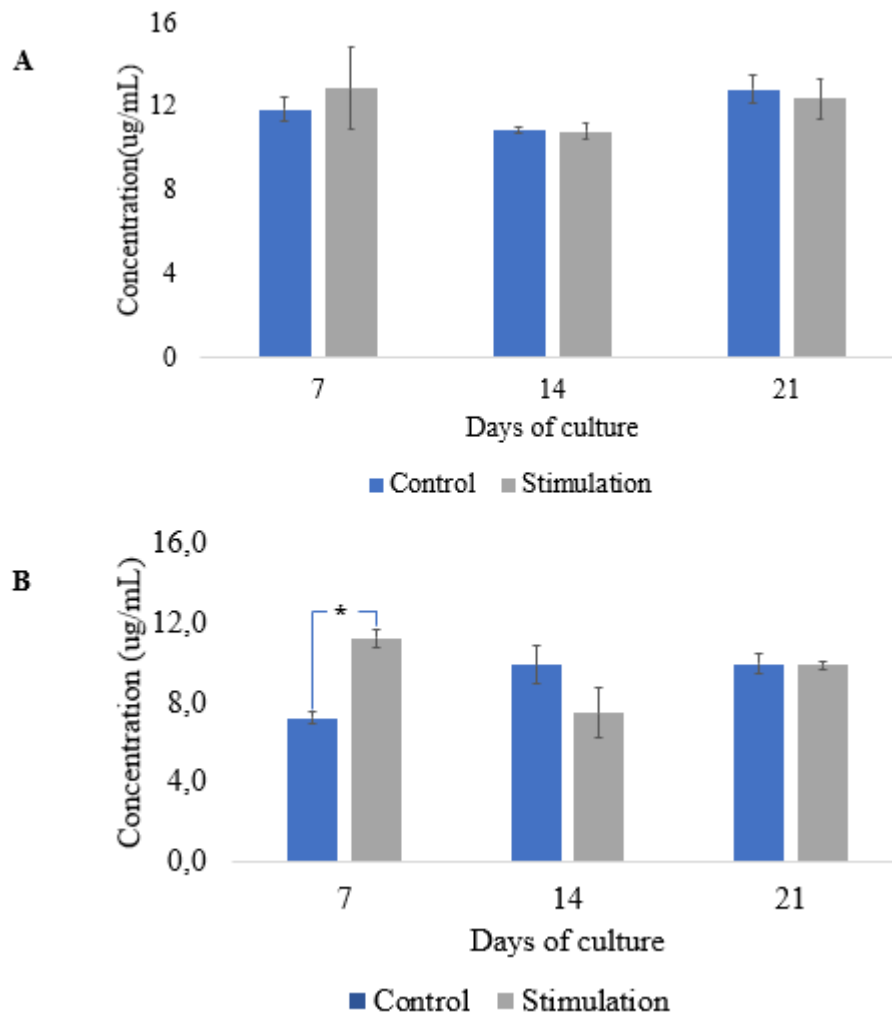


Figure 4-10: Glycosaminoglycans concentration of controls and stimulated hydrogels. A) Electric field of 7,7 mV/cm. B) Electric field of 8,7 mV/cm

4.4 Discussion

The aim of this research was to evaluate the effect of electric fields over chondrocytes cultured in hydrogels. Results showed that different EFs applied can decrease cell proliferation but can increase the production of important biomolecules in the extracellular matrix of articular cartilage, such as GAGs. Therefore, an adequate electrical stimulation could be established to optimize the chondrocyte behavior during in vitro cultures, depending on the applied electric field, the number of cells in biological samples can be maintained, while the production of extracellular matrix can be increased. If the aim is to stimulate the synthesis of GAGs, it is feasible to apply an EF of 8.8 mV/cm. Accordingly, therapies such as the autologous chondrocyte implantation may be enhanced using this methodology.

Regarding the stimulation method, there are two possible schemes to apply the EFs. In the first case, the stimulation may be applied using a direct coupling system in order to assess cell migration and orientation [147]. However, the physicochemical features of the cell culture medium may be altered due to the insufficient biocompatibility of the electrodes that are in contact with the biological material; furthermore, there are potential changes in the pH and a reduction in the levels of molecular oxygen [175]. Taking such limitation into account, the second method, which consists of an indirect coupling system that uses external parallel electrodes, was implemented in this study. This stimulation method has reported to increase cell population and molecular synthesis in chondrocytes [155]–[159], [161], [162], [167]. The custom-designed device implemented in this research is based on the same principle as used by other authors, which consists of two parallel electrodes connected to a power supply [176], nevertheless, some improvements to the device were made. For instance, potentiometers were implemented to vary the frequency of the oscillator. The results show that it is a useful tool to achieve a biological effect, as evidenced by the changes observed in chondrocyte cell dynamics. In this context, the main advantages of using this device are that it is a noninvasive capacitive coupling system, it is an adaptive system to stimulate any cellular type and it is a device that applies external EFs as it is currently used in medical stimulation. Thus, we proposed a high-performance product that could be used in the industry to modulate chondrocytes behavior.

Experimental analyzes showed that cell behavior is affected by the EFs intensity. A decrease in cell proliferation was observed using an EF of 7,8 mV/cm and 8,8 mV/cm. Cells show inhibition in proliferation since cell population is stable during the whole cell culture period. This finding contrasts with those observed by other authors, who have reported an increase in cell population continuously stimulated for 24 h [155], [157], [158]. In addition, it was observed that cell death has a similar behavior in stimulation scheme; cell death does not increase or decrease in stimulated cultures or controls. Based on these findings, it is possible to hypothesize that the applied EFs do not cause cell death. *In vitro*, it was found that culture of chondrocytes in the gelatin hydrogels showed that chondrocytes had round morphology while cell proliferation was hampered. The effects should be due to the 3D microenvironment in hydrogels combined with electrical field stimulation that are beneficial for molecular synthesis rather than cell proliferation. In contrast to the initial hypothesis, which proposed that electrical stimulation of chondrocytes cultured in gelatin hydrogels could enhance cell proliferation, the study did not find a significant increase in proliferation. However, several studies have demonstrated that electrical field stimulation and the use of hydrogels can promote chondrocyte proliferation. Therefore, it is necessary to evaluate the effect of different electrical stimulation parameters and to adjust the properties of hydrogels, such as microporosity, on chondrocyte proliferation.

In terms of molecular synthesis of chondrocytes, in this study, GAGs synthesis presented an increase at day 7 with a EF intensity of 8,7 mV/cm. These results suggest that a short-term exposition of chondrocytes cultures to high EFs favors the preservation of GAGs synthesis. Based on the results obtained for the proliferation and GAGs synthesis using the different stimulation schemes (50 Vpp and 100Vpp at 60 kHz), we hypothesize that stimulating a cell culture with high EFs keeps the chondrocytes in a low-proliferative state, maintaining its natural morphology and continuing with the production of molecules of interest. However, further studies using a wider range of EFs from 2 to 10 mV/cm, summed to the analysis of the expression of other molecules are needed to confirm this affirmation, since as observed in our results and some other literature reports, chondrocyte behavior is very sensitive to different electrical stimulation schemes [155], [156]. In addition, there were no changes in the pH of the cell culture medium; therefore, this variable was not considered when assessing the mechanism of change in cell behavior.

4.5 Concluding remarks

This research evidences changes in cell dynamics when chondrocytes are stimulated with different EF intensities. Within this context, depending on the EFs, in vitro procedures can be focused either on cell population is stable during the whole cell culture period or enhancing synthesis of GAGs. Furthermore, the procedures carried out in this research are suitable to be extrapolated to study cell behavior in different cell types, representing a new way in tissue engineering and regenerative medicine research.

5 Conclusions and perspectives

Articular cartilage has been extensively studied from different fields such as medicine, physiotherapy and engineering in order to find an alternative therapeutic approach to treat cartilage pathologies. Given the complex morphophysiological structure of cartilaginous tissue, current techniques such as physiotherapeutic treatments, surgical interventions, and biocompatible materials to replace cartilage defects are not definitive therapies to restore cartilage tissue. Nowadays, cartilage tissue engineering has focused on the combination of regenerative medicine techniques with the application of biophysical stimuli to improve the dynamic of the molecular structure of cartilage. Electrical stimulation has been used as external biophysical stimuli to evaluate the effect of chondrocyte proliferation rate and stimulate matrix proteins synthesis. Promising results have been obtained with the application of this kind of stimulation in terms of ECM production enhancement and chondrocyte morphophysiology modulation; however, a definitive treatment either to restore cartilage completely or to replace native cartilage with a biomimetic material with similar biochemical and biomechanical features has not been achieved yet.

The cellular dynamics of chondrocytes encapsulated in gelatin hydrogels was evaluated under electric field stimulation. The implementation of computational simulation is relevant since it allows predicting the distribution of the electric field in the capacitively coupled system. The scheme implemented to stimulate cells encapsulated in hydrogels allowed us to conclude that to maintain stable proliferation and increase the synthesis of important biomolecules in the extracellular matrix of articular cartilage, such as GAGs, an electric field of 8.8 mV/cm is needed. , a stimulation time of 30 minutes every 6 hours for 7 days.

In vitro, it was found that culture of chondrocytes in the gelatin hydrogels showed that chondrocytes had round morphology while cell proliferation was hampered. The effects should be due to the 3D microenvironment in hydrogels combined with electrical field stimulation that are beneficial for molecular synthesis rather than cell proliferation. In contrast to the initial hypothesis, which proposed that electrical stimulation of chondrocytes cultured in gelatin hydrogels could enhance cell proliferation, the study did not find a significant increase in proliferation. However, several studies have demonstrated that electrical field stimulation and the use of hydrogels can promote chondrocyte proliferation. Therefore, it is necessary to evaluate the effect of different electrical stimulation parameters and to adjust the properties of hydrogels, such as microporosity, on chondrocyte proliferation.

In conclusion, electrical stimulations allow controlling the cellular dynamics of chondrocytes cultured in gelatin hydrogels: Cell proliferation and molecular synthesis. Furthermore, if the appropriate parameters, electric field intensity and stimulation time per day, are used, the desired responses can be obtained, whether it is an increase in the cell population or molecular synthesis. This allows focusing the electrical stimulation of biomaterials according to the required therapy, optimizing *in vitro* procedures focused on the regeneration of articular cartilage.

The main findings of this research show that electrical stimulation on chondrocytes cultured in gelatin hydrogels increases the synthesis of GAGs when applying a certain electric field. Therefore, performing a more detailed analysis of the synthesis of molecules such as type II collagen and aggrecan in the presence of an electric field would allow us to complement this study.

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