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OXYGEN-GENERATING SMART HYDROGELS SUPPORTING CHONDROCYTES CELL SURVIVAL IN HYPOXIC ENVIRONMENTS

HIDROGEIS INTELIGENTES GERADORES DE OXIGÊNIO APOIANDO NA SOBREVIVENCIA DOS CONDRÓCITOS CELULARES EM AMBIENTES DE HIPOXIA

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Teresina - Piauí
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DEDICATION

Dedicated to my parents, Raul Carrasco and Marlene Montesdeoca and my brothers Raúl Bernardo and Cristian who have put all their trust and have been able to support me all the time. And dedicate to my beautiful country that I love with all my heart. I am Ecuadorian, yes sir!

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“La vida no es fácil para nadie. Y qué importa? Debemos tener perseverancia y confianza en nosotros mismos, creer que tenemos un don para algo y que ese algo debe ser realizado”.

(Marie Curie)

LIST OF ABBREVIATIONS AND SYMBOLS

GelMA- Gelatin Methacryloyl

CPO- Calcium Peroxide

MA- Methacrylic Anhydride

DI- Deionized Water

PBS- Phosphate Buffered Saline

TGA- Thermogravimetry Analysis

DSC- Differential Scanning Calorimetry

DTGA- Derivative thermal gravimetric

FTIR- Fourier Transform Infrared

SEM- Scanning Electron Microscopy

EDX- energy-dispersive X-ray spectroscopy

mm- Millimeters

μL- Micro Liters

mg/L- Micro grams/Liter

g/L- Grams/ Liter

T_{Onset}- Initial degradation temperature

T_{Endset}- Final decomposition temperature

T_P- Peak temperature

U- Up

D- Down

L- Left

R- Right

mBMSCs- Bone marrow stromal cells

OD21 cells- Odontoblast cells

Huh-7.5 cell- Cellulosaurus cell

RGD- Arginine-glycine-aspartic acid

HCS-High Content Screening

ROS- Reactive Oxidant Species

PLGA-Poly Lactic-co-Glycol Acid

NIPAAm-Hydroxyethyl methacrylate oligo (hydroxybutyrate)

AAC-N-isopropylacrylamide and acrylic acid

CDCs-Cardiosphere-derivedcells

PLA-Polylactic acid

PVP- Poly(2-vinlypyrridione)

PEG- Glycol–poly lactic

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RESUMO

O papel e os benefícios do uso de biomateriais inteligentes que liberam oxigênio para aplicações na regeneração de tecidos têm recebido interesse nos últimos anos. As abordagens são os tecidos que não conseguem se regenerar completamente devido ao ambiente de hipóxia no qual as células encontram-se na estrutura de biomateriais. A cartilagem é um desses tecidos que é criticamente difícil para-se reparar por si só. Por esse motivo, pesquisas buscam criar materiais que ajudem na entrega de quantidades suficientes de oxigênio controlando a liberação para não causar morte celular ou dano aos tecidos, sendo o cartilaginoso um deles. Desta forma, o objetivo principal desta dissertação foi desenvolver hidrogéis inteligentes que liberam oxigênio. Os biomateriais inteligentes foram baseados em hidrogéis de gelatina metacrilada (GelMA) carregados com partículas de peróxido de cálcio (CPO) em concentrações de (0,5; 1 e 3% w/w). Os hidrogéis foram caracterizados e a liberação de oxigênio monitorada em ambiente de hipóxia por um período de até 6 dias. Ao mesmo tempo, células de condrócitos foram incorporadas à estrutura dos hidrogéis e avaliou-se a sobrevivência delas in vitro em um ambiente de hipóxia. Conseguiu-se desenvolver um hidrogel capaz de liberar oxigênio por 6 dias e que ao mesmo tempo proporcionou um microambiente propício para a sobrevivência de condrócitos em ambiente de hipóxia podendo assim ser utilizado em engenharia de tecido para regeneração de cartilagem.

Palavras chaves: Gelatina metacrilada (GelMA), peróxido de cálcio (CPO), liberação de oxigênio, regeneração de tecidos, células condrócitos.

ABSTRACT

The role and benefits of using smart biomaterials that release oxygen for tissue regeneration applications have received interest in the last years. Approaches are tissues that cannot completely regenerate due to the hypoxia environment in which cells are found in the structure into biomaterials. Cartilage is one of those tissues that is critically difficult to repair on its own. For this reason, research seeks to create materials that help deliver sufficient amounts of oxygen by controlling release so as not to cause cell death or tissue damage. Thus, the main objective of this dissertation was to develop oxygen release smart hydrogels. The smart biomaterials based on gelatin methacryloyl (GelMA) loaded with calcium peroxide (CPO) particles in concentrations of (0.5; 1 and 3% w / w). Hydrogels were characterized and oxygen release monitored in a hypoxia environment for a period of 6 days. At the same time, chondrocyte cells were incorporated into the structure of the hydrogels and cell survival in vitro was evaluated in a hypoxia environment. It was possible to develop a hydrogel capable of releasing oxygen up to 6 days, and at the same time, it provided a microenvironment conducive to the survival of chondrocytes in a hypoxia environment, thus being able to be used in tissue engineering for cartilage regeneration.

Key-Words: Gelatin Methacryloyl (GelMA), calcium peroxide (CPO), oxygen release, tissue regeneration, chondrocytes cells.

1. INTRODUCTION

Tissue loss can be caused by diseases, age, blows, and treatments. Some tissues lack the ability to regenerate, especially compared to certain animals with excellent regenerative abilities (Fa-Ming & Xiaohua, 2016). Thus, tissue engineering, biomedicine, medicine, and other research fields have explored mechanisms to help the injured tissues regenerate themselves (El-Sayed Kaoud, 2018).

Some tissues can self-regenerate when they suffer some types of injury, and the new cells replace dead or damaged cells so that the tissue returns to its original form (Krafts, 2010). This can cause problems associated with the lack of muscle movement and severe bone pain. Cartilage is a type of tissue that does not have enough oxygen within its structure for cells to self-replicate and differentiate leading to limited vascularization.

For bioengineering, materials engineering, medicine, and other sciences, tissue regeneration has recently become attractive for biologists, engineers, and doctors. Regeneration varies widely between organs and organisms; therefore, a range of materials and models with different technical characteristics and innovative strategies should be studied. The use of biological materials (biomaterials) allows combining scaffolds, cells and biologically active molecules of functional tissues (El-Sayed Kaoud, 2018).

Within the range of biomaterials are natural and synthetic polymeric materials, in which the main difference is the price and the purity. Synthetic scaffolds can easily be designed. However, a disadvantage typically includes poorer cellular affinity compared to natural polymers and the risk of foreign body stimulation response by polymer or degraded products (Yang, et al., 2014). Biomaterials can act as a support (scaffolding) to promote cell growth and order differentiation to promote the healing of some tissue injury types (Dolcimascolo, et al., 2019).

Synthetic polymers have been chosen due to their higher purity at the processing time unlike many polymers of natural origin, although some polymer have the risk of stimulating a foreign body response. (Yang, et al., 2014) Within the applications, such materials have been used for skin and bone regeneration, as well as implants. Some polymers have also been used to transport drugs and particles. (Zhang, Wang, & Zhou, 2018)

To be admitted by the body, polymeric materials must comply with physical, chemical, mechanical, biodegradability, and biocompatibility properties. (Asadi, et al., 2019)

Biopolymers based of gelatin that have been used because they have similar characteristics as the native extracellular matrix, showing amide groups I, II, and III from the collagen (Sneha, et al., 2016). Anhydrous groups of gelatin substitute forms modified by gelatin methacryloyl (GelMA). The presence of methyl groups in the structure allow cross-linking to obtain a gel with greater mechanical properties (Sun, et al., 2018).

GelMA is used for cell culture and transport material by the presence of hydrophilic properties (Miri, et al., 2018) to allow encapsulating nanoparticles, which can be released by external stimuli and mechanisms, such as diffusion, swelling, and osmosis. At the same time, porosity is a main properly in the matrix that allows exchange between the internal and external environment.

Cartilaginous tissue needs additional oxygen because a hypoxic environment does not allow the cells sufficient amounts of oxygen for cell differentiation and in regeneration of turn injured cartilage. Researchers have sought a mechanism to develop hybridized materials based on polymeric biomaterials with oxygen-releasing particles, which allow controlled release of sufficient oxygen within a hypoxic environment.

Particles are generally derived from perfluorocarbons and solid peroxides (Gholipourmalekabadi, et al., 2016), however, some parameters must be fulfilled to control release. The quantities of oxygen released must not be excessive or insufficient during the release. For injured tissue regeneration, the ideal time for released would be at least 21 days, which is the time that the tissues need for regeneration (Huang, et al., 2019).

Calcium peroxides (CPO) are particles with the most advantages compared to the others solid peroxides, due to controlled release time and presence of the H_2O_2 in the catalase enzyme that originated during the chemical reaction between CPO. The water can be broken down into molecular oxygen, resulting in a complete decomposition of the CPO (Gholipourmalekabadi, et al., 2016).

Therefore, this dissertation contains two chapters. The first chapter described concepts as a literature review. The second chapter is a research project, with the objective to fabricate hydrogel matrices as smart materials that transport released oxygen particles to allow cellular survival.

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CHAPTER 1: LITERATURE OF REVIEW

SMART OXYGEN-DELIVERY HYDROGELS FOR CARTILAGE TISSUE REGENERATION

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RESUMO

Até o momento é evidente a busca por novos hidrogéis capazes de fornecer oxigênio com propriedades simuladas e semelhanças com a matriz extracelular nativa que permite que células de crescimento em ambientes hipóxicos reparem tecido cartilaginoso. Dessa maneira, hidrogéis com propriedades químicas e físicas ideais foram pesquisados para responder a estímulos externos para liberar vários agentes bioativos melhor para promover ainda mais uma resposta tecidual desejável. Por exemplo, gelatina metacrilada (GelMA) é um tipo de hidrogel modificado que permite o encapsulamento de nanopartículas liberadoras de oxigênio que na presença de meio aquoso e por porosidade controlada, bem como inchaço, permitem trocas ambientais internas e externas. Nesta revisão, mostramos diferentes tipos e características de hidrogéis e, especificamente, a família de hidrogéis GelMA. Além disso, métodos de síntese de biomateriais liberadores de oxigênio, finalmente, o mecanismo de liberação foi discutido.

Palavras-Chaves: Biomateriais Inteligentes, Regeneração de Tecido Cartilaginoso, Gelatina Metacrilada (GelMA), Nanopartículas liberadoras de oxigênio

ABSTRACT

New hydrogels able to deliver oxygen with properties similar to the native extracellular matrix that allow cell growth in hypoxic environments to repair cartilage tissue. In this way, hydrogels with optimal chemical and physical properties have been researched to respond to external stimulus to release various bioactive agents to further promote a desirable tissue response. For instance, methacryloyl gelatin (GelMA) is a type of modified hydrogel that allows the encapsulation of oxygen-releasing nanoparticles that, in the presence of aqueous medium and through controlled porosity and swelling, allows internal and external environmental exchange. This review explores different types and characteristics of hydrogels and, specifically, the family of GelMA hydrogels. In addition, synthesis methods of oxygen-releasing biomaterials and their release mechanism were discussed.

Key-Words: Smart Biomaterials, Cartilage Tissue Regeneration, Gelatin Methacryloyl (GelMA), Oxygen-Releasing Nanoparticles

1. INTRODUCTION

Cartilage tissue is one of the most critical tissue for repair. It cannot regenerate itself because of the small amount of oxygen due to a lack of blood vessels, nerves, and lymphatics vessels form a hypoxic environment. Thus, cartilage cells do not have sufficient amounts of oxygen to proliferate and differentiate, often causing cell death after injury (Huang, Xu, & Zhang, 2019). A period of 4–6 weeks is required to reach the vasculature with 83% patency within a damaged tissue (ashneh-Tala, MacNeil, & Claeyssens, 2015). Cartilage tissue regeneration takes 21 days to completely vascularize, but the most critical period is after post-injection that requires oxygen supplementation (Huang, et al., 2019; Londono, et al., 2017).

Tissue engineering has developed biological materials (biomaterials) based on hydrogels made of natural or synthetic polymers with composition and mechanical properties comparable to the native extracellular matrix of cartilage, which are used to generate scaffolds for cartilage regeneration (El-Sayed Kaoud, 2018). A major problem with traditional scaffolds is that they are unable to form an environment with adequate concentrations of oxygen inside, thus impairing potential tissue regeneration.

Some technologies have been construct to supply oxygen and promote cell growth, such as perfusion bioreactor, microfluidics techniques, incorporation of scaffolding matrices formed by angiogenic cells for rapid neovascularization and to ease blood vessel formation. Other research has used porous interconnected scaffold matrices that release growth factors. However, excellent result have not been achieved due to the inhomogeneity of 3D growth factors, the poor vasculature formed both in vitro and in vivo, incomplete vasculature, and lack of oxygen (Suvarnapathaki, et al., 2019).

In recent years, different materials of natural and synthetic origin, such as gelatin, chitosan, chondroitin sulfate, hyaluronic acid, poly (vinyl alcohol) (PVA), and methacryloyl gelatin (GelMA), have been used to fabricate oxygen-releasing biomaterials for cartilage repair (Sánchez, Téllez, & Rodríguez, 2017).

Studies about supplying oxygen have been development by loading from oxygen-releasing particles inside hydrogels, which break down into aqueous medium forming functional biological scaffolds that can evaluate in vitro the cell proliferate and cell survival in natural conditions. Some materials shaped like particles that release oxygen have been synthetized, including, perfluorocarbon (PFC) emulsion (Camci-Unal, et al., 2013), hemoglobin particles (Xiong, et al., 2013), calcium peroxide (CPO), hydrogen peroxide (H_2O_2), and sodium percarbonate particles (SPO) (Ward, et al., 2013), which can form oxygen inside or next to created tissues to enable cell migration, neovascularization, and ideal tissue growth (Khorshidi, Karkhaneh, & Bonakdar, 2019).

Ideally oxygen-releasing materials must deliver oxygen supply for 1–2 week, which is the range needed for vascularization post-implantation and is a critical period in which cells need oxygen that must be satisfied by external supply (Li, Guo, & Guan, 2012). One of the important parameters is the controlled release, which mean the amounts of oxygen must not exceed the percentage of oxygen released, because the excess can inhibit vascularization, differentiation, or cause tissue damage.

The vascularization is followed by three stages. First, hypoxia will allow angiogenesis, followed by the hyperoxia, and finally the recovery when the tissue needs extra oxygen amounts but without producing the radioactive species or changes in the pH to avoid inducing the apoptosis and allow complete vascularization (Rademakers, et al., 2019).

Calcium peroxide nanoparticles (CPO) are an oxygen releasing material to be loaded inside hydrogels, because CPO have some better characteristics than other compounds, such as higher purity and more controllable oxygenation (Khorshidi, Karkhaneh, & Bonakdar, 2019), and the initial supersaturation does not occur as in the liquid peroxides. In the presence of a catalase enzyme, the chemistry reaction fully avoids the formation of reactive oxidant species (ROS) and previous research has verified the controlled and sustained release of oxygen by CPO.

In this context, many investigations seek to generate intelligent materials that combine polymeric materials that have similar characteristics as extracellular matrix and particles that allow oxygen release as well as control the time it takes to regenerate the tissue without forming negative toxicity responses within the tissue. These issues are the main focuses of this review paper. Therefore, this literature review aims to present some concepts, mechanisms, and nanoparticles loaded inside polymers to create smart materials that can delivery amounts of oxygen for cartilage tissue regeneration.

1.1 OXYGEN AND CARTILAGE TISSUE

During the tissue process, a great supply of the oxygen is essential, because the oxygen is a prerequisite for cell proliferation and differentiation and formation of new tissues.

Although, the articular cartilage cells are adapted to survive in a hypoxia environment, an adequate oxygen level is essential for their normal metabolic activity. Oxygen levels below 1% significantly alter the cell activity of chondrocytes inhibiting glucose uptake and cellular ribonucleic acid (RNA) synthesis. In addition, oxygen levels down to 1% cause cell to produce calcified cartilage (Silver, 1975).

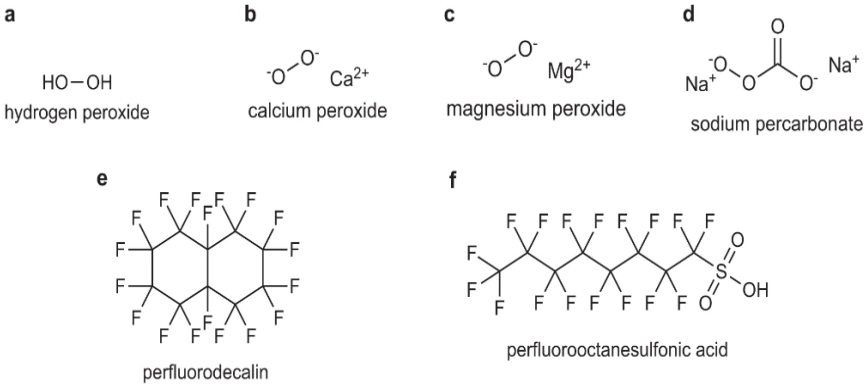
Grimshaw & Mason also showed the role of oxygen in metabolism of chondrocytes. They compared the comportment of bovine articular chondrocytes cultured in alginate beads for a period of 7 days in medium, maintained at different oxygen levels (<0.1, 5, 10, and 20%). The results demonstrated that the articular chondrocytes must be cultured at oxygen levels between 5 to 10%, since the cells showed greater activity in this range (Grimshaw & Mason, 2000).

1.2 OXYGEN-RELEASING MATERIALS

Biomaterials with oxygen-releasing nanoparticles are considered smart materials with potential applications in the biomedical market especially for cells and tissues with more metabolic activity, and as carrier materials for oxygen supplementation to maintain healthy tissues (e.g., cardiac, pancreas, muscle, cartilage, skin) (Asadi, et al., 2018). Various types of nanoparticles and their compounds in combination with hydrogels not only generate structural diversity but also improve mechanical strength and responses to stimulus plurality (Thoniyot, et al., 2015).

Oxygen-releasing smart materials can be created through the incorporation of peroxides of solid, liquid, or fluorination types (Suvarnapathaki, et al., 2019) incorporated into constructs of different forms, such as microspheres, nanoparticles, films, electrospun, nanofibers, electrospray, and scaffolds (Gholipourmalekabadi, et al.,2016). Figure 1 shows the chemical structures of different oxygen-releasing elements.

Figure 3-Chemical structures of oxygen-releasing elements. **a)** Hydrogen peroxide, **b)** calcium peroxide, **c)** magnesium peroxide, **d)** sodium percarbonate, **e)** perfluorodecalin, and **f)** perfluorooctanesulfonic acid (PFOS).



Reference: Suvarnapathaki (2019)

1.2.1 Solid Inorganic Peroxides

Sodium, calcium, and magnesium peroxides are the most used solid inorganic peroxides. Hydrolysis is the main mechanics for oxygen release when the nano/microparticles interact with the water, as shown these equations.

Calcium Peroxide $CaO_2(s) + 2H_2O \rightarrow Ca(OH)_2(s) + H_2O_2$

Magnesium peroxide $MgO_2(s) + 2H_2O \rightarrow Mg(OH)_2(s) + H_2O_2$

Sodium percarbonate $(Na_2CO_3) * 3H_2O \rightarrow 4Na^+ + 2CO_3^{-2} + 3H_2O_2$

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

Generally, MgO_2 has a faster reaction rate that in turn causes supersaturation. A high saturation is not efficient, because the death of the cells occurs as the oxygen rate delivery is not sufficient to allow cell proliferate and survival. This is what happens when perfluorocarbonates are used (Camci-Unal, et al., 2013).

Calcium-based nanoparticles have already been used in different applications, including tissue regeneration, and allow a sufficient amount of oxygen delivery with a

sufficient reaction rate for cells to proliferate. This type of reaction in the presence of an enzyme such as catalase allows complete decomposition of the peroxide and does not cause secondary toxic products, such as H_2O_2 (Gholipourmalekabadi, et al., 2016; Alemdar, et al., 2017). Table 1 summarizes some types of oxygen-generating composites, their solubility coefficients, and approximate oxygen-release amounts.

Table 1. Types of oxygen-generating composites, theirs solubility coefficients and oxygen-release to tissue regeneration are summarized

Compound	**Solubility coefficient**	**Amount of oxygen release**
Calcium peroxide	1.65 g/L at 20 ºC	22±3.3 mg/L
Magnesium peroxide	0.086 g/L at 18 ºC	44.38 mg/L
Sodium percarbonate	120 g/L at 20 ºC	40 mg per 100 mL of O_2, i.e., 57.16 mg per 100 mL O_2

Reference: Suvarnapathaki (2019)

1.2.2 Liquid Inorganic Peroxides

Some biomedical applications have used liquid peroxides to form oxygen-releasing particles. They present excellent solubility in aqueous medium, as the water permits fast oxygen release. Their decomposition can be into oxygen and water. When catalase enzyme of the human body is present in the liver, the blood is transformed into water and oxygen. The decomposition follows these reactions:

$$H_2O_2\,(aq) + 2Fe^{3+}(aq) \rightarrow O_2(g) + 2Fe^{2+}(aq) + 2\,H^{+}\,(aq)$$

$$H_2O_2\,(aq) + 2Fe^{3+}(aq) + 2\,H^{+}(aq) \rightarrow 2\,H_2O(l) + 2Fe^{2+}(aq)$$

The reaction velocity is a problem when hydrogen peroxides are used, due to the major reaction at the initial super-saturation of cells. Abdi et al. encapsulated H_2O_2 into Poly Lactic-co-Glycol Acid (PLGA), which was coated by a secondary layer composed of alginate hydrogel with the catalase incorporated to allow a total decomposition of the hydrogen peroxide into oxygen and water, avoiding generation of harmful radicals that influence viability of cell scaffolds but released oxygen (Abdi, et al., 2011).

Choi et al. fabricated polymeric microspheres for sustained oxygen-release and developed a sponge through embedding microspheres into alginate-based hydrogel that can supply oxygen to wound healing *in vitro* and *in vivo*. A double emulsion method was used with PLGA to form a porous oxygen-releasing hydrogel sponge (ORHS). The results showed that oxygen release induced the neovascularization and cell proliferation, helping wound healing. On day 7, a complete formation of the skin layer was observed (Jeongyeon, Gyeongsik, Taegyun, & Jeong, 2018).

1.2.3 Methods and Kinetics of Released Oxygen

The release of oxygen from nanoparticles that are hybridized in polymeric matrices, as well as drug release, is controlled via diffusion, swelling of the hydrogel, reversible nanoparticle-polymer interactions, and degradation of labile covalent bonds, osmosis, and erosion (Doblado Ponce de León, 2013).

The diffusion mechanism along with matrix porosity plays important roles. Other important roles include the size of the nanoparticles in the hydrogels, where results have shown that a larger size of incorporated nanoparticles produces a lower release rate and also increases insolubility. The release rate increases with insoluble or poorly soluble substances (Doblado Ponce de León, 2013).

In most hydrogel matrices, the release is controlled by diffusion of the nanoparticles to outside of the matrix. The release of nanoparticles is time dependent; when the time increases, the release of nanoparticles decreases. While the release rate decreases with time, there may be a saturation point where diffusion can no longer be controlled and is instead controlled by dissolution of the nanoparticles in the presence of a liquid medium. This is called zero order release kinetics (Doblado Ponce de León, 2013; Zarzycki, Modzejewska, & Nawrotek, 2010).

For hydrophilic matrices, the main variables that affect the release are the type of polymer, the nanoparticle/polymer ratio, the solubility of the nanoparticles, the size of the nanoparticles, and other hydrogel factors, such as viscosity, compression force, incorporation, and distribution of nanoparticles in the matrices. The matrices in contact with an aqueous medium are rapidly hydrated and form the solid-liquid interface (Doblado Ponce de León, 2013).

When the liquid medium enters the hydrogel matrix, swelling, rearrangement, and relaxation of the polymer chains occurs, increasing the volume and causing the outermost layers to undergo an erosion process. The release of oxygen will occur in the transition from the crystalline state of the matrices, due to water penetrating the layers. In the case of solid peroxide nanoparticles, a chemical reaction occurs, and the oxygen molecules are released through the pores of the matrix (Doblado Ponce de León, 2013).

The oxygen rate is important to understand for particles tissues as the rate of vascularization into particular tissues because enough oxygen in cells is crucial to allow the cellular survival in hypoxic conditions and avoid inhibiting the vascularization, differentiation, or tissue damage from excess release of oxygen (Colton, 2014). Thus, the control of oxygen release has four reasons: 1) oxidative damage for cells is cause by hyperoxia and by reactive oxygen species (ROS); 2) the differentiation is affects by the

ROS; 3) the inflammatory process is produced by the ROS which acts as a mediator; and 4) adequate hypoxia stimulate vascular infiltration (Farris, Rindone, & Grayson, 2016).

To help cell proliferate, hydrogels must be hybridized with another types of nanoparticle that are responsible for releasing sufficient and controlled amounts of oxygen to help the cells to proliferate, avoid apoptosis, and necrosis (Klotz, et al., 2016).

Nanoparticles composed of peroxides are preferred because they respond successfully in aqueous medium in combination with hydrogels (Gholipourmalekabadi, et al., 2016). The adhesion of calcium nanoparticles also helps improve porosity and swelling. Alemdar et al. used different concentration of CPO at 1, 2, and 3% w/w in GelMA hydrogels. This study showed that porosity and swelling are directly related. The swelling ratio increased from ∼17 ±0.8 to ∼27 ±0.7 for the GelMA hydrogels upon incorporation of a 3% CPO, and cell survival was 80% at 3% CPO at day 5 in comparison with the control which was 60% (Alemdar, et al., 2016).

Another study by Li Z et al encapsulated hydrogen peroxide and poly(2-vinlypyrridione) (PVP) into PLGA to form microparticles, which were loaded into a thermosensitive hydrogel from hydroxyethyl methacrylate oligo (hydroxybutyrate), N-isopropylacrylamide (NIPAAm), and acrylic acid (AAC). Cardiosphere-derived cells (CDCs) were also loaded to form a hybrid hydrogel. The results showed a homogeneous distribution of cells in the 3D structure and a significant increased of cell viability with the oxygen-generating hydrogels placed in a hypoxic environment for up to 2 weeks. Therefore, this can serve as a biomaterial for cardiac tissue.

1.3 HYDROGELS TO DELIVERY OXYGEN FOR CARTILAGE TISSUE

Hydrogels are polymeric, hydrophilic, and have three-dimensional networks. This special type of material can absorb large amounts of water or fluids, in addition to having excellent biocompatibility (Xia, et al., 2018).

These hydrogel properties turn out to be highly promising for medical application delivery and tissue regeneration. The lack of biodegradability of polymeric materials had been a difficulty in the past. However, some natural and synthetic polymers have been used as a basis for constructions that promote angiogenesis in tissue engineering, such as collagen, gelatin, chitosan, silk, and fibroin, due to the high flexibility they present (Nosrati, Pourmotabed, & Sharifi, 2018). Hydrogels that are hybrid with synthetic and natural polymers have shown to be better for the encapsulation of cells (Asadi, et al., 2018).

1.3.1 Natural Polymers

Natural polymers are cheaper and often mimic the extracellular matrix (ECM) providing better biocompatibility and adhesion with cells. However, the variation in the quality of naturally occurring polymers and the portions that are extracted from different sources are obstacles to study reproducibility (Hoque, et al., 2015). Natural hydrogels exhibit some limitations, as they do not present strong mechanical properties and cannot easily be controlled. Engineered scaffolds have used protein-based materials, such as hyaluronan, collagen, gelatin, and fibrin, because they present advantages in extracellular environments (Catoira, et al., 2019; Li, et al., 2019).

Hyaluronan can provide cells in three-dimensional environments very similar to the natural matrix because it is a component of extracellular matrix. La Gatta et al. synthesized sponge-based scaffolds by lysine methyl-ester crosslinking at different amounts and

hyaluronan to obtain materials that closely resemble elements in physiological cellular environments. Their results demonstrated that the water-uptake, mechanical, morphological, and stability properties were comparable or superior to solely hyaluronan scaffolds, and the chondrocytes cultured were maintained for 3 weeks. Thus, these are promising hydrogels for cartilage repair. (La Gatta, et al., 2017).

Collagen is other natural material with outstanding properties, such as low antigenicity, biodegradability, biocompatibility, and cell adaptation, which allow it to be commonly used in biology and medicine. However, the degradation rate and mechanical stability of the natural collagen are insufficient to fulfill the requirements of the tissue engineering; therefore, collagen needs to be modified and its properties cross-linked (Lan, et al., 2019). Zhenhui et al. conjugated biocompatible carbon dot nanoparticles (CP NPs) onto collagen through a natural product crosslinker (genipin) to prepare an injectable hydrogel. They demonstrated that increased stiffness due to the cross-linking presented a 21-fold higher compression modulus and a 39.3% lower degradation rate than the pure collagen, and the hydrogel increased the proliferation by 205.1% on day 21(Zhenhui, et al., 2019)

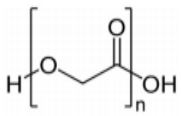
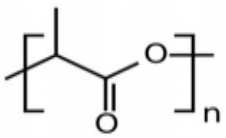
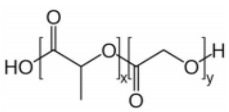
Fibrin is also a material used in biomaterial for tissue engineering. It has been investigated as a cell vehicle and for therapeutic drug delivery system. However, fibrin is not chondro-permissive and clearly requires further functionalization to be a versatile injection hydrogel system used in cartilage repair therapies (Almeida, et al., 2016). Gelatin is another hydrogel currently being used as a biomaterial with advantages in its biocompatibility, biodegradability, and inexpensive price. The gelatin is extracted from a process of collagen hydrolysis and can also absolve water 5-10 times its own weight. However, despite the versatility of gelatin within different biomaterial fields, it has weak

mechanical stability and durability; therefore, gelatin needs to be polymerized with anhydride groups to obtain GelMA hydrogels with cross-linked chains for a free movement of the cells within the matrix, and through the use of a photo-crosslinking, the degradation properties are improved (Kaleem, et al., 2019).

1.3.2 Synthetic Polymers

Synthetic polymers present an acceptable processing flexibility and do not have immunological concerns compared with natural polymers (Liu, Holzwarth, & Ma, 2012). Cartilage tissue engineering has used a variety of synthetic polymers, such as polyglycolic acid (PGA), polylactic acid (PLA), poly (ethylene glycol) (PEG), poly (vinyl alcohol) (PVA) polydioxanone, and methacryloyl gelatin hydrogels (GelMA) (Fatemeh., et al, 2017, Li, et al., 2019), due to their mechanic, porosity, swelling, and lubricating properties similar to natural cartilage (Sun, et al., 2018). Some properties of synthetic biodegradable polymers are shown in Table 2.

Table 2. Properties of synthetic biodegradable polymers for cartilage tissue engineering

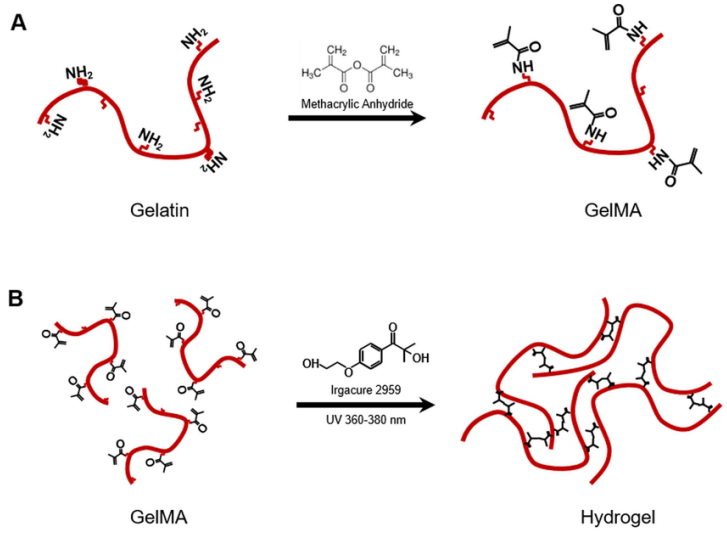
Polymers	**Molecular Formula**	**Melting Point**	**Abbreviation**	**Glass Transition Temperature**	**Fundamental Chemical Structure**
Polyglycolic acid	$(C_2H_2O_2)n$	225–230 ºC	(PGA)	35–40 C	
Polylactic acid	$(C_3H_4O_2)n$	150–160 ºC	(PLA)	60–65 ºC	
Poly (lactic-co-glycolic acid)		Depend on the percent compositio n (PLA, PGA)	(PLGA)	40–60 ºC	

Reference: (Fatemeh., et al, 2017)

1.3.3 Gelatin Methacryloyl (GelMA) hydrogel

GelMA is derived from gelatin (porcine or fish) and presents important characteristics, such as lower immunogenicity than its precursor (denatured collagen) (Mũnoz, Shih, & Lin, 2014). Arginine-glycine-aspartic acid (RGD) is the bioactive part of the gelatin, which stimulates the adhesion and growth of cells, and the matrix of metalloproteinase (MMP) is used for cell remodeling. Gelatin modification is obtained when the gelatin reacts with methacrylic anhydride and is optically crosslinked in the presence of photo-initiators (Yue K., et al., 2015; Shining, et al., 2019). Figure 2 shows the syntheses of GelMA hydrogels.

Figure 4- Synthesis and fabrication of gelatin methacryloyl (GelMA) photo-crosslinked. **a)** Gelatin was reacted with methacrylic anhydride (MA) to introduce a methacryloyl substitution group on the reactive amine and hydroxyl groups of the amino acid residues. **b)** GelMA photo-crosslinked



Reference: (Yoon, et al., 2016)

Mechanical strength as well as porosity, degradation, and swelling are critical properties for scaffold materials used to repair cartilage tissue. The mechanical strength of GelMA hydrogels is essential to hold and allow the encapsulated cells. This property can be adjust by change the degree of substitution of methacrylic anhydride, photo-crosslinking time, and the concentration of GelMA. Table 3 shows some of the main variables to synthesize hydrogels.

Zhao et al. informed that with an increase of GelMA concentration, the mechanical strength increased but the swelling and degradation decreased (Zhao, et al., 2016). Schuurman et al. reported similar results in which the effective swelling ratio depended to the UV exposure radiation. The swelling ration of hydrogels decreased by 60% for 5 min exposure. They confirmed a negligible swelling after at least 25 minutes of UV exposure and demonstrated that the gels attained about two thirds of their maximum modulus after approximately 10 min of UV exposure (Schuurman, et al., 2013).

Celikkin et al. reported obtaining 80±10% porosity for 5% GelMA scaffolds and confirmed that the porosity decreased at 60±10% when the GelMA concentration was increased to 10% w/v (Nehar, et al.,2017).

Table 3. The synthesis of hydrogels depending on desirable characteristic.

Regulating Variables	**Effects on Mechanical Properties**	**Cell Types**	**Results**	**Ref**
Concentration of GelMA	Higher compressive modulus and lower swelling properties	Bone marrow stromal cells (mBMSCs)	At 10% (w/v) GelMA hydrogels. 60 ±10% of porosity and with average pore size: 250±65 mm	(Nehar, et al.,2017)

Photo-exposure time	Cell viability decreased with increasing exposure duration (5–20 s)	Odontoblast cells (OD21 cells)	Cell viability decreased at 60% after 24h at 20s of photo-crosslinking time.	(Monteiro, et al., 2018)
Degree of methacrylate degradation	Increasing the degree of substitution in GelMA increased the storage modulus of the resulting hydrogel.	Cellulosaurus cell (Huh-7.5 cell)	High degree of methacrylate substitution promoted cell extrusion from 67.6 to 1.9 kPa to 94.9% and 14.8% DS respectively.	(Lee, et al., 2015)

Reference: Shining (2019)

2. CONCLUSION

Synthetic polymeric materials with similar characteristics to the native extracellular matrix (ECM) such as porosity, biocompatibility, and biodegradability can be loaded with nanoparticles acting as oxygen-releasing biomaterials which respond to external stimulates to repair tissue damage in cartilage engineering application by cell ingrowth, and they have been demonstrated to be good candidates for use in in vitro and in vivo assays.

Additional research is still needed about biomaterials for cartilage tissue repair. Development of smart biomaterials for cartilage repair with controlled oxygen-releasing would help tissue engineering and be innovative to replace commonly used tissue regeneration techniques as debridement and lavage, microfracture, as well as autografts (cell and tissue transplantation). These type of therapies have been shown to efficacy repair

cartilage defects. There are some limitations still, such as lack integration with healthy cartilage, little existing nutrients, and fibrous tissue formed instead of hyaline cartilage that present a morphology and function consistent in clinical applications. Possibly coating oxygen-releasing nanoparticles with biomaterials from gelatin could, in hypoxia environments, generate oxygen to allow the survival of chondrocyte cells.

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CHAPTER 2: ARTICLE 1

OXYGEN-GENERATING SMART HYDROGELS SUPPORTING CHONDROCYTES CELL SURVIVAL IN HYPOXIC ENVIRONMENTS

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RESUMO

A cartilagem é um dos tecidos do nosso corpo que não é reparado automaticamente por si só. Os problemas associados à cartilagem são muito comuns em todo o mundo e são consideradas as principais causas de dor e incapacidade. Os biomateriais inteligentes ou biomateriais "quadridimensionais" (4D) começaram a emergir como um candidato adequado, que são principalmente materiais tridimensionais (3D) que alteram sua morfologia ou geram uma resposta medida no espaço e no tempo a estímulos fisiológicos. Nesse contexto, a liberação de oxigênio através de hidrogéis em contato com a água é considerada biomaterial 4D. O objetivo deste estudo é desenvolver estratégias para liberar oxigênio de maneira sustentável e prolongada através de sistemas de hidrogéis para promover a sobrevivência das células de condrócitos em ambiente hipóxico. Os biomateriais 4D são desenvolvidos a partir hidrogéis de gelatina metacrilada (GelMA) carregada com peróxido de cálcio (CPO) e têm a capacidade de gerar oxigênio de maneira controlada e sustentada por até 6 dias. A incorporação de CPO no sistema de hidrogel forneceu materiais com propriedades mecânicas e de porosidade aprimoradas. Além disso, os hidrogéis promoveram a sobrevivência dos condrócitos e reduziram a morte celular em condições hipóxicas.

Palavras-Chaves: Gelatina metacrilada (GelMA), Peróxido de Calcio (CPO), Materiais inteligentes liberadores de oxigênio, Células Condrócitos

ABSTRACT

Cartilage is one of our body tissues that is not repaired automatically. Problems associated with cartilage are very common worldwide and are considered the leading causes of pain and disability. Smart biomaterial or “Four dimensional” (4D) biomaterials has started to emerge as a suitable candidate, which are principally three-dimensional (3D) materials that change their morphology or to generate a response measured in space and time due to physiologic stimuli. In this context, hydrogels that release of oxygen through contact with water are considered as 4D biomaterials. The objective of this study is to develop strategies to release oxygen in a sustainable and prolonged manner through hydrogel systems that promote chondrocytes cell survival in hypoxic environment. The 4D biomaterials are made from gelatin methacryloyl (GelMA) loaded with calcium peroxide (CPO) and have the ability to generate oxygen in a controlled and sustained manner for up 6 days. The incorporation of CPO into the hydrogel system provided materials with enhanced mechanical and porosity properties. Furthermore, the hydrogels promoted chondrocyte survival and reduced cell death under hypoxic conditions.

Keywords: Gelatin Methacryloyl (GelMA), Calcium peroxide (CPO), Smart materials oxygen release, Chondrocytes cells

1. INTRODUCTION

The lack of adequate oxygen levels limits the cellular therapy; however, the defective tissue can be replaced by autologous cells. Nevertheless, the presence of low oxygen levels limits the vascularization (Novosel, et al., 2011). This is a great challenge, particularly when it comes to repairing cartilage that lacks spontaneous regenerative capabilities (Wang, et al., 2018). The continuous supply of oxygen is essential to maintain life, cell metabolism (Mallepally, et al., 2014), and complete regeneration of tissues (Seekell, et at., 2016). Therefore, the engineering of biomaterials with the ability to providing sufficient and sustainable oxygen to the microenvironment in the body would provide a great benefit for the health and healing ability of tissues (Harrison, et al., 2007).

In this context, smart biomaterial or "Four dimensional" (4D) biomaterials, which are three-dimensional (3D) materials with the additional ability to change their morphology and response in time to physiological stimulus, have been extensively employed for tissue engineering and regenerative therapy (Ashammakji, et al.,2020). Several fabrication technologies can be employed to design 4D biomaterials, such as solvent casting, spin/dip coating, photolithography, and printing (Ionov, 2018).

Moreover, peroxide-based solids have been proven to deliver oxygen for a prolonged and sustained manner to tissues, promoting cellular survival and tissue repair (Steg, et al., 2015; Morais, et al., 2020). Calcium peroxide (CPO) has proven to be the most promising oxygen-generating material due to its two-step decomposition process, leading to sustained release of oxygen for a longer period of time compared to the liquid peroxide that almost immediately undergoes complete molecular oxygen decomposition (Gholipourmalekabadi, et al., 2016).

Furthermore, Gelatin methacryloyl (GelMA) has been used to design nanogels to transport oxygen and control release because it has characteristics very similar to the native extracellular matrix (Yue, et al., 2015). These hydrogels are 3D cross-linked networks that have the capacity to absorb large amounts of water (Wang, et al., 2018). These types of biomaterials are widely used due to their unique properties, such as biocompatibility, controllable degradability, swelling capacity, and ability to transport various agents. In addition, they provide interactions, such as Van der Waal forces and hydrogen bonds, that results in rapid dissociation and release of the encapsulated agents (Sarfraz, et al., 2017).

Herein, we disclose the design of smart oxygen-releasing hydrogels with the ability to release oxygen in a prolonged and sustained manner. The engineered hydrogels maintained the survival of chondrocyte cells in hypoxic environment in a 3D microenvironment with the potential to promote cartilage tissue regeneration.

2. MATERIALS AND METHODS

2.1 MATERIALS

Gelatin, type A (porcine skin, 300 g Bloom), methacrylic anhydride (MA), Calcium peroxide (CPO), phosphate buffered saline (PBS) and 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, 98%.) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chondrocytes cells were obtained in full accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Animal Care Committee guidelines at Federal University of São Paulo (protocol no. 2478130315).

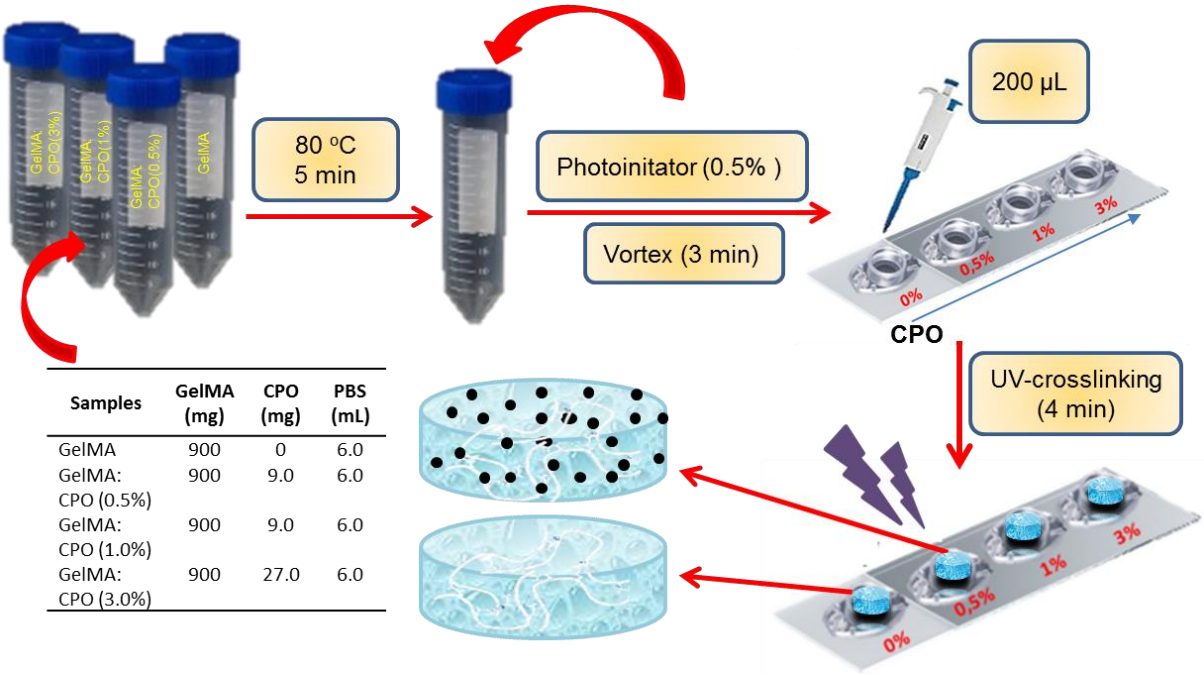
2.2 SYNTHESIS OF GELMA

The gelatin modification was synthetized following previously established protocol. (Alemdar, et al., 2016) Briefly, gelatin was mixed at 10% (w/v) with PBS (pH = 7.4) at 50 °C and stirred until completely dissolved. Subsequently, MA (8.0 mL) was added dropwise at 50 °C and stirring continued for 3 h. Afterwards, the reaction mixture was quenched by diluting with PBS. The mixture was filtered, and the solution was dialyzed against distilled water using 12-14 kDa for 1 week (the water was changed every day) at 50 °C to remove unreacted residues. The solution was lyophilized and stored at −80 °C.

2.3 FABRICATION OF CPO LOADED GELMA HYDROGELS

The smart hydrogels were synthesized following a previously demonstrated methodology (Alemdar, et al., 2017). Briefly, GelMA, 15% (w/v) solution was mixed with the desired concentrations of CPO (0.5, 1.0, and 3.0 wt%), and the mixture was dissolved in PBS (pH = 7.4) and incubated at 80 °C for 5 min. Afterwards, the photoinitiator Irgacure 2959 (0.5% (w/v) was added and then stirred in a vortex for an additional 3 min to form a homogenous solution (**Figure. 1**). From this stock solution, 200 μL was taken and photocrosslinked by placing it under a UV light (360−480 nm) source at 7.7 mW/cm^2 and with a distance of 7.3 cm for 4 min, generating a photocrosslinked hydrogel with a 4 mm thickness and 5 mm diameter (**Figure. 1**).

Figure 1- Schematic representation of the fabrication technology to design the smart oxygen-releasing hydrogels and the various compositions. GelMA solution was mixed with the CPO and the photoinitiator. Then 200 µL of the mixture was UV-crosslinked to provide crosslinked hydrogels with the thickness of 4 mm and diameter of 5 mm.



Samples	GelMA (mg)	CPO (mg)	PBS (mL)
GelMA	900	0	6.0
GelMA: CPO (0.5%)	900	9.0	6.0
GelMA: CPO (1.0%)	900	9.0	6.0
GelMA: CPO (3.0%)	900	27.0	6.0

2.4 CHARACTERIZATIONS OF HYDROGELS

2.4.1 Fourier Transformed Infrared Spectroscopy (FTIR) analysis.

The FTIR analysis of pure CPO and GelMA:CPO composite were obtained using an FTIR model Vertex 70 from Bruker. Spectra were obtained within the range 500–4000 cm^{-1} and measured with 60 scans.

2.4.2 Morphology and Porosity Analysis

The microstructure of the GelMA:CPO hydrogels with different CPO concentrations were characterized by scanning electron microscopy (SEM) (Quanta FEG, with 10 kV voltage accelerator), equipped with EDX of SDD (Silicon drift detectors),

Ametek brand, HX-1001 model (detector Apollo X-SDD). Prior to the SEM analyses, the hydrogels were crosslinked, freeze dried, and then coated with gold (Au) in the Quorum brand Q150R metallizer for 30 s at 20 mA. The microstructure and porosity of the samples were analyzed using ImageJ software (NIH Image, USA) and percentages for Ca^{+2} weight were obtained for each GelMA:CPO composite (0.5, 1, 3% w/w).

2.4.3 Thermogravimetry Analysis (TG) and Differential Scanning Calorimetry (DSC)

The thermal properties of the systems were evaluated to analyze the influence of CPO on the thermal stability of the crosslinked GelMA composite. TG was performed on a TA Instruments model SDT Q600 equipment, operating at a heating rate of 10 °C/min between 24–400 °C, under argon gas flow. Moreover, from the DSC curves, the melting enthalpy (ΔH_m) was calculated as the area of the endothermic peak. The melting temperature (T_m) of solely GelMA and the GelMA:CPO composite (0.5, 1, 3 %) were determined as the onset peak observed upon heating. The endothermic transition widths (ΔT) were estimated as the difference between the onset and the offset temperatures.

2.4.4 Mechanical Properties

The mechanical properties of the various hydrogels were measured by compression tests. The mixture solutions of GelMA, various concentrations of CPO, and photoinitiator were added to a specific cylindric mold (8 mm of diameter and 1 mm of thickness) and placed under UV light (360–480 nm, 6.9 mW/cm^2) for 60 s. Subsequently, the crosslinked hydrogels were incubated in PBS for 24 h at 37 °C (Sun, et al., 2018). A mechanical tester (TA.XTplus brand, Surrey, London) was used to compress the samples to obtain the stress-strain curve of the hydrogels with the uniaxial rate of 1 mm/min. The compressive modulus

was determined as the slope of the linear region corresponding to 0–5% strain. At least three specimens were used for each tested conditions (Nichol, et al., 2010).

2.4.5 Swelling studies

The swelling tests for the hydrogels were performed by immersing the samples in PBS for 24 h at 37 ºC and the weight changes of the swollen hydrogels were recorded (Alemdar, et al., 2017). At least three replicates was performed for each test. The swelling percentage was calculated by using equation 1:

$$Swelling\ (\%) = \left|\left(\frac{Wo-Wi}{Wi}\right)\right| * 100 \qquad (1)$$

where, *Wo* = Weight of swollen gel

Wi = Weight of dry gel

2.4.6 Oxygen Release Kinetics Analysis of the Hydrogels

Oxygen release analysis was performed in a completely hypoxic environment, while passing nitrogen in the system. The oxygen release kinetics experiments were performed for up to 7 days, employing an optical sensor (PRESENS, Germany). Measurements were made in deionized water (DI) following the previous established methodology (Alemdar, et al., 2017).

2.4.7 Degradation studies of the hydrogels

The various hydrogels with a diameter of 6 mm and a thickness of 1 mm were evaluated by incubation in PBS solution containing collagenase I (1.0 µg/mL) for 3 h at 37 °C. For each time point, the samples were freeze dried, and the dry weight was determined. The weight loss at the various time points was determined following equation 2.

$$\text{Weight loss} = \left[\frac{W_0 - W_t}{W_0}\right] \times 100 \qquad (2)$$

W_t: The dry weight of each hydrogel at time t; W_0: The initial dry weight of each hydrogel at time 0.

2.5 IN VITRO CELL STUDIES OF THE HYDROGELS

2.5.1 Chondrocytes isolation

Primary chondrocytes were harvested from articular cartilage of the knee joints of Wistar rats (130–150 g). Briefly, articular cartilage layers of the knee joints were sliced and minced and digested in 0.25% type I collagenase (Sigma-Aldrich, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, USA) overnight at 37 °C in a 5% CO_2 incubator overnight. Then, cells were collected from the digestion solution and seeded onto tissue culture flasks, for expansion in monolayer, and kept as sub-confluent monolayers in growth medium, DMEM supplemented with 1.5 mL glutamine, 10% fetal bovine serum (FBS), and 100 units/mL penicillin–streptomycin (Gibco-Invitrogen, USA).

The cells were expanded using standard tissue culture techniques, in which the incubations occurred in a 5% CO_2 atmosphere at 37 °C and culture medium was changed every 3 days. After 80% confluence, the cells were trypsinized and replaced at the same density until the third passage (P3). The P3 chondrocytes were used in the following experiments.

2.5.2 Cell Encapsulation and 3D Culture

To evaluate the cell viability, chondrocytes were encapsulated in the gelatin methacryloyl hydrogel containing different concentrations of CPO: 0% (control), 0.5%, 1%, and 3% w/v. Briefly, chondrocytes were trypsinized, collected by centrifugation,

counted with a hemocytometer, and resuspended in the DMEM solution containing GelMA (10% w/v) and photoinitiator Irgacure 2959 (0.5% w/v) at a final cell density of 6×10^6 cells/mL.

Subsequently, the various CPO concentrations were added. The four different solutions were pipetted into 96 well plate and cured with UV light (wavelength: 360-480 nm; 6.9 mW/cm^2; Omnicure Series 2000, EXFO, Canada) for 4–5 min, providing crosslinked cell-laden hydrogels. Culture medium, composed of DMEM supplemented with 1.5 mL glutamine, 10% fetal bovine serum (FBS), and 100 units/mL penicillin–streptomycin, was added immediately after the gel formation. The cell-laden hydrogels were maintained at 37 °C in an incubator with an atmosphere comprised of 95% air, 5% CO_2, and 100% relative humidity. ***Cell viability.*** Cell viability in the various cell-laden hydrogels was determined on days 1 and 5 after using Hoechst 33342 (Thermo Fisher Scientific, USA) and Propidium Iodide (Thermo Fisher Scientific, USA) to stain the cells. Briefly, the cell-laden hydrogels were washed with PBS and incubated in culture medium with a concentration of 0.2 μg/mL Hoechst 33342 and 0.2 μg/mL Propidium Iodide for 30 min at 37 °C. Afterwards, the hydrogels were rinsed again with PBS, and stained cells were viewed using an ImageXpress Micro® Confocal High-Content Imaging System (Molecular Devices, USA).

The images were captured with a 10× objective (9 images per well) and analyzed by the Multi-Wavelength Cell Scoring Application module for MetaXpress® Software (Molecular Devices, USA). The cell viability was based on the percentage of live cells, which was determined by excluding the dead cells (red fluorescence) from the total amount of cells (blue fluorescence).

2.6 STATISTICAL ANALYSIS

All data were reported as the mean ± standard deviation (SD) of three independent samples of each CPO concentration (triplicate wells). Statistically significant differences between groups of samples were evaluated by one-way ANOVA followed by Tukey's post hoc analysis, and p-values <0.05 were considered statistically significant. All statistical analyses were executed using Minitab version 17 software (Minitab Inc., USA).

3. RESULTS AND DISCUSSION

The photocrosslinking of the different hydrogels with varying CPO concentrations under UV radiation provided self-standing hydrogel discs with smooth and homogeneous surfaces. The sample comprised of solely GelMA was transparent, while GelMA with CPO had opaque color that became more turbid as the CPO concentration increased (Rahali, et al., 2017) (**Figure. 2a**). Furthermore, the crosslinked hydrogels were characterized by FTIR, and the methacrylate groups of the GelMA could be detected at 1637 cm^{-1} of the amide I (C=O stretching), the amide II band at 1550 cm^{-1} from the N-H stretching (Aldana, et al., 2017), and the amide III at 1448 cm^{-1}, mainly attributed to the C-N stretching (**Figure. 2b**) (Dallas, et al., 2007; De Paula, et al., 2018; Lobo, et al., 2018). Moreover, the presence of the CPO could also be confirmed by peaks from 1490 to 1384 cm^{-1} of the bending vibrations associated with the O-Ca-O group. The peak at 875 cm^{-1} corresponds to the peroxide group (O-O) bridge of CPO (Albuquerque, et al., 2008; Yan, et al., 2009) and at 711cm^{-1} is associated with the vibration of carbonate ion in calcite (**Figure. 2b**) (Zeglin, et al., 2006; Rastinfard, et al., 2018; Oxley, et al., 2008) The absorption band at 3000 to 3700 cm^{-1} and peak at 917 cm^{-1} is attributed to the elongation mode of the -OH group of the CPO (Morais, et al., 2020). For the GelMA:CPO hydrogels, the exhibited peaks at 1440 cm^{-1}, which was superposition in amine groups, and the new peak at 1031 cm^{-1} are possibly assigned to stretching and strains of C-O and C-H bonds as well as to deformation in the O-H bond, and this could be derived from the presence of Ca in the exchangeable complex adsorbed on the surfaces (Lopes, et al., 2017; Goydaragh, et al., 2019). An absorption peak located at 2971 cm^{-1} is due to the stretching vibration of C-H possibly because of H_2O_2 formation when the samples are displaced from molds and interact with

the water use to displace them. Additionally, the presence of peaks of hydroxyl confirmed the Ca present in each sample at ~3734 $cm^{-1}$and 937 cm^{-1} (Raja, et al., 2013).

The CPO incorporated inside GelMA hydrogels was confirmed through Energy-dispersive X-ray spectroscopy (EDX). As anticipated, hydrogel with solely GelMA did not showed any traces of calcium, nevertheless calcium was present in the GelMA:CPO (0.5, 1.0, and 3.0%), confirming that as the CPO increased the Ca weight also increased. The Ca values were 0.6, 1.6, and 5.7%, respectively (**Figure. 2c**).

The hydrogels must have optimal mechanical properties to be used as cartilage substitutes; therefore, these parameters were further evaluated. The compressive stress-strain curves and percentage of compression of the hydrogels are presented in **Figure. 2d,e**. The curves demonstrate an increased stress response with the addition of CPO – GelMA ~ 101 kPa at 73 strain (%) and GelMA:CPO (3.0%) ~ 185 kPa at 108 strain (%) (**Figure. 2d**). The highest stress was observed for the GelMA:CPO (1.0%) ~ 264 kPa at 100 strain (%) indicating that the system becomes saturated with increased CPO content up to 3%.

Moreover, the compressive percentage significantly increased with the addition of CPO (**Figure. 2e**). These could be explained by the increased interactions and physical crosslinking within the hydrogel with added CPO (Jung & Oh., 2014) (Sun, et al., 2010; Visser, et al., 2015).

The observed mechanical properties of the devised hydrogels make them an ideal candidate as oxygen-releasing implant materials for cartilage regeneration (Loy, et al., 2018). The presented technology could be an alternative approach to improve the application of GelMA in cartilage tissue engineering since one of its limitations is weak mechanical property. Several approaches have been demonstrated to improve the mechanical properties of GelMA by adding other components. For instance, Jingyi et al. combined GelMA with nanohydroxyapatite (nHA) as a scaffold to repair osteochondral

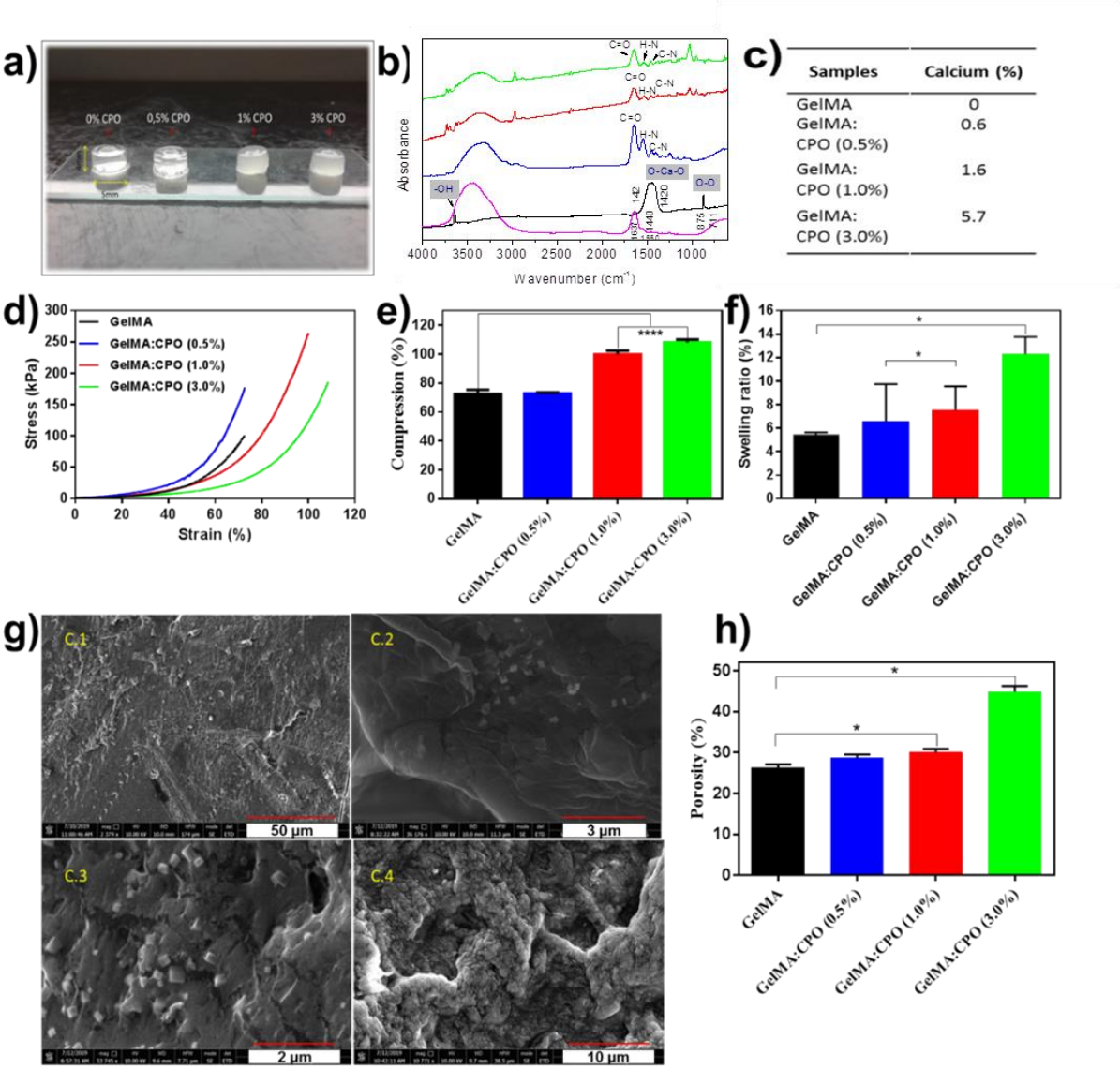
defect. The authors observed that the addition of 3% w/v nHA increased the compressive modulus compared to solely GelMA (GelMA ~ 209 kPa and GelMA:nHA (3%) ~ 625 kPa (Liu, et al., 2019). Gan et al. prepared GelMA hydrogels intercalated with dopamine oligomer, which exhibited five times higher compression strength than GelMA alone (2.5 MPa at the maximum compressive strain of 75%) (Gan, et al., 2019). Moreover, Gaharwar et al. combined GelMA with nanosilicates (0.5, 1, and 2%) to increase the mechanical properties, which increased the modulus to ~ 4.7, ~ 8.9, and ~ 12.9 kPa (GelMA ~ 3.3 kPa), respectively (Xavier, et al., 2015).

Furthermore, swelling is an important parameter connected with the porosity of the material and, therefore, impacts the oxygen-release kinetics and cell viability (Jung & Oh., 2014). It is well known that cartilage tissue defects display limited self-recovery, and to promote the initial repair by chondrocyte cells, oxygen supplementation is critical (Dai, et al., 2019). Within this framework, the addition of CPO to the GelMA hydrogel resulted in higher swelling ratio, which increasing with the greater amount of CPO employed (~ 6.3, ~ 7.5, and ~ 12.3% and for solely GelMA ~ 5.5%) (**Figure. 2f**). The trend of increased CPO into GelMA augmenting the swelling ratio was also demonstrated by Alemdar, et al., who synthesized matrices of GelMA: CPO (1, 2, 3% w/w) and confirmed that 3% CPO in GelMA allowed more swelling of ~ 27% than with ~ 17% of solely GelMA. Nevertheless, increasing the gel concentration decreased the swelling ratio, which is important to control the oxygen release and provides greater cell viability (Yoon, et al., 2016; Alemdar, et al., 2017).

The morphology and porosity of the hydrogels were evaluated through the imageJ software, to quantify the percentage of porosity based on scanning electron microscopy (SEM) images. Through SEM images, the CPO could clearly be observed with increased agglomerations as CPO concentration increases, and at 3% CPO, completely saturated the

system (**Figure. 2g**). The porosity for each matrix increased with increasing CPO content (~ 29, ~ 30, and ~ 45%, respectively and solely GelMA ~ 26%) (**Figure. 2h**). Correspondingly, Alemdar et al. presented similar trend; when content of CPO in GelMA hydrogel was increased to 3% CPO, there was an 60% increase and greater porosity. On the other hand, increased GelMA concentration lead to lower porosity due to increased crosslinking density (Alemdar, et al., 2017). Porosity and swelling are two primary parameters due to the direct relationship between them, an increase of porosity and swelling will allow oxygen release for more time, and also determine the degree of diffusion of the medium allowing an exchange of nutrients and oxygen to the cells.

Figure 2- **a)** The images of the various crosslinked hydrogels GelMA, GelMA:CPO (0.5%), GelMA:CPO (1.0%), and GelMA:CPO (3.0%) with 5 mm diameter and 4 mm thickness. **b)** The Fourier Transform Infrared (FTIR) spectra of the various samples including solely CPO. **c)** A table presenting the percentage of oxygen and calcium in the hydrogels confirmed by energy-dispersive X-ray spectroscopy (EDX). **d)** Mechanical properties of the hydrogels presented through the stress-strain curve, and the **e)** compression results. **f)** The swelling ability of the various hydrogels. **g)** The scanning electron microscopy (SEM) micrographs of the various hydrogels C.1:GelMA, C.2:GelMA:CPO (0.5%), C.3:GelMA:CPO (1.0%), and C.4: GelMA:CPO (3.0%). **h)** The porosity of the hydrogels based on the analysis of the SEM micrographs and using ImageJ software. Values are mean ± SD, N=3. ANOVA ($p< 0.05$) following by Tukey's multiple comparisons test. (*) $p< 0.05$, (**) $p< 0.01$, (****) $p< 0.001$ mean statistical differences.

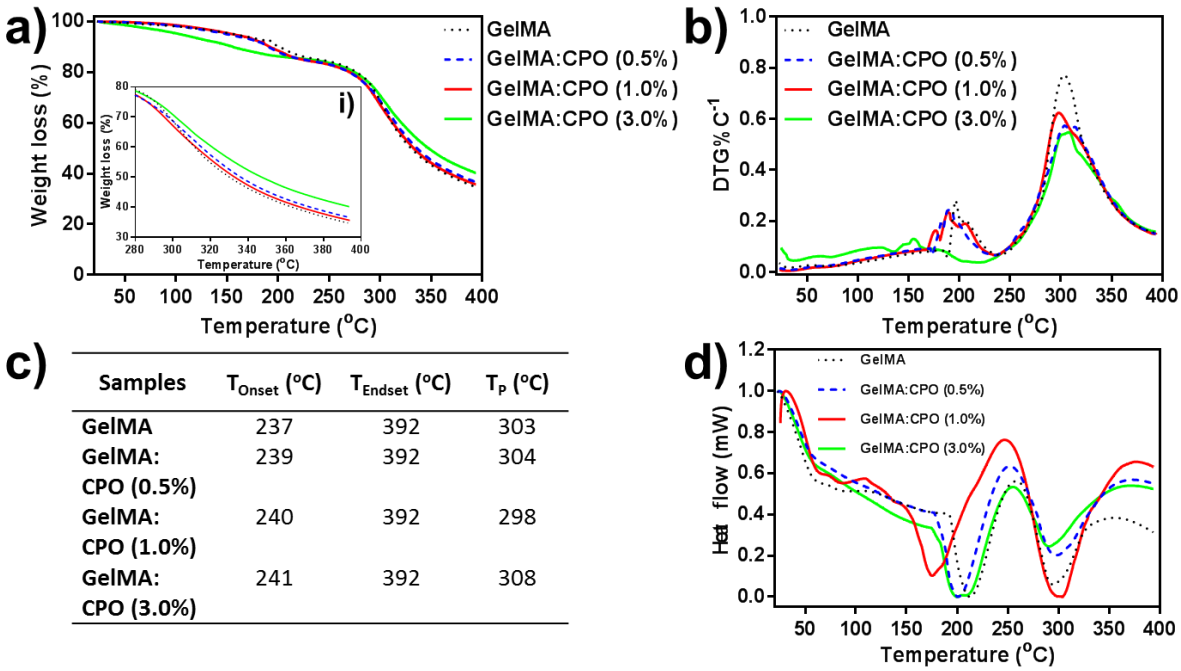


Thermal characterization of the hydrogels was performed through TG analysis. The TG and DTG curves (**Figure. 3a,b)** indicate that between 200 and 400 °C the hydrogels start losing integrity and, therefore, increase weight loss (%). Prior to these temperatures, the hydrogels only exhibited only a small weight loss, which corresponds to the dehydration of the GelMA, a characteristic thermal behavior of GelMA based hydrogels (Kim, et al., 2009). The weight loss exhibited two events; the first degradation of the hydrogels was (T_{Onset}) approximately between 237–241 °C, and all the hydrogels displayed final decomposition temperature values (T_{Endset}) at 393 °C (**Figure. 3c**), corresponding to the structural decomposition of GelMA and burning of the organic components. At temperatures beyond 393 °C, the remnant mass was almost stable with only trace amounts of final residue left at 540 °C, which may be related to the decomposition of calcite (Morais, et al., 2020). CPO had greater thermal stability with weight loss between 350 °C and 410 °C attributed at oxygen release. The final mass loss of the various hydrogels was ~ 65.0% GelMA, ~ 64.2% GelMA:CPO (0.5%), ~ 62.7% GelMA:CPO (1.0%), and ~ 59.4% GelMA:CPO (3.0%), respectively, at 324 °C. The observed thermal behaviors of the hydrogels are in accordance with previously disclosed performance, where the addition of nanoparticles into polymeric matrices did not significantly change the thermal property of the polymers because the degradation process for pure GelMA stopped approximately at 393 °C, while for GelMA:CPO hydrogels, the process ended at the same temperatures as the pure GelMA, indicating a large weight loss of the entire sample. However, the CPO incorporation was confirmed due to the remaining mass loss for samples (Heo, et al., 2014).

Moreover, the DSC curves show multiple endotherms, and compared with solely GelMA, a small dislocation was observed. The first peak between 50 and 70 °C corresponded to the glass transition of amino acids groups in the protein and the amorphous

regions of gelatin (**Figure. 3d**).(Aldana, et al., 2019) The endothermic peaks on the DSC curves were at about 202 and 325 °C, and with the incorporation of CPO, the first endothermic peak (202 °C) slightly shifted towards a lower temperature (**Figure.3c**). For instance, the GelMA:CPO (3%) exhibited a peak at 175 °C, due to the increased CPO content within the hydrogel (Heo, et al., 2014).

Figure 3- Thermal property analysis of the various crosslinked hydrogels. a) The thermal gravimetric (TG) and b) the derivative thermal gravimetric (DTGA) analysis. c) A table providing the thermal stability parameters of the various hydrogels. T_{Onset}: Initial degradation temperature, T_{Endset}: Final decomposition temperature and T_P: Peak temperature. d) The differential scanning calorimetry (DSC) curves of the various hydrogels.



Samples	T_{Onset} (°C)	T_{Endset} (°C)	T_P (°C)
GelMA	237	392	303
GelMA: CPO (0.5%)	239	392	304
GelMA: CPO (1.0%)	240	392	298
GelMA: CPO (3.0%)	241	392	308

Oxygen within the tissues is very important to avoid cell apoptosis and necrosis caused by hypoxic environments. From this perspective, the chondrocytes cells are exposed to this type of environment and, therefore, require supplementary oxygen (Wang, et al., 2018). The CPO incorporated into GelMA hydrogels converts to peroxide upon contact with water, followed by releasing oxygen as demonstrated in equation 3.

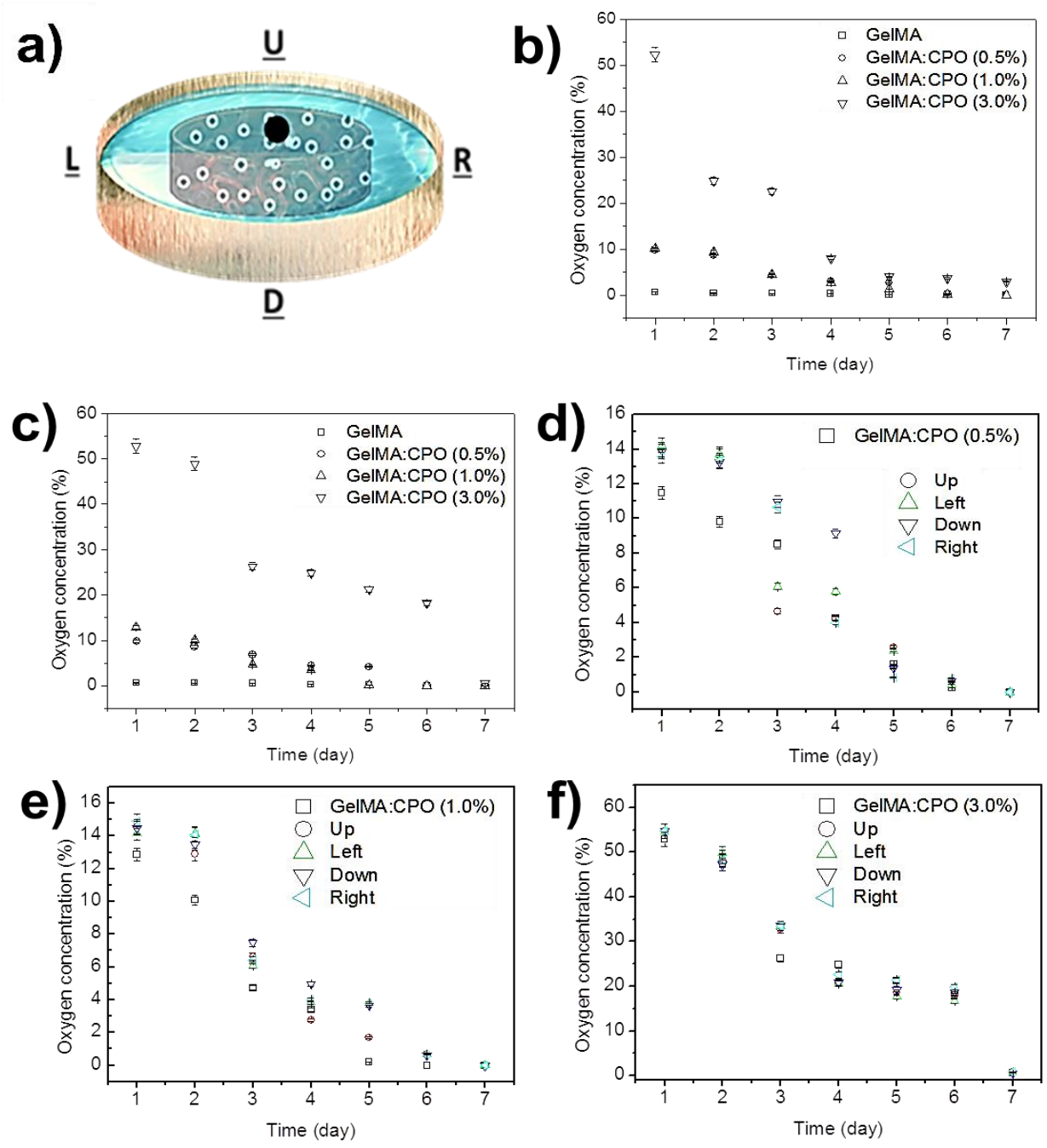
$$CaO_2 + 2H_2O \rightarrow Ca(OH)_2 + H_2O_2 \quad (3)$$

$$2H_2O_2 + catalase \rightarrow 2H_2O + O_2$$

The release of the CPO from the hydrogels occurs through swelling and diffusion mechanisms upon the interaction with the liquid medium and promoting an exchange between the internal and external environment (Razavi, Qiao & Thakor., 2019). Due to this mechanism, the hydrogels can be oxygen-releasing smart biomaterials (Bodenberger, et al., 2016). Subsequently, the oxygen release kinetics from the designed hydrogels was evaluated. To demonstrate a more representative accuracy of the oxygen concentration release, the oxygen level measurements were performed in various areas of the samples, within the hydrogels and also in various areas outside the hydrogels (**Figure. 4a)**. On the first day, the oxygen concentration measured inside the hydrogel was 9.83 ± 0.30, 10.09 ± 0.30, and 52.33 ± 1.57% for the hydrogels loaded with 0.5, 1.0, and 3.0% CPO, respectively (**Figure. 4b**). The following day the concentrations decreased to 8.66 ± 0.26, 9.47 ± 0.28, and 24.87 ± 0.76%, indicating oxygen release. The oxygen release stopped at day 6 for all the hydrogels except for the hydrogel with 3% CPO, which showed 2.97 ± 0.08% oxygen level at day 6. These results confirm that the increase of CPO concentration within the hydrogel prolonged the oxygen-release for an additional 2 days. Moreover, the concentration of oxygen released was measured in liquid by placing the hydrogels in DI water, which found an initial burst release followed by a decrease at day 3 (**Figure. 4c**). Moreover, the oxygen release kinetics was measured from 4 positions outside the hydrogels in DI water (**Figure. 4a**). The results from the various positions were similar; however, the measurements performed in the central point of the hydrogels found an initial burst release for the GelMA:CPO (0.5%) and GelMA:CPO (1.0%) (**Figure.4d-f**). Subsequently, the release rate decrease, probably due to the slow process that water molecules suffer when trying to penetrate the GelMA matrix hydrogels to reaction with the

CPO for oxygen release (Novosel, et al., 2011; Gholipournalekabadi, et al., 2016; Ionov, 2018 & Sarfraz, et al., 2017). The functional setting type linear or polynomial for the oxygen release measurement in liquid medium is showed in the **Annex A.**

Figure 4- Oxygen release kinetic studies of the various hydrogels for 7 days. a) Figure illustrating the measurements of the oxygen release from the various positions inside and outside the hydrogels (the black spot demonstrates measurements inside the hydrogels and U: up, L: left, D: down and R: right side outside the hydrogels). N = 10 for each measurement at the specified positions. The oxygen release kinetics under hypoxic conditions measured b) at the center point of the liquid media and c) on the hydrogels. d)-f) The oxygen release kinetics of the various hydrogels measured from different positions.

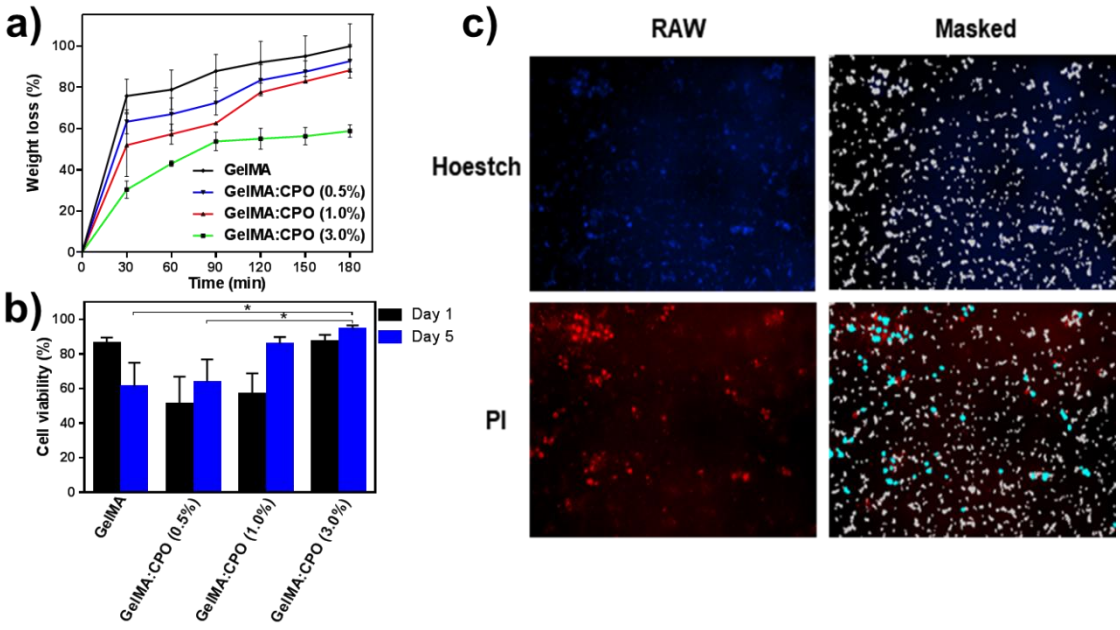


Several other researchers have reported the use of CPO loaded in polymeric materials for the delivery of oxygen. Shiekh et al. demonstrated the synthesis of a matrix based on antioxidant polyurethane cryogel scaffold loaded with 1.0, 2.0, and 3.0% of CPO (Shiekh, Singh & Kumar., 2018). The oxygen-releasing materials provided a sustained oxygen release for a period of 10 days in hypoxic condition; thus, it is a conceivable candidate for skin regeneration application (Shiekh, Singh & Kumar., 2018).

The stability of the engineered hydrogels was evaluated through enzymatic degradation analysis with collagenase Type I. After 3 h, the GelMA hydrogel already fully degraded; however, the incorporation of CPO provided more stable hydrogels. The GelMA:CPO (0.5%) resulted in ~ 93% degradation, GelMA:CPO (1.0%) in ~ 88% degradation, and the GelMA:CPO (3.0%) in only ~ 59% degradation after 3 h (**Figure. 5a**).

The incorporation of CPO into the GelMA hydrogels probably provides further interaction within the gel, which increases its stability resulting in slower degradation. (Zhao, et al., 2016) Moreover, the *in vitro* biocompatibility and functionalities of the hydrogels was further evaluated against chondrocyte cells. The cells were encapsulated within the various hydrogels for up to 5 days, and the live and dead cells were quantified. At the dynamic of cells survival (**Figure. 5b)**, the solely GelMA hydrogel showed a decrease of cell survival along the 5 days, while the samples containing CPO showed positive impact on the cell survival. At day 5, a significant viability was observed for the hydrogels containing 1 and 3% CPO (~ 86 and ~ 94%) compared to the GelMA and GelMA:CPO hydrogels (0.5%) (~ 61 and ~ 64 %), respectively (**Figure. 5b**). The positive impact from the CPO of the GelMA:CPO (3%) on the cells was also confirmed in the images captured of the cells (**Figure. 5c**). These initial results indicate that the presence of oxygen was essential to positively change the bias of cell behavior, which confirmed the importance of having the oxygen presence in a sustained and controlled manner.

Figure 5- **a)** The degradation study of the various hydrogels. Cell viability studies of the hydrogels from day 1 and day 5 under hypoxic conditions. **b)** The confocal micrographs of the live cells. **c)** Selected example of captured images by High Content Screening (HCS) equipment in three wavelength excitation (370, 535, and 485 nm) illustrating the emission of Hoestch (blue), PI (red), and ROS (green), respectively. "Raw" denotes the captured images and "Masked" the way the equipment mark and recognize the cells. (WHITE- Hoestch+; BLUE- Hoestch+ and PI+; ORANGE- Hoestch+ and ROS+). The images are for the GelMA:CPO (3.0%) hydrogel.



4. CONCLUSION

In summary, we successfully engineered oxygen-releasing smart materials comprised of CPO loaded GelMA hydrogels suitable for survival of chondrocyte cells. The incorporation of the CPO enhanced properties, such as swelling, slower degradation, porosity, and compression, over hydrogels made of solely GelMA. These characteristics further allowed controlled oxygen-releasing in hypoxic environment for up to 5 days. Furthermore, the *in vitro* cell study showed that the hydrogels containing CPO had a safe environment and improved chondrocyte cell survival. Hence, hydrogel can serve as an oxygen-releasing biomaterial scaffold for cell survival in a hypoxic environment and has great potential for cartilage tissue regeneration.

Acknowledgements

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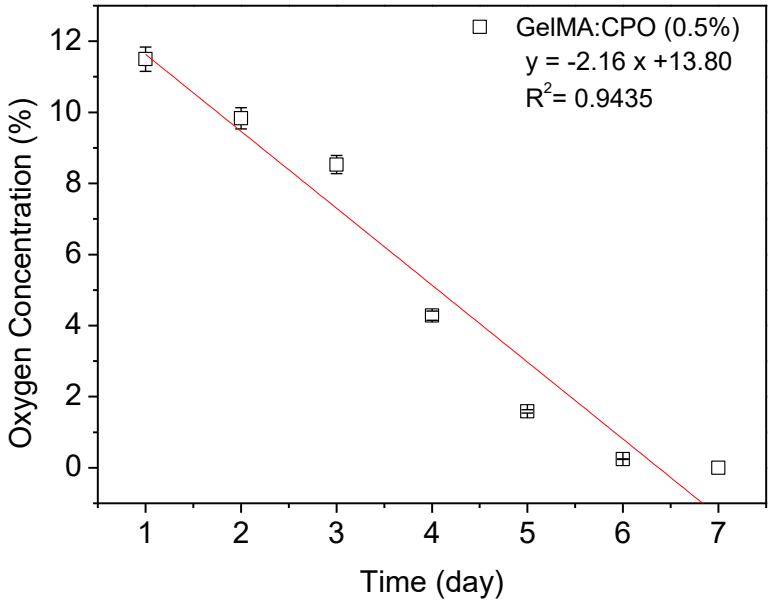
FINAL CONSIDERATIONS

Given the results, discussions, and conclusions reported in this research, some considerations for future work are discussed below:

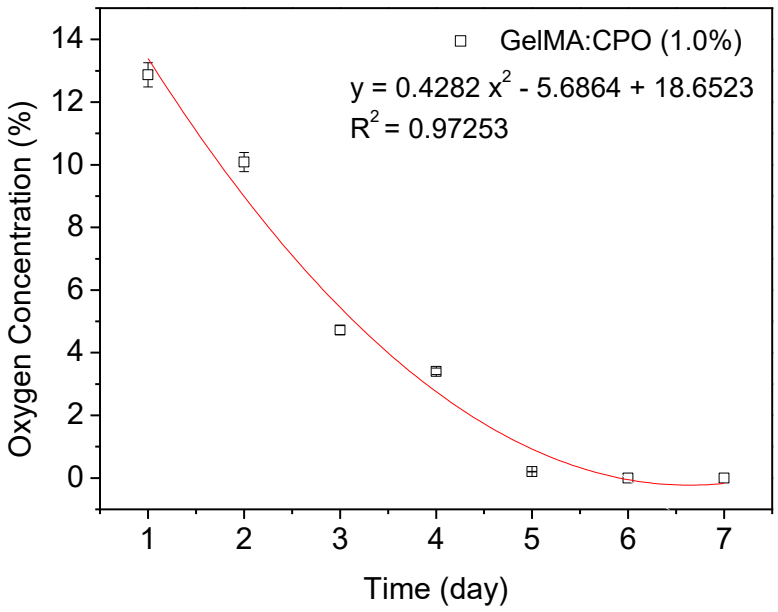
- Utilize a better system to generate an optimal hypoxia system.
- Use other methods to synthesize oxygen-releasing nanoparticles as a core shell to induce more extensive release.
- Bioprinting GelMA-based hydrogel arrays together with the chondrocyte cells.
- To continue with the in vivo study, two strategies may be carried out, the first one would involve the ingestion of the hydrogels loaded with CPO synthesized following the protocol for GelMA: CPO in cartilage that has been induced to injury, and the second involves multi-layered scaffolds printing.

ANNEXES

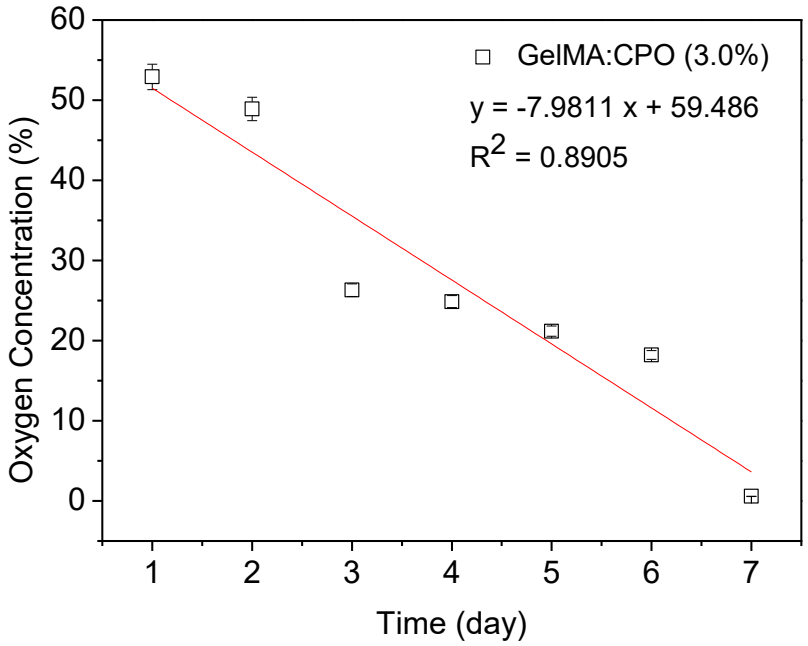
ANNEX A. Functional setting



Independent variable (Position)	Algebraic expression of the function	Coefficient determination	Type of function
GelMA:CPO (0.5%)	y = -2.16 x + 13.80	0.94	Linear
GelMA:CPO (0.5%) UP	y = -2.50 x + 15.70	0.84	Linear
GelMA:CPO (0.5%) DOWN	y = -2.74 x + 17.99	0.90	Linear
GelMA:CPO (0.5%) LEFT	y = -2.61 x + 16.53	0.90	Linear
GelMA:CPO (0.5%) RIGHT	y = -2.705 x + 16.99	0.88	Linear



Independent variable (Position)	**Algebraic expression of the function**	**Coefficient determination**	**Type of function**
GelMA:CPO (1.5%)	$y = 0.4282\ x^2 – 5.6864\ x + 18.6523$	0.97253	Polynomial
GelMA:CPO (1.5%) UP	$y = 0.4374\ x^2 – 6.1056\ x + 21.282$	0.95251	Polynomial
GelMA:CPO (1.5%) DOWN	$y = 0.2291\ x^2 – 4.4437\ x + 19.5869$	0.95484	Polynomial
GelMA:CPO (1.5%) LEFT	$y = 0.3071\ x^2 – 5.0192\ x + 20.0379$	0.89157	Polynomial
GelMA:CPO (1.5%) RIGHT	$y = 0.3336\ x^2 – 5.3212\ x + 20.8839$	0.9185	Polynomial



Independent variable (Position)	**Algebraic expression of the function**	**Coefficient determination**	**Type of function**
GelMA:CPO (3.0%)	y = -7.9811 x + 59.486	0.8905	Linear
GelMA:CPO (3.0%) UP	y = -8.4465 x + 61.423	0.9283	Linear
GelMA:CPO (3.0%) DOWN	y = -8.3638 x + 61.314	0.9272	Linear
GelMA:CPO (3.0%) LEFT	y = -8.7089 x + 62.4749	0.9331	Linear
GelMA:CPO (3.0%) RIGHT	y = -8.2484 x + 61.7271	0.9232	Linear