Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences

"On the improvement of the Process of Synthesis of Gelatin Methacryloyl (GelMA) Hydrogels and development of a hybrid nanoparticle-GelMA-based Bioink for Tissue Engineering"

A thesis presented by

Víctor Hugo Sánchez Rodríguez

Submitted to the School of Engineering and Sciences
in partial fulfillment of the requirements for the degree of

Master of Science
in
Nanotechnology

Monterrey, Nuevo León, May 2019

Instituto Tecnológico y de Estudios Superiores de Monterrey

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The committee members, hereby, certify that have read the thesis presented by Víctor Hugo Sánchez Rodríguez and that it is fully adequate in scope and quality as a partial requirement for the degree of Master of Science in Nanotechnology.

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Declaration of Authorship

I, Víctor Hugo Sánchez Rodríguez, declare that this thesis titled, "On the improvement of the Process of Synthesis of Gelatin Methacryloyl (GelMA) Hydrogels and development of a hybrid nanoparticle-GelMA-based Bioink for Tissue Engineering" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
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- Where I have consulted the published work of others, this is always clearly attributed.
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Víctor Hugo Sánchez Rodríguez
Monterrey, Nuevo León, May 2019

Dedication

To my parents:
my endless source of inspiration.

To my brothers:
my life accomplices

To the memory of Maxi, Turis, and Pachis:
second mothers whom I will remember forever.

Acknowledgements

To Dr. Grissel Trujillo de Santiago for accepting me in her research group, for being an intellectual mentor and incredible scientific inspiration.

To Dr. Mario Moisés Álvarez for being an admirable scientist and for sharing with me his advice full of wisdom, experience, and motivation.

To Dr. Ivonne González Gamboa for being a role model, a remarkable scientist, and an admirable friend. For teaching me that the best experiences of life can be lived on the side of science.

To Dr. Ali Tamayol for opening the doors of his laboratory and giving me the valuable opportunity to do science in an international environment.

To BSc. Sara Pedroza for being the best lab partner and for all her support in the laboratory.

To the Nanoamiguitos: Matías, Manuel and Israel who, with their moral and intellectual support, contributed with many grains of sand to successfully complete the master's degree.

To my friends and colleagues in the laboratory, Ada, Brenda, Caro, David, Diego, Isaac, Jorge O, Jorge T, Mauricio, and Salvador.

To my parents, for being my driving force, my greatest inspiration and the best parents, for supporting me throughout my life and projects, for giving me life, for their love and sacrifice in all these years. I am proud to be their son.

To my brothers for being my life partners, for giving me their love and unconditional support. Ph.D. Pablo, for showing me the most valuable path of academic inspiration and ambition to knowledge. BSc. Jesus, for inspiring me with his inexhaustible charisma, optimism and leadership.

To LIMB LAB in University of Nebraska Lincoln: Carina, Jacob, Zack, Azadeh, Fariba, Hossein, Jai and Morgan for their patience and the meaningful know-how exchange.

To Tecnológico de Monterrey for the financial support on this project and for these ten year of lessons learned. To CONACYT for the sustenance grant.

“On the improvement of the Process of Synthesis of gelatin Methacryloyl (GelMA) Hydrogels and development of a hybrid nanoparticle-loaded-GelMA-bioink for Tissue Engineering”

By
Víctor Hugo Sánchez Rodríguez

Abstract

Gelatin methacryloyl (GelMA) is a semisynthetic biomaterial that conserves some relevant advantages of native collagen such as the presence of cell-binding domains with protease-cleavage sites. GelMA-based hydrogels are currently used as biomaterials to develop cell-laden systems for several biomedical applications. GelMA provides the necessary physiological microenvironment and biocompatibility for tissue engineering studies. However, currently used methods to produce GelMA do not consider a strict control of the key parameters of the reaction process (i.e., mixing, location and rate of addition of methacrylic anhydride, and pH) which leads to batch to batch inconsistencies and low yields.

In this work, a semi-automated process to synthesize and purify GelMA is presented. Briefly, the method considered (a) the use of a custom-made jacketed reactor with temperature, agitation, and pH control, (b) the addition of methacrylic anhydride (MA), the key reagent of the synthesis, by a controllable syringe pump, and (c) a continuous dialysis stage, carried through a set of peristaltic pumps, follows the procedure shortening effectively the time needed for methacrylic acid removal.

Automated equipment and pH control reduce synthesis and purification times and enhance reactivity, leading to a higher degree of substitution. Using this synthesis and purification strategy, we conducted multiple sets of GelMA production and correlated reaction conditions to final yield and quality of the product. As a result of this process intensification, the required time of synthesis and purification was significantly reduced, and the amount of water required for the removal of cytotoxic residues (i.e., methacrylic acid) was minimized. While conventional protocols require a 2 weeks preparation time and result in an inconsistent quality of the final product, the methodologies presented here yield consistent quality GelMA in 5 days.

Moreover, this work presents results on the rates of methacryloyl functionalization during the reaction stage and the rate of methacrylic acid removal during the purification stage. The degree of methacrylation (DoM), the exposure to UV light (intensity and time) during the

photo-crosslinking time of GelMA hydrogels, as well as the gelatin and photoinitiator type and concentration have a strong and direct influence on the mechanical, physicochemical, properties of this biomaterial, which at the same time influence cellular behavior.

Finally, we developed a proof-of-principle strategy to use our hydrogel as a nano-composite bio-ink for 3D printing. To fulfill the goal of proper rheology for GelMA extrusion, a variety of bioinks were prepared by mixing GelMA, gelatin, and halloysite-nanoparticles (HNT). The concentration of GelMA, gelatin, and nanoparticles was varied to create an executable 3D ink at room temperature taking advantage of a two-step thermal- / photo-crosslinking strategy. This approach demonstrated to obtain reproducible cell-laden scaffolds by using extrusion as a tool for 3D printing.

Keywords: GelMA, nanocomposite, scaffold, 3D printing, optimized process, standardization, bio-ink, parameters

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Rationale

This thesis mainly focuses on the standardization and implementation of a semi-automated method to synthesize Gelatin Methacryloyl (GelMA) hydrogel and the performance assessment of nanoparticle-loaded-GelMA hydrogels for tissue engineering applications, for instance, as a bio-ink in 3D bioprinting to fabricate microtissues. The semi-automated method involves the use of a custom-made jacketed reactor for gelatin solution preparation, an automatic syringe pump for continuous methacrylic acid addition and a continuous-flow system using peristaltic pumps to conduct GelMA dialysis.

The main challenge in GelMA synthesis is to achieve reproducibility between batches. Every variable involved in the process is important, and minimal modifications to the method can result in meaningful changes to GelMA quality. Variables such as the gelatin batch, reaction temperature, pH, agitation speed, the rate of methacrylic anhydride addition, and others, must be carefully selected and monitored in every batch to achieve reproducible GelMA qualities. This thesis aims to tackle this problem by first standardizing the semi-automated method to synthesize GelMA that substantially reduces human intervention, dialysis time and water consumption with respect to actual reported protocols. Second, by conducting an adequate final product characterization which will lead to a guaranteed functional and high-quality GelMA. And third, by investigating the effect of nanoparticle loading to improve the properties of GelMA as a bio-ink in extrusion bioprinting.

The reproducibility of our GelMA samples synthesized with our semi-automated system were assessed by multiple characterization methods and assays such as FTIR, NMR, photo-crosslinking time, cell proliferation, OPA (*o*-phthaldialdehyde) assay as well as mechanical tests. The biological performance of the GelMA hydrogels will be evaluated in terms of the cell growth rate and proliferation activity of connective tissue cells. Additionally, bioprinting tests will be conducted to assess amenability of the nanoparticle-loaded hydrogel as bio-ink for the development of 2D and 3D constructs. The previously mentioned methods were used to compare our final GelMA and its commercially available analogous.

All in all, hydrogel characterization and nanoparticle-functionalization results will bring up a big study opportunity towards its application and feasibility as bio-ink for cell-laden fibers and microtissue scaffolds.

Justification

GelMA-based hydrogels are biomaterials widely used in tissue engineering applications due to several advantageous characteristics. For instance, GelMA conserves essential properties of collagen which makes it bio-responsive and biocompatible, also, it is photo-crosslinkable, transparent and amenable to be used as a raw material for different microfabrication technologies. The current established protocol to produce GelMA hydrogels, although simple, is not very robust or reproducible, is highly technician-dependent, and its purification requires long times. The implementation of a procedure that assures the reproducibility of this material, that reduces its production time and that is easy to implement, will be highly appreciated by the academic community that uses GelMA on daily basis.

GelMA hydrogels are commercially available, both as a raw material (Sigma Aldrich) and as bioinks (Cell Ink, Allevi). Most of the sellers offer these products at high cost ($190 USD per gram) and they do not guarantee the final DoM of the hydrogel. Aiming to produce high quality, reproducible GelMA for our lab needs, we chose to produce and characterize our own hydrogels at lower costs and accurate synthesis standardization. In the future, our improved and standardized process can represent an entrepreneurial opportunity to produce this valuable high-end product for our local market.

Recently, there has been a great interest in nano-composite hydrogels to design bioinspired nanomaterials used in tissue-engineering applications. Nanocomposite hydrogels may exhibit enhanced properties and physical and biological functionalities relative to their pristine-hydrogels counterparts.

A wide range of nanoparticles, including carbon-based, metallic, ceramic, polymeric, among others, can be laden to hydrogels to enhance different properties (i.e.: mechanical, rheological, biological, etc.). In this project, the fabrication of nano-composite hydrogels using GelMA and halloysite nanotubes aims to improve its biological and physical properties inspired in a biological tissue which can be helpful in several biomedical applications.

Hydrogels are the most popular choice to be used as bioinks for extrusion-3D-bioprinting. GelMA has been widely used as a bioink, however, it's naive version shows a challenging rheology to extrude as a self-standing ink. By using the nano-composite hydrogel we aim to get a bioprintable and biocompatible ink.

The control and standardization of the synthesis of GelMA, and the rational design of a nano-composite bioink, will provide us with a solid platform to tailor bioinks for any desired biomedical application in the future. With this in mind, this thesis proposes a new way to design nanostructured hydrogels by harmonizing the combination of nanotechnology and GelMA hydrogels which will bring promising benefits in tissue engineering.

Introduction

Hydrogels are generally polymer networks that retain water within them. Due to their high-water content, some hydrogels do not fulfill the required mechanical properties for several tissue engineering applications. Strategies to improve their mechanical properties, include combination with other polymers or nanoparticles [1], [2].

GelMA is a hydrogel that belongs to the group of semisynthetic biomaterials. It is synthesized from the reaction of gelatin and methacrylic anhydride. GelMA is attractive because it resembles some essential properties of the native extracellular matrix (ECM). GelMA tunability is determined, partway, by the degree of methacrylation (DoM) that occurs during hydrogel synthesis. An advantage of GelMA, over other biomaterials, is that it can be cross-linked when exposed to light irradiation. This reaction is initiated by mixing a photocurable hydrogel precursor with a photoinitiator. This mix is next exposed to light to finish the crosslinking [3].

Moreover, GelMA hydrogels can be easily laden with cells or mixed with nanoparticles to modify their physical and bio-responsive properties [4]. GelMA hydrogels are widely used in biomedical applications due to its biocompatibility and tunability of physical features. Also, these versatile hydrophilic materials have also been used as drug-delivery vehicles, adhesives and injectable fillers [5].

Often, naive GelMA hydrogels need to be combined with other components to tailor their properties for a desired purpose. A frequent aim, when designing composite-materials, is to enhance the mechanical strength, however, some biomedical applications require other properties, for instance, conductive hydrogels [5]. For example, Zhang *et al*. (2011) developed graphene oxide/polyacrylamide-hydrogels (GO/PAM) that were responsive to pH and electric fields. They observed that addition of GO resulted in significant improvement of the compressive strength [6]. Furthermore, Shin *et al*. (2013) engineered cardiac constructs by culturing cardiomyocytes onto carbon nanotubes-GelMA hydrogel (CNT-GelMA). The CNT-GelMA composite successfully

increased mechanical integrity and improves electrophysiological functions by obtaining higher beating rates on the composite hydrogel than in the pristine GelMA [7]

This photo-crosslinkable material and its hybrid hydrogel variants allow the construction of microconstructs via bioprinting representing a promising opportunity to mimic native tissues via bioprinting. Also, new development of smart materials could enhance this hydrogel properties to increase the therapeutic and clinic possibilities of tissue engineering.

Background

The first publications about GelMA were in the 2000s, almost 20 years ago. However, it is in the most recent years that GelMA's potential for different biomedical applications has been explored. The first method reported for the synthesis of GelMA was published for Van Den Bulcke et al. (2000)[8]. They reported a synthesis method of this material, which consisted on adding methacrylic anhydride directly to gelatin aqueous solution. Years later, Hoch et al. (2012), reported the synthesis of GelMA of different degrees of methacryloyl substitution, using different concentrations of methacrylic anhydride.[9] This last report, that demonstrated the versatility of GelMA, boosted this hydrogel within the community of biomaterial, tissue engineering and micro-biofabrication.

In 2006 GelMA was first used to encapsulate different types of cell[10]. This was given as a first photograph of the idealization of the GelMA as a bioink for cell encapsulation [4][10]. Later, the encapsulation of cells in GelMA hydrogels was coupled with different microfabrication technologies aiming to develop sophisticated tissue-like constructs. Examples of these technologies are: photopatterning or photolitography, micromolding, self-assembling, and bioprinting[4] More recent reports also examined the interaction of cells in 3D culturing with the hydrogels, which involves cell proliferation, migration, and differentiation[11], [12]. Moreover, the use of GelMA has expanded to be used in applications as: photo patterning, micro molding, microfluidics, and bioprinting [13]–[16].

GelMA has been frequently used as the main component of hybrid hydrogels. Some materials that have been used in combination with GelMA are biopolymers, inorganic particles, and synthetic polymers. The selection of the blend-components will depend on its intended biomedical application. For instance, hydroxyapatite particles have been used in combination with GelMA to promote osteoinductivity, hyaluronic acid-GelMA to mimic cardiovascular tissue, and poly-ethylene glycol to reinforce the GelMA constructs intended for connective tissue [4]

Recently, the development of nanotechnology has made extensive efforts to develop new nanoparticles for different tissue engineering applications. Nanoparticles represent an attractive platform, depending on the surface and core properties, they can be engineered for multimodal applications [17]. Hydrogels can be mixed with nanoparticles to create nano-composite hydrogels. For example, they can be mixed with mineral NPs, polymeric NPs, metallic NPs, magnetic NPs, and carbon-based NPs. Often, NP are combined with hydrogels aiming to mimic the ECM in animal tissues.

Extracellular Matrix

The extracellular matrix (ECM) is a 3D network composed of biomolecules that support cells in tissues and provide necessary support for cell growing, proliferation and differentiation. The ECM is formed by collagen, elastin, fibronectin, enzymes, proteoglycans (PGs), glycosaminoglycan (GAGs), laminins and some other glycoproteins. All these components bind each other in an organized architecture, to form a complex network in which cells reside forming tissues [4].

Cells that are encapsulated or embedded into ECMs-like hydrogels, interact with macromolecules through surface membrane receptors. Cells interact with cytoskeleton receptors such as: integrins, discoidin domain receptors (DDRs), PGs from the cell surface, and hyaluronan receptors CD4. These receptors help to mediate adhesion with functional Arg-Gly-Asp (RGD) sequences present on the ECM. According to Rozario and DeSimone (2010), all embryogenic cells types synthesize components of the ECM [19]. ECM-cell interactions play an important role in regrowth and tissue healing remodeling processes[20].

Collagen is the most abundant protein in the ECMs of animal's tissues. It constitutes around 30% of the total proteins in humans and it is synthesized and secreted by most of the mammalian adherent cells[21], [22]. Collagen is highly modified at the post-translational level in the endoplasmic reticulum through hydroxylation, glycosylation, and formation of disulfide bonds prior to the triple helix formation [23]Collagens are associated with other proteins and PGs to render long fibrillar structures. All these molecules associate to form a 3D complex and functional matrix network [18][24].Different materials have been engineered aiming to recapitulate the essential properties of this complex matrix, and to use them as scaffolds to design and produce artificial tissues. Most of these engineered biomaterials are hydrogels.

Hydrogels

According to Warren et al., gels and hydrogels are a network of polymer chains that are hydrophilic sometimes found as a colloidal gel in which water is the dispersion medium, a three-dimensional solid results from the hydrophilic polymer chains being held together by cross-links.[25] Hydrogels must be "gelled", solidified or cross-linked, to change from their liquid or "pre-gel" form to their semi-solid form. The crosslinking methods for hydrogels are ionic crosslinking, physical crosslinking mediated by temperature and self-assembly crosslinking. Physical cross-linking methods are less toxic for use with cells, while chemical cross-linking methods are more likely to have cell toxicity.

Hydrogels are the most popular option to be used as bioinks for 3D bioprinting. The high-water content in hydrogels, facilitates the diffusion of nutrients, allowing proper feeding and survival of encapsulated cells. These polymeric networks have demonstrated to be effective as matrixes for 3D cell culture [26]. Three-dimensional cell culture mimics better the natural interactions between the cells and the micro-environment, as it occurs in our tissues. According to Zhang et al., cells in a 2D culture have restricted interaction with the neighboring cells and have less access to the culture media, limiting the intracellular signaling and phenotypic expression [27], [28].

Some natural polymers used to fabricate hydrogels are agarose, alginate, chitosan, collagen, fibrin, hyaluronic acid (HA) and gelatin. Since these materials are derived from natural

sources, they are biocompatible and bioactive [29]. The choice of the material depends on the cell type to be cultured, the microfabrication technique to be used, and the intended tissue to be engineered. The pristine version of natural hydrogels is often week and difficult to handle. Therefore, different engineering strategies have been developed to surpass these shortcomings. Chemical modification of natural polymers has been reported to be an effective way to convey mechanical tunability, stability and processability to these hydrogels. For instance, methacrylation has been widely used in different natural polymers (i.e., gelatin, tropoelastin, and hyaluronic acid) to provide the ability to cross-link colloidal solutions into covalently crosslinked and solid networks upon light exposure.

GelMA Synthesis

Gelatin methacryloyl (GelMA) also known as methacrylated gelatin, methacrylamide modified gelatin or gelatin methacrylamide, is a photo-crosslinkable hydrogel based on gelatin, the hydrolyzed version of collagen. Pure gelatin is not stable at body temperature, which decreases the chances to do research in the biomedical field. To preclude this shortcoming, researchers functionalize the polymer chains with unsaturated methacryloyl groups to form GelMA. This methacryloyl groups by photo-initiated polymerization form covalently crosslinked hydrogels [8], [30]. The synthesis method involves adding MA (usually 10% v/v) to gelatin in phosphate-buffered saline (PBS) at a temperature of 50 °C for 1h [8]. It is worth mentioning that pH is a critical parameter since methacrylic acid is being generated as a byproduct and can reduce the solution below gelatin's isoelectric point (where the amine- residues are not easily available for reaction)[9].

An advantage of gelatin is that after collagen hydrolysis, it conserves the arginine-glycine-aspartic acid (RGD) sequences that mediates cell attachments in native tissues [31][8], [32]. GelMA is synthesized from the combination of gelatin and methacrylic anhydride (MA). Briefly, methacryloyl groups monomers react with lysine and hydroxyl lysine groups from the polypeptide chain of the gelatin[8].

One of the most interesting features of GelMA is that it can be covalently photo-crosslinked into a stable hydrogel within seconds, when a pre-gel solution (liquid) is shined with light. Additionally, the mechanical and chemical properties of gelatin can be modified using various kinds of crosslinking agents[33]–[35]GelMA polymerization is mediate by a free-radical reaction, which is initiated upon UV light exposure in the presence of a photoinitiator. Compared with the use of crosslinking chemicals, photo-crosslinking methods provide fast, uniform in situ curing[36].

Photoinitiators are generally added to improve photosensitivity. Common photoinitiators include 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and an Eosin Y-based photoinitiator. Irgacure 2959 is a common photoinitiator with a peak absorbance around 275 nm, since this wavelength is extremely damaging to DNA (peak absorbance 260 nm). Irgacure 2959 at a concentration at 0.05–0.5 wt % has been used with no effect on cell [37]. LAP, on the other hand, has an absorbance peak around 380 nm, with a tail that reaches well into the visible spectrum. This photoinitiator allows for the photo-polymerization with commonly available UV-A LEDs and is thus a popular choice for extrusion-based bioprinters[38].

This photo-crosslinking ability of GelMA allows it to be shaped, molded and photo-patterned into microstructures or morphologies. This ability has been widely capitalized by many research groups around the globe, to fabricate cell-laden constructs that recapitulate real and complex tissues.[8] GelMA ability to fabricate 3D scaffolds could elicit a physiologically relevant response and result into improved funtionality of the cultured tissue

.

Bioprinting

Bioprinting has been defined as the bio-manufacturing process that involves the layer by layer deposition of a biomaterial with cells under controlled conditions of space and time for the recreation of a 3D tissue or organ architecture [39]. Bioprinting also includes the utilization of 3D printing techniques to combine cells, growth factors and biomaterials to fabricate tissues that imitate the natural characteristics[40].

There are three different types of bioprinting techniques: extrusion, inkjet, and laser assisted. Extrusion bioprinting is the most common and cheapest method. It deposits the biomaterial layer by layer using pressure[41][42]. Inkjet bioprinting are used for fast and large-scale products, an inject bioprinter extrudes the biomaterial using a syringe pump and crosslinks the bioprinted construct with UV light. Laser assisted bioprinting technique uses light from stereo lithography providing the best resolution, however these bioprinters are very expensive.[43]

The growing use and interest in bioprinting, has trigger the interest to develop different bioinks with different materials, either synthetic or natural, according to the needs of each case [44]. Given the nature of the GelMA, with its ability to crosslink in a matter of seconds, and its resemblance to animal tissues, it has become one of the preferred biomaterials to be used as a bioink [45].

Objectives

General Objective

To evaluate the performance of hybrid GelMA hydrogel with nanoparticles in cell viability towards its application as bioink in cell laden fibers and microtissue scaffolds.

Specific Objectives

1. To develop and standardize a semi-automated method for the synthesis and continuous purification of GelMA setting up different strategies for both, the reaction and the methacrylic acid removal stages.

2. To characterize every resulting GelMA hydrogel batches in terms of their degree of chemical functionalization, photo-crosslinking time, and mechanical and biological performance.

3. To evaluate the performance of nanoparticle-loaded-GelMA hydrogel in cell viability towards its application as bio-ink in cell-laden fibers and microtissue scaffolds.

Experimental Strategy

Based on the objectives outlined in the previous section, the experimental strategies carried out to achieve them are outlined schematically. The objectives are shown to the left of each diagram and remain in the same order in which they were mentioned.

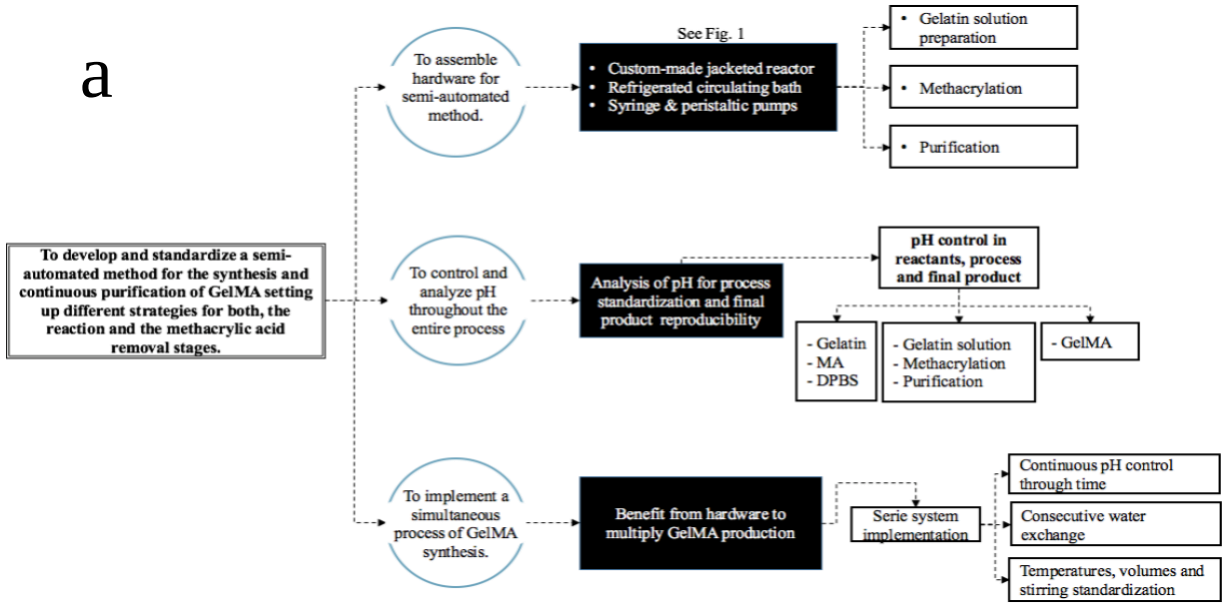


Figure 1a. Experimental strategies for the improvement of the synthesis and characterization of GelMA and the development of a hybrid nanoparticle-GelMA-based Bioink.

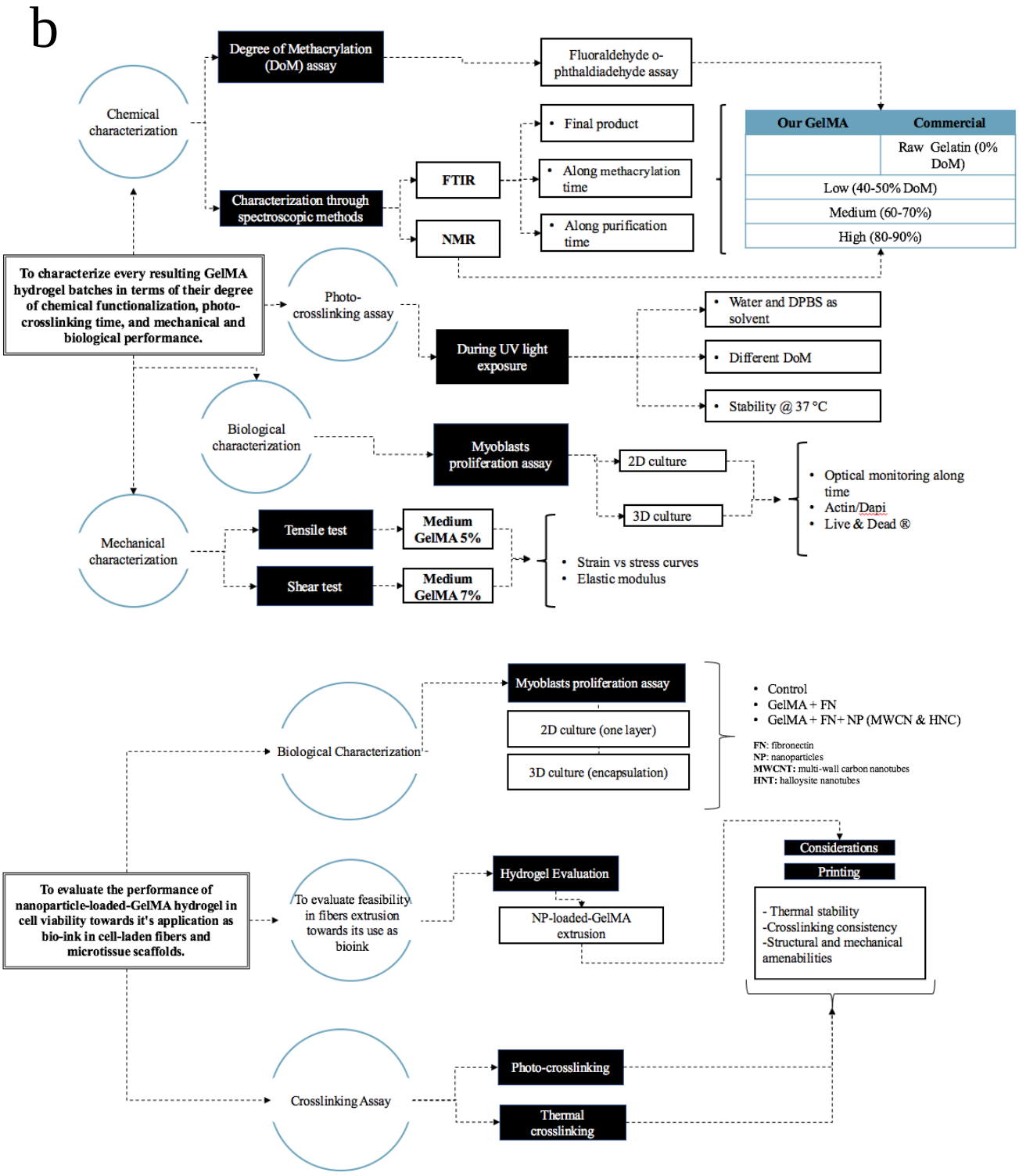


Figure 2b. Experimental strategies for the improvement of the synthesis and characterization of GelMA and the development of a hybrid nanoparticle-GelMA-based Bioink.

Materials and Methods

Reagents

Gelatin from Porcine Skin Type A (Sigma-Aldrich, cat. no. G2500- St. Louis MO, USA), Dulbecco´s Phosphate Buffer Saline or DPBS (Life Technologies, cat. no. 100100- Gran Island, NY, USA), Methacrylic Anhydride or MAA (Sigma-Aldrich, cat. no. 276685- St. Louis MO, USA), Dialysis Membrane 12-14 kDa (Sigma-Aldrich, St. Louis MO, USA), Deionized Water 0.22µ bottle top vacuum filter (Corning, cat.no. CLS430624- St. Louis MO, USA), $NaHCO_3$ (Research Organics, Inc. Cleveland, OH, USA), NaOH (Merck, Germany), Fluoraldehyde *o*-phthaldialdehyde reagent solution (Life Technologies, cat. No 10977-015), 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) (Sigma Aldrich, NY, USA), Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Allevi, Philadelphia, PA, USA), Halloysite Nanoclay- HNT-HNP (Sigma- Aldrich, cat. No. 685445, USA), Dulbecco´s Modified Eagle´s Medium (Life Technologies, cat. no.11320033 - Gran Island, NY, USA), Bovine Fetal Serum (Sigma-Aldrich. cat. no. F2442- St. Louis MO, USA), 0.22 µm syringe paper filter (Corning. cat. no. CLS431222 - St. Louis MO, USA), Trypsin 0.25% (Life Technologies, cat. no. 25200056- Gran Island, NY, USA), Antibiotic Antimycotic Solution 100 X (Sigma-Aldrich, cat. No. A5955, USA), LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, cat. No. L3224, USA), DAPI (Sigma-Aldrich, Cat. No. D9542, USA), Actin Phalloidin-iFluor 405 Reagent (ABCAM, cat. No. Ab176753, USA), Phalloidin-iFluor 647 Reagent (ABCAM, cat. No. Ab176759, USA)
Krazy Glue (Eler´s Products, Inc, Ohio, USA), Paraformaldehyde (Sigma-Aldrich, St. Louis MO, USA), TritonX-100 (Sigma-Aldrich, St. Louis MO, USA)

Materials

Dialysis Membrane 12-14 kDa (Sigma-Aldrich, St. Louis MO, USA), Custom-made jacketed reactor, refrigerated Heated Circulating Bath (VWR, USA), syringe pump (Chemyx, USA), pH meter monitor (VWR Symphony, USA), Centrifuge (VWR, USA), Peristaltic pump (Masterflex, USA), 0.22 µm syringe paper filter (Corning. cat. no. CLS431222 - St. Louis MO, USA, Freeze-dryer LABCONCO (USA), Synergy HTX (BioTek, USA), FT-IR Nicolet™ iS™ 10

(Thermo Fisher, USA), Bruker Avance III-HD 300 MHz NMR (Bruker, USA), FEI Quanta 200 Environmental SEM (Thermo Fisher, USA), Uniaxial Testing (UniVert, Canada), Bio X (Cellink, Sweden), Axiovert (Zeiss, Germany), JowBeam Pen Flashlight (JowBeam, n.d.), ElectroForce 3200 (TA Instruments, USA), Ultrasonic Cleaner (Branson, USA), Vortex Mixer (Labnet, USA)

GelMA Synthesis

Gelatin Methacryloyl was synthesized by mixing gelatin from porcine skin at 10% (w/v) into Dulbeccos Phosphate Buffer Saline or DPBS. In these synthesis experiments, key process parameters (the methacrylation reaction time, the rate of MA addition, the pH and pH control strategy, the temperature of addition) were varied to assess the effect of these parameters in the different GelMA quality indicators to be evaluated. For some batches, the DPBS was substituted by deionized water. The gelatin solution was mixed in our custom-made jacketed reactor (connected to a refrigerating-heating circulating bath) set at 50 ºC at 300 rpm for 1 hour or until the gelatin was fully dissolved (Figure 6, A). While maintaining the previous conditions of temperature and agitation speed in the reactor, a MA volume was added to the gelatin solution in continuous fashion using a syringe pump. This mix was allowed to react for the desired time. In this experimental stage, the volumes of MA varied from 0.25% to 20% (v/v) to modify the fraction of reacted amine groups. Additionally, the reaction time for the gelatin methacrylation ranged from 1 h to 24 h at 50 ºC and at 300 rpm During the process of addition of MA, pH was monitored with pH meter (VWR Symphony. USA). Following a dilution with additional warm DPBS (5 times the gelatin solution volume) at 40ºC under continuous stirring for 15 minutes to stop the reaction. Some GelMA batches were centrifuged for 3 minutes at 3500 rpm (Figure 6, B). Brief information about modified variables in GelMA synthesis is shown in Table 1.

Supernatant was recovered and dialyzed against distilled water at 40 ºC for 4-5 days using a 12-14 kDa cutoff dialysis tubing to remove salts and generated methacrylic acid. For the dialysis procedure, GelMA was placed into dialysis membranes. Membranes were placed into a 2l Buchner flask with preheated water at 40 °C. Then, a peristaltic pump (Masterflex) was used to continuously replace dialysis water by distilled water working at a flow of 5 ml/min. In this stage, pH was monitored, and the purification process was stopped when the pH of the distilled water remained

stable (Figure 6, C). At the end of dialysis stage, some of the GelMA batches were adjusted to a final pH 7.4 with 2M NaHCO3. The solution was lyophilized for 5 days to generate a white porous foam that was stored at -80 ºC until further use (Figure 6, D).

Table 1. Modified variables modified throughout the experimental strategy for the synthesis of GelMA.

Variable	Level 1	Level 2	Level 2	Level 3	Level 4
Solvent of Gelatin	dH_2O	DPBS	-	-	-
MA Volume (v/v)	0.25%	1.25%	5%	10%	20%
MA Addition Rate	0.5 ml/min	X_{Total} / h	-	-	-
ReactionTime	1 h	3 h	24 h	-	-
pH control	Initial pH to 9	During MA addition to 7.4	Final product to 7.4	No pH control	-
Photo-initiator	Irgacure 2959	LAP	-	-	-
Centrifugation	Yes	No	-	-	-

GelMA Characterization

Pre-polymer solution preparation

We used two different photo-initiators (namely 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) and Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)) for the preparation of the GelMA pre-polymer solutions.

For the preparation with *Irgacure 2959*, half of the total desired volume of DPBS was added to freeze dried GelMA to hydrate it (See Table 2 for most common concentrations and weights). The rest of the volume of DPBS was mixed with 0.5% (w/v) of Irgacure 2959 at 80ºC for 10 minutes in a water bath. Both parts were mixed together under dark conditions and incubated in a water bath for 10 minutes at 80ºC. Occasional mixing in vortex is recommended to enhance

GelMA dissolution. The sample was covered with aluminum foil and refrigerated at 4 ºC until further use.

For the preparation of *LAP hydrogels*, freeze dried GelMA was dissolved in DPBS and then mixed with 0.067% (w/v) of LAP. This mix was dissolved in a water bath at 60ºC for 10 minutes or until the white powder had completely disappeared. Th GelMA solution was covered with aluminum foil and refrigerated at 4 ºC until further use. Both, *Irgacure 2959* and *LAP* are light sensitive reactants. For best results, it is recommended to weight and maintain them under dark conditions. SEM micrographs of GelMA-Irgacure and GelMA-LAP hydrogels were obtained at different concentrations of GelMA. To do that, a volume of 50 µl of GelMA hydrogel was placed over a glass slide, and this sample was freeze dried for two days, nanocoated with gold, and scanned in FEI Quanta 200 Environmental SEM.

Table 2. Amounts of GelMA and photo-initiators per milliliter to attain different concentrations of GelMA solutions.

		Photo-initiator	
Concentration (%)	Weight of freeze-dried GelMA (mg/ml)	Ircacure (mg/ml)	LAP (mg/ml)
5	50	5	0.67
7	70	5	0.67
10	100	5	0.67
15	150	5	0.67

Chemical Characterization

Degree of Methacrylation Assay

The GelMA samples selected for characterization were dissolved in DPBS at 0.5 mg/ml. Next, these solutions were mixed with Fluoraldehyde OPA reagent solution at 1/9 (v/v) ratio and allowed to react for one minute to generate blue fluorescent derivatives. Samples were pipetted to

a 96-well plate designed for fluorescence assays only. The fluorescence intensity of the samples was monitored in a Synergy microplate reader (excitation = 360 nm, emission=460 nm). Gelatin added with fluoraldehyde OPA in DPBS was used as a standard, and pure DPBS added with Fluoraldehyde OPA was used as the blank. The DoM, which is calculated from the conversion of amine groups, was calculated as indicated below:

$$DoM = 1- (I_{sample} – I_{blank})/ (I_{standard}- I_{blank}) \quad \text{(eq.1)}$$

FTIR

Freeze-dried samples for FTIR characterization were weighed (20 µg) and placed in a micro-vial for easy transportation. The samples spectra were obtained in a FT-IR Nicolet™ iS™ 10 from Thermo Fisher. Data collected was processed using Spectragryph® software.

H-NMR

GelMA samples were dissolved in deuterium oxide (D_2O) at a concentration of 10 mg/ml at room temperature. Data was collected and processed in a Bruker Avance III-HD 300 MHz NMR. Data spectra were processed using Mnova software from Mestre Lab. To obtain the degree of methacrylation (DoM), equation 2 was used over the integrated areas of the lysine residues [46].

$$DoM [\%] = (1\text{-A (Lysine methylene of GelMA)/A (Lysine methylene of unmodified gelatin))}* 100 \quad \text{(eq.2)}$$

Photo-crosslinking Assay

GelMA samples were prepared at 5% (w/v) with two different photo-initiators (namely, Irgacure 2959 and LAP). In addition, a set of nano-composite samples were prepared with dispersed HNT. The nano-composite hydrogel was prepared using a concentration of 1 mg/ml of HNT. Briefly, the HNT were dispersed into 10% of the total volume of GelMA 5% (w/v) (previously dissolved in DPBS at 60 ºC and without initiator) using ultrasonic cleaner. This

solution was then added to the remaining GelMA to complete the total volume. LAP photo-initiator was added at a concentration of 0.067% (w/v) at 60 ºC. The solution was then mixed in a vortex mixer for 5 min to dissolve LAP and to incorporate the aliquot of HNT + GelMA to the main solution. Once the samples were prepared (GelMA + Irgacure, GelMA + LAP, nano-composite suspension + Irgacure, nano-composite suspension + LAP), small cylinders of 4 mm height and 3.5mm radius were photo-crosslinked in a PDMS mold using a 365 nm JowBeam UV-light. The UV-light exposure time was 20 s for LAP samples and 240 s for Irgacure samples.

Mechanical Characterization

For the GelMA mechanical two standards were followed with modifications: adhesion tests ASTM F2458-05 [47] and shear test ASTM F2255-05 [48].

For the adhesion tests, the interface adhesion between the pork muscle tissues of 2 mm x 1cm x 1 cm was filled with 200 µl of GelMA. Twelve samples at 5%(w/v) and 7% (w/v) of GelMA were carried out using hydrogel of medium DoM with 0.067% LAP photo-initiator. The adhesion interphases were over exposed to 365 nm using JowBeam UV-light for 120 s and placed in the testing holder of ElectroForce 3200 using a speed of 0.166 mm/s and stopped until failure.

Shear tests were run in the same equipment as the adhesion tests setting up a speed of 0.166mm/s. Two pieces of pork muscle tissue (1cm x .5cm x .1 cm) were glued to the glass slides. The samples of GelMA that were prepared at the same conditions than used in the adhesion tests were sealed between two glass slides. For this experiment a set of TMSPMA treated glass slide samples were added. For glass slides treatments the protocol from Allevi was followed [49] In total 32 samples were tested, 24 with GelMA (12 for untreated glass samples and 12 for treated glass samples) and 6 for the control (tissue on tissue). Approximately a 2mm thick single layer of GelMA consisting of 250 µl was used for the samples.

Biological Characterization

Cell Preparation

Myoblast cells of *Mus musculus* pass 15 (C2C12) were cultured at 37 ºC with 5% of CO_2. Cells were cultured in Dulbecco`s Modified Eagle´s Medium (DMEM) in 10% of Fetal Bovine Serum (FBS). Cell passes were done every 4 days. To perform the biological assays, we waited for cells to reach a confluence around 80-100%. After desired confluence was reached, the medium of C2C12 cells pass 12 was removed, cells were washed with PBS and trypsinized for 15 min at 37 ºC. Cells were centrifuged for 5 minutes at 1000 rpm and then counted in a Neubauer Chamber. Cells were resuspended in DMEM with 10% of FBS until their use.

2D assay

For the 2D assay GelMA of medium and high DoM was prepared at 5% (w/v) using LAP. GelMA was sterilized using a 0.22 µm syringe paper filter. After preparation, 40 µl of GelMA were disposed by triplicate in a 96-well plate. GelMA samples were photo-crosslinked at 365 nm UV light from the BioX bioprint. In each well, 5x10^3 C2C12 cells were added. The constructs were then covered with DMEM, 10% of FBS and 1% of antibiotic antimycotic solution 100 X. The constructs were monitored for 15 days while de culture media was changed every 3 days.

3D assay

A set of 5 x 10^5 of C2C12 cells was transferred to the prepolymer solutions consisting of 5% medium and high GelMA with 0.067% LAP photo-initiator. In a 96-well plate, 40 µl of the solutions of GelMA were added by triplicate. The construct of cells and GelMA were exposed to 365 nm UV-light using JowBeam for 20 s. The constructs were covered with DMEM, 10% of FBS and 1% of antibiotic antimycotic solution 100 X. The cells were monitored for 15 days while culture media was changed every 3 days.

Cell Viability Assay

Cell Viability Assay was carried out using the LIVE/DEAD ® kit from Invitrogen. The performance of the experiment was done according to manufacturer`s instructions. Constructs were washed using PBS. Staining solutions were added to the constructs and incubated at room

temperature for 15 minutes. Later, they were observed in Axiovert microscope from Zeiss. Live (green) staining was observed at 488/515 nm (emission/excitation) a red staining (red) was observed at 570/602 nm (emission/excitation).

Actin/DAPI Staining

Actin and DAPI staining were performed according to the manufacturer`s instructions. First, culture media was removed from the wells, then constructs were washed three times with PBS. 3D constructs were fixed with 4% of paraformaldehyde. Then, they were washed three times with PBS. A 0.1% solution of TritonX-100 was added to the constructs and then, the constructs were incubated for 5 min. They were washed again with PBS. Actin and DAPI staining were added until they covered the constructs. The samples were incubated under dark conditions for 90 minutes at room temperature. Later, they were observed in Axiovert of Zeiss. Phalloidin 405 staining (blue) was observed in 400/421 nm (emission/excitation) and DAPI staining was observed in 385/410 nm (emission/excitation).

Nano-particle Loaded GelMA Characterization

Pre-polymer solution preparation

For the preparation of the nano-particle loaded GelMA or nano-composite GelMA a set of pre-polymer solutions were prepared. For the cross-linking tests LAP and Irgacure 2959 were used. In the experiments conducted for bioprinting extrusion and biological characterization only LAP photo-initiator was used.

The preparation of pure GelMA without nanoparticles was conducted as following: freeze dried GelMA was dissolved in DPBS and then mixed with 0.067% (w/v) of LAP. It was dissolved in water bath at 60ºC for 10 minutes or until white powder had completely disappeared. The sample was then covered with aluminum foil and it was refrigerated at 4 ºC until further use. *LAP* is a light sensitive reactant, for better results it is recommendable to weight and maintain it under dark conditions.

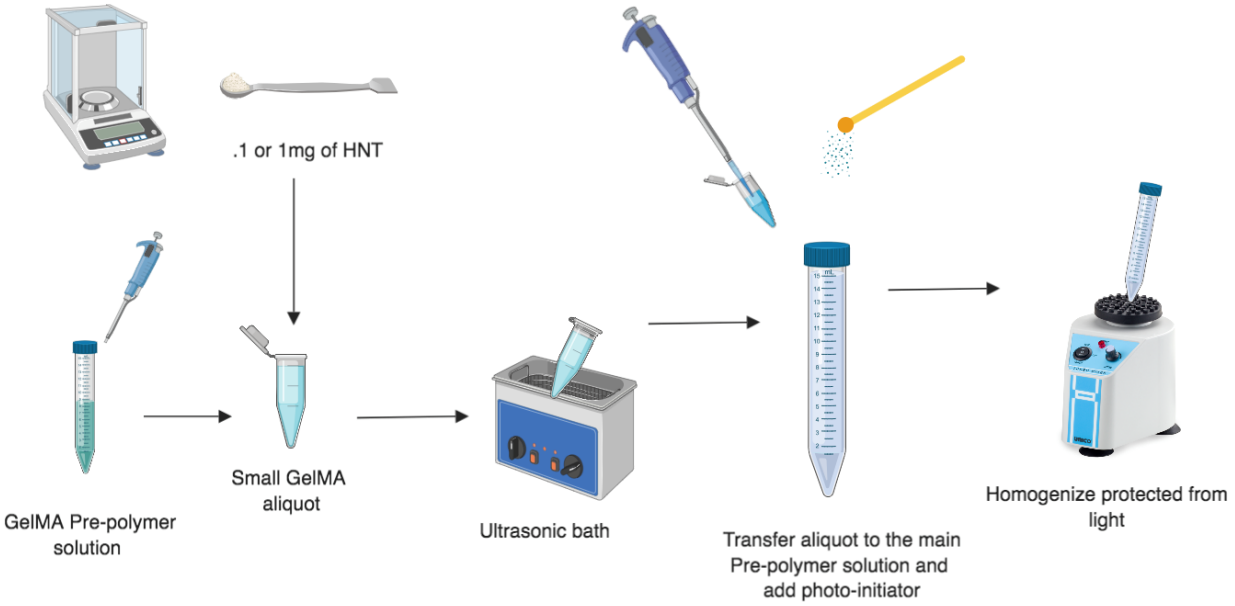


Figure 3. General diagram of nano-composite GelMA solution preparation prior to use in characterization. A small aliquot of the GelMA solution was dispersed with the nano-particles and then it was incorporated again to the main solution. Finally, it was homogenized no create a nano-composite hydrogel.

Photo-crosslinking Assay

As previously mentioned in the GelMA characterization, the photo-crosslinking assay was conducted as following: GelMA samples were prepared at 5% (w/v) with two different photo-initiators 8 (Irgacure 2959 and LAP). A set of nano-composite samples were also prepared with dispersed HNT. The nano-composite hydrogel was prepared as following: using a concentration of 1 mg/ml of HNT, the HNT were dispersed using ultrasonic cleaner into 10% of the total volume of GelMA 5% (w/v) (previously dissolved in DPBS at 60 ºC), taken from the total volume without photo initiator, this solution was then added to the remaining GelMA solution to complete the previous total volume. LAP photo-initiator was added at a concentration of 0.067% (w/v) at 60 ºC. The solution was then mixed in vortex mixer for 5 min to dissolve LAP and to incorporate the aliquot of HNT + GelMA to the main solution. Once the samples were prepared (GelMA + Irgacure, GelMA + LAP, nano-composite + Irgacure, nano-composite + LAP), small cylinders of

4 mm height and 3.5mm radius were photo-crosslinked in a PDMS mold using 365 nm JowBeam UV-light. The UV-light exposure time was 20 s for LAP samples and 240 s for Irgacure samples.

Mechanical Characterization

Compression test were conducted in a Uniaxial Testing machine. The GelMA prepared for the experiments was 5% (w/v) and medium DoM. The compression strength of the control samples was compared to the nano-composite hydrogel which was prepared using HNT. The nano-composite hydrogel was prepared as mentioned in the photo-crosslinking assay section. Once the nano-composite hydrogel was prepared, several cylinders of 155 µl (4 mm height and 3.5mm radius) of GelMA and nano-composite hydrogel were photo-crosslinked in a transparent PDMS mold. The compression speed was set up to 0.1 mm/ s.

Bio-ink Extrusion Evaluation

We prepared bioinks added with Halloysite nanotubes at two distinct GelMA concentrations, 7% namely 5 and 7 %(w/v). For these experiments, medium and high DoM GelMA were used. The control solutions were naive GelMA at 5% (w/v) and 7% (w/v). For the nano-composite bioink we followed the procedure stated previously (in the *Photo-crosslinking assay* section). Briefly, from the main pre-polymer solution an aliquot of 10% of the total volume was taken and mixed with HNP. This aliquot was sonicated for 10 minutes. Then, the HNP solution was resuspended into the GelMA pre-polymer solution with LAP (0.067%). Bioinks were filtered through a 0.22 µm syringe filter and refrigerated until use. The bioinks were dosed into sterile cartridges and then loaded into the printhead of the BioX bioprinter. Extrusion parameters were set to a pressure of 20kPa and an extrusion rate of 10 mm/s. The bioprinter was configured to print a toroidal structure (O-ring shape) designed in. stl (Standard Triangle Language) format in a 24-well plate. The extrusion printing process was monitored.

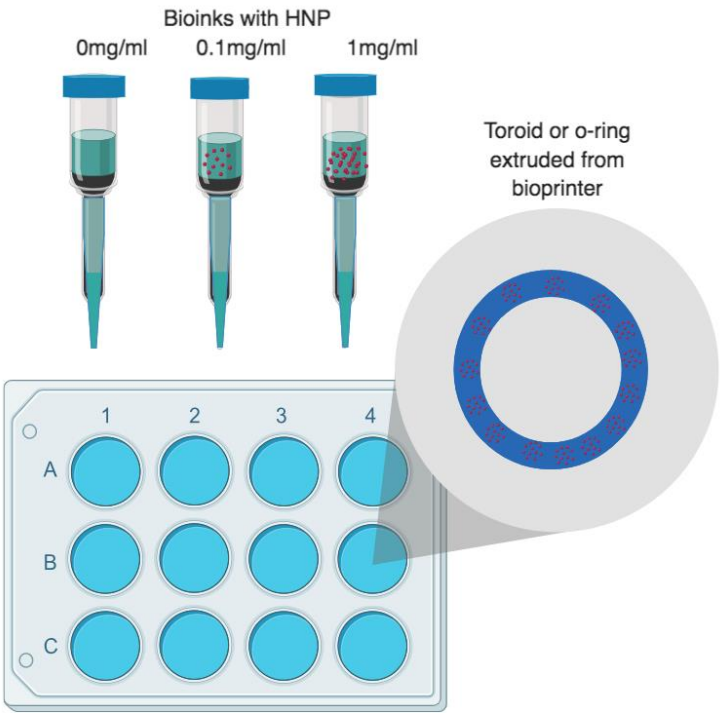


Figure 4. General scheme of extrusion bioprinting of GelMA-based nano-composite bioinks in a plate. A cell-laden toroidal volume (o-ring shape) was extruded.

Biological Characterization

Cell Preparation

Myoblast cells of *Mus musculus* (C2C12; pass 15) were cultured at 37 ºC in a 5% CO_2 atmosphere. Cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) added with 10% of Fetal Bovine Serum (FBS). Cell passes were done every 4 days. To perform the biological assays, we waited for cells to reach an 80-100% of confluence. After desired confluence was reached, the medium was removed, and cells were washed with PBS and trypsinized for 15 min at 37 ºC. Cells were centrifuged for 5 minutes at 1000 rpm and then counted in a Neubauer Chamber. Cells were resuspended in DMEM with 10% of FBS until use.

2D and 3D Bioprinting

Two types of 2D cell culture assays were conducted, one using the nanoparticle-GelMA bioink and other using a nanoparticle-GelMA-Gelatin bioink. Since these experiments involves cell culture, all the procedures were done under sterile conditions. For both assays, the bioinks was transferred to the bioprinting cartridge using a syringe. The printheads with the cartridges were cooled down at 15 ºC for 30 min prior to be used in the bioprinter. Then, the printhead was attached to the plug-in of the BioX bioprinter. An extrusion rate of 10 mm/s and a pressure of 15kPa was set for the nanoparticle-GelMA bioink. The configuration for the nanoparticle-GelMA-Gelatin bioink was 10mm/s and 20kPa. After bioprinting in a 24-well plate, constructs were photo-crosslinked for 30 s using a 365 nm UV light (from the bioprinter). Previously prepared cells ($5x10^4$) were added to each well. Constructs were covered with DMEM added with 10% of FBS and 1% of antibiotic and antimycotic solution (100 X). 2D constructs were made by triplicate and monitored for 15 days. Culture medium was changed every 3 days.

3D assay

Similarly, two types of 3D cell culture assays were conducted, one using nanoparticle-GelMA bioink and other using nanoparticle-GelMA-Gelatin. A concentration of 5×10^5 cells per milliliter was transferred to an Eppendorf tube and centrifuged at 1000 rpm for 5 minutes. Medium was removed, and cells were resuspended into the bioinks with a pipette. For both assays, the bioink was transferred to the cartridge using a syringe. Cartridges were cooled down at 15 ºC for 30 min. Bioinks cartridges were incorporated to the printhead and then plugged to the bioprinter. A 3D model of a toroidal volume was printed by triplicate. For the nanoparticle-GelMA bioink, a configuration of 15 kPa and 10 mm/s was used. For nanoparticle-GelMA-Gelatin inks, a setting of 20 kPa pressure and a 10 mm/s speed was selected. After the cell-laden 3D constructs were bioprinted in a 24-well plate, they were photo-crosslinked for 30 s and 365 nm using the UV light of the bioprinter. Constructs were covered with DMEM added with 10% of FBS and 1% of antibiotic antimycotic solution (100 X). Constructs were monitored for 15 days and the culture media was changed every 3 days. An extra 3D assay (a control), using naive GelMA was implemented. A small square of zein-nanofibers was used underneath an extruded bioink drop (Figure 5).

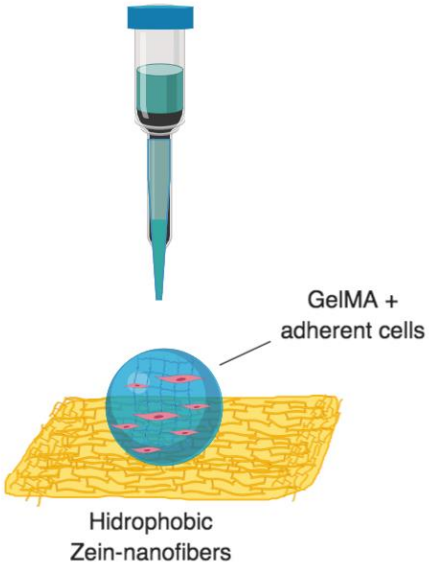


Figure 5. Bioink extrusion over a hydrophobic surface formed by a square of zein-nanofibers.

Cell Viability Assays

Cell viability assays was carried out using a LIVE/DEAD ® kit from Invitrogen. The experiment was done according to manufacturer`s instructions. Constructs were washed with PBS, stained, and allowed to incubate at room temperature for 15 minutes. Later, they were observed in an Axiovert inverted optical microscope (from Zeiss, Germany). Live (green) staining was observed at 488/515 nm (emission/excitation) and dead staining (red) was observed at 570/602 nm (emission/excitation).

Actin/DAPI Staining

Actin and DAPI staining were prepared according to the manufacturer`s instructions. First, culture media was removed from the wells and constructs were washed three times with PBS. 3D constructs were fixed with 4% of paraformaldehyde. Then, they were washed three times with PBS. Constructs were then added with and added with a 0.1% solution of TritonX-100 and incubated for 5 min. Then, they were washed twice with PBS, and covered with actin and DAPI staining solutions. Samples were incubated under dark conditions for 90 minutes at room

temperature. Later, they were observed under the microscope (Axiovert from Zeiss; Germany). Phalloidin 405 staining (blue) was observed at 400/421 nm (emission/excitation) and DAPI staining was observed at 385/410 nm (emission/excitation).

Results and Discussion

In this section, the results obtained from our experimental plan are presented and discussed. We followed a three-stage experimental strategy. First, we developed and standardized a semi-automated method for the synthesis and continuous purification of GelMA. Second, we characterized the resulting GelMA batches in terms of their degree of chemical functionalization, photo-crosslinking time, and mechanical and biological performance. Finally, we assessed the performance of the nanoparticle-loaded-GelMA hydrogel towards its application as a bio-ink in bioprinted microtissue scaffolds.

GelMA Synthesis: Standardization and Semi-automation

Different protocols for GelMA synthesis have been reported with minimum variations from the originally reported by Van den Bulcke et al. (2000) Gelatin Methacryloyl is prepared by reaction of gelatin with methacrylic anhydride. After dissolution of gelatin in phosphate buffer (pH 7.5) at 50 ºC, methacrylic anhydride is added while vigorously stirring. After 1 hour the reaction is diluted and dialyzed for 5 days against distilled water at 40 ºC. Then the product is freeze-dried to obtain a white solid foam. [8][37].

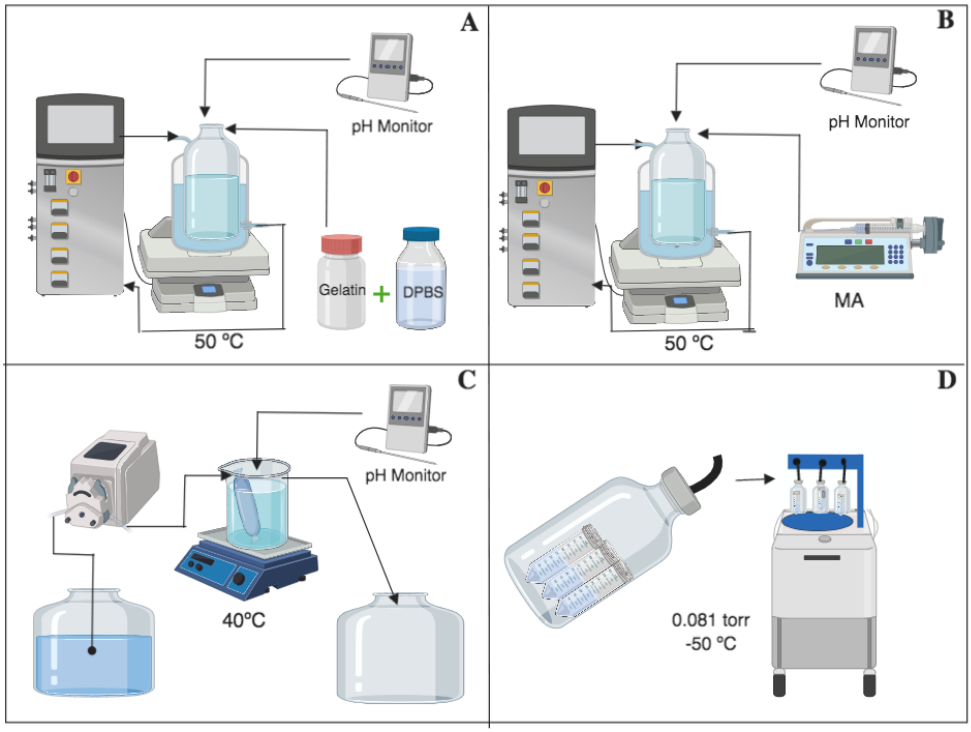


Figure 6. General diagram of our semi-automated process for GelMA synthesis. (A) Gelatin solution preparation in our custom-made jacketed reactor with pH monitoring. (B)Methacrylic anhydride continuous addition using a syringe pump with pH monitoring. (C)Purification through continuous dialysis process using peristaltic pump for water exchange. (D) Freeze-drying process.

We aimed on improving the traditional method for GelMA synthesis by assembling a set of semi-automated hardware for synthesis and purification, and by pH analysis through the entire process. For the initial gelatin solution preparation and the MA addition, we used a custom-made jacketed reactor connected to a refrigerated heating circulating bath (VWR; Figure 6, A). The custom-made jacketed reactor maintained the temperature as well as the proper agitation, and pH control. We controlled the addition of MA volumes by a configurable automatic syringe pump while the pH was monitored along the addition (Figure 6, B). A continuous dialysis to purify GelMA from was successfully implemented by using two peristaltic pumps (Figure 6, C). This implementation improved the MA removal and effectively shortened the time required for purification.

Gelatin is a soluble polypeptide mixture of denatured and partially hydrolyzed collagens, derived from animal tissues, such as skin tendons and bones [50]. As mentioned in the background section, collagen is the main component of the ECM in human tissues. In our case we used porcine gelatin for GelMA fabrication, which is water soluble, and forms a thermo-reversible hydrogel via physical crosslinking between collagen molecules [8]. For the gelatin solution preparation, our custom-made jacketed reactor allowed us to maintain a proper temperature, above the melting temperature of 32 ºC [51], while vigorously mixing with DPBS.

Among the process variations that we tested, we used dH_2O instead of DPBS (Table 3). However, the use of dH_2O for GelMA preparation did not result in proper crosslinking, probably because the gelatin was denatured due to the radical pH change when MA was added. The use of buffers may protect the protein from changes in pH, which can cause denaturation, aggregation or fragmentation [52]. We also tried to increase the DPBS pH by NaOH 1M addition prior to gelatin dissolution, but the final product did not remain stable and didn't crosslink correctly (Table 3).

In the second step of GelMA synthesis, we added MA dropwise to the previously prepared solution into the custom-made jacketed reactor using a syringe pump. The volume of the MA addition was modified to obtain different degrees of methacrylation (DoM). We used MA volumes ranging from 0.25 to 20 % (v/v). MA was added until the target volume was reached at different rates ranging from 0.04166 ml/min to 0.5 ml/min. Slow addition of MA to gelatin solution induces methacryloyl substitution groups on the reactive amine and hydroxyl groups of the amino acid residues [8]. In this set of experiments, we kept the pH constant to 7.4 by the continuous addition of $NaHCO_3$. The GelMA hydrogels obtained from these experiments exhibited poor crosslinking. This suggests that the excess of $NaHCO_3$ may have caused a protein denaturation and lower the extent of methacryloyl substitution in the gelatin polymer chain (Table 4). When MA is added to the gelatin solution, it immediately reacts exothermically with water molecules resulting in the formation of methacrylic acid which inhibits the methacryloyl substitution. Moreover, methacrylic acid has been reported to be cytotoxic, hence, not suitable for cell culture.

As previously stated, the mechanical properties of GelMA based hydrogels are easily tunable by simple varying the DoM, the polymer and photoinitiator concentration, and photo-

crosslinking time [8][53]. In our methodology, we modified the DoM, while the photoinitiator concentration (Irgacure 2959 or LAP) remained constant in our samples. The photo-crosslinking time was set up to 4 min for the samples crosslinked with Irgacure and 20 s for the samples crosslinked with LAP. The volume used of MA to modify the DoM directly influences the highest achievable crosslinking density of the GelMA matrix, the hydrogel porosity and the mechanical properties [53].

Table 3. Modifications in the conventional GelMA synthesis procedure and their effect on crosslinking.

Modification	Use of H2O as solvent for gelatin solution	Use of DPBS as solvent for gelatin solution	Increase initial pH of gelatin solution to 9	Maitain pH at 7.4 in MA addition
Crosslinking	×	✓	×	×

We assess the effect of different MA addition strategies on the DoM (degree of methacryloyl groups substitution in the gelatin chain). We varied stirring time to promote methacryloyl groups substitution between 0 and 24 hours. We analyzed the DoM using either FTIR analysis or H-NMR analysis (Figure 10, Figure 11). The results of the effect of stirring time will be further discussed.

In all our GelMA synthesis experiment, regardless the addition strategy, the reactions were stopped by diluting with 1-5X volumes of pre-heated DPBS at 40 ºC. The exact volume to be added was calculated based on the volume of the initial gelatin solution. Subsequently the diluted GelMA was stirred for additional 15 minutes. Next, we dialyzed de GelMA solution against distilled water using a 12-14 kDa cutoff dialysis tubing for 5 days at 40 ºC. In the purification stage we implemented a set of peristaltic pumps to provide a continuous water exchange using a flow rate of 5 ml/min. This purification was implemented to substitute the manual water exchange used conventionally in GelMA preparation. We monitored the pH of the dialysis solution continuously. We stopped the purification process when pH reached a plateau stage or when reaching the pH of the inlet distilled water. At the end of dialysis process, we adjusted some of the GelMA solutions

to a final pH 7.4 using 2M $NaHCO_3$. This pH adjustment aims to rich a cell culture friendly pH value, compatible with cell culture applications. Finally, for freeze-drying stage, we froze the GelMA samples in 50 mL falcon tubes for 24 h. The GelMA solution was lyophilized for 5 days and then stored for further use. Freeze-drying process is responsible to sublimate all water by creating a vacuum atmosphere at -50 ºC.

Using this synthesis and purification strategies, together with some hardware implementation, we conducted multiple sets of GelMA production experiments. Automated equipment and pH control effectively reduced synthesis and purification time from two weeks to 10 days. This means that we were able to produce up to 12 g of freeze-dried GelMA every 10 days. As previously mentioned, this procedure can be conducted simultaneously resulting in a total time synthesis of 5 days. To implement a simultaneous process of double synthesis also allowed us to reduce time, equipment and water usage in comparison to previously reported GelMA preparation protocols.

GelMA Characterization

Pre-polymer solution preparation

The preparation of the pre-polymer consisted of the use of two different photo-initiators. The use of photo-initiators is based on the microstereolitography technology which its principal resource is the use of photosensitive materials that can solidify as a result of UV-light exposure. Photo-polymerization of hydrogels involves the use of free radicals to initiate monomers or oligomers crosslinking[54], [55]. Both, LAP and Irgacure conduct on GelMA a free radical polymerization and the concentration of these reactants must be kept in mind. In some of our first experiments we try to increase the concentration of *Irgacure 2959* in our samples, but we noticed that the photo-polymerization wasn´t successful. LAP manufacturer suggests a concentration of 0.5 %(w/v) to use with the polymer, however we decreased that concentration to 0.067% (which is 7X times lower than the suggested) to save reactant and to increase cell viability. Eventually, free radical polymerization forms covalent bond with each other. J.M. G. Cowie and V. Arrighi

mention, as a rule that the greater the free radical concentration, the shorter the chain length. This factor is important for the final stiffness and microstructure of the hydrogel matrix[56].

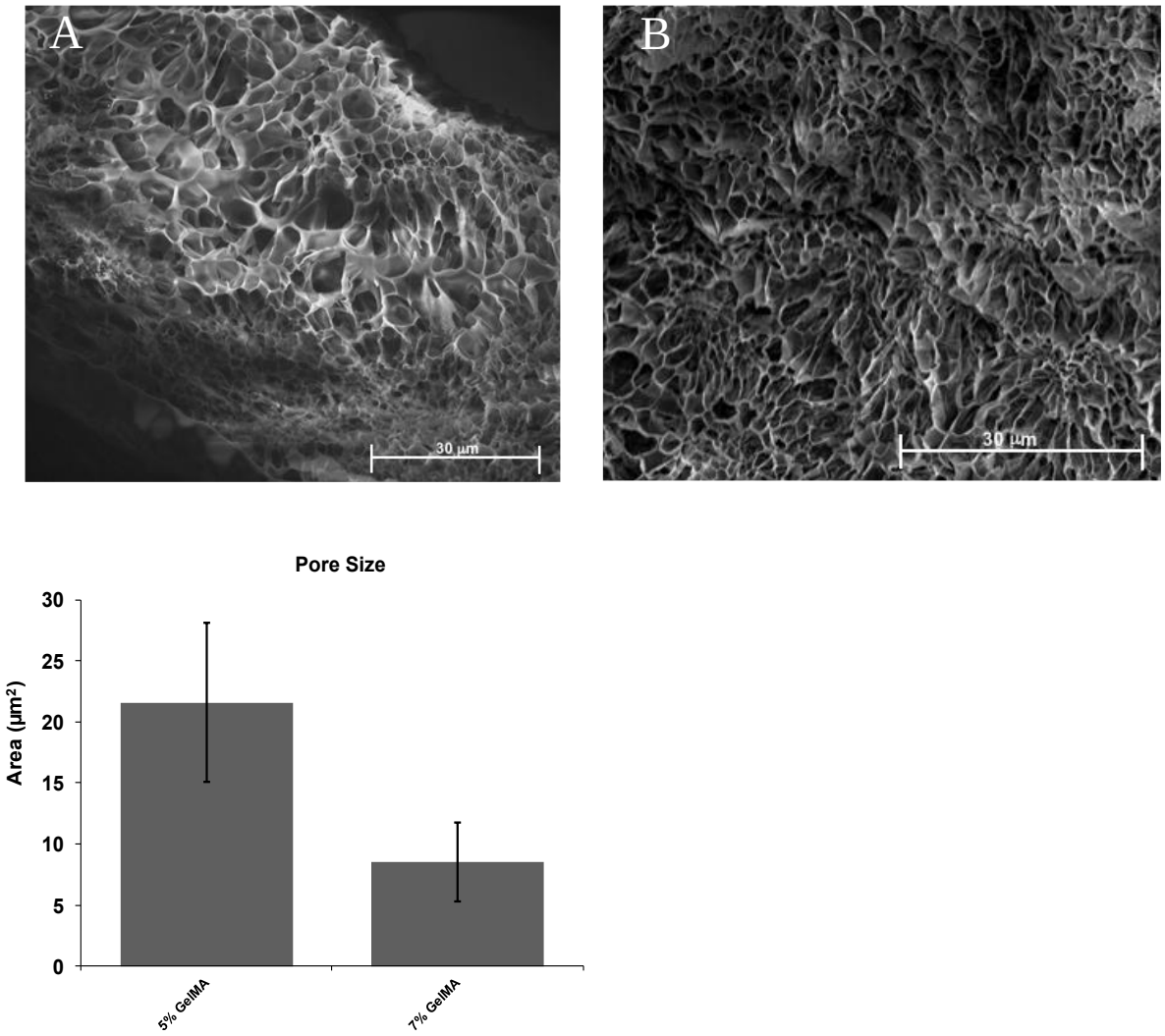


Figure 7. SEM images of GelMA samples prepared using different concentrations, 20 s of UV-light exposure and medium DoM. A. GelMA medium 5%. B. GelMA medium 7%. C. Average pore size (measured from the SEM images). Error bars represent standard errors; A is +6.53, B is, + 3.23.

One of the classifications of photo-initiators is by the wavelength at which they most strongly absorb. Our photo-initiators were activated in the UV- range, however there exists other photo-initiators which activate at visible light [57], [58]. The advantage of working with UV-

activated photo-initiators is that the user decides the easiest ambient conditions, however care must be taken when working with encapsulated cells in the gel. Our photo-initiators were used due to the high cytocompatibility. Irgacure-2959 is characterized for having a low solubility in water (0.5% wt) and showing a low molar absorption at 365nm ($\varepsilon<10M^{-1}cm^{-1}$). This means that lower molar absorption values of a photo-initiator involve large amounts of photo-initiator and longer exposures must be used. Consequently, Irgacure is not recommendable when using in polymers with encapsulated cells. On the other hand LAP has a higher absorption at 365 nm ($\varepsilon\approx200M^{-1}cm^{-1}$) and is very water soluble (at least 8.5 wt%), these features make LAP a more promising option when working with encapsulated cells solutions [58], [59]. O´Connel et al.,2018, used a concentration of 0.005% (w/v) *Irgacure 2959* and found a maximum absorption profile at 365 nm using a UV-light intensity of 150 mW/cm^2. They also noted an accelerated crosslinking process at 140 s when using the same UV-light intensity. In this accelerated process, the viscosity increases, and the mobility of the free radicals decreases resulting in a polymerization. They also mention that the rates of crosslinking are not affected by the concentration of GelMA and the highest crosslinking rate is reached at 140 s using a 100 mW/cm^2 intensity. Finally they found that they highest rate of biocompatibility when using Irgacure is reached at lowest UV-light intensities ($10mW/cm^2$), however, the crosslinking time was found at 316 s[60]. When we prepared pre-polymer solutions and tried to photo-crosslink by UV-light exposure using Irgacure, we found that our optimal UV-light wavelength was 302 nm from a UV lamp with selectable wavelengths (254, 302 and 365 nm). Nevertheless, the minimal time exposure for the samples was 2 min which resulted to be harmless for cells (See Table 4 and Figure 16). For our convenience we used LAP for the mechanical tests and biological characterization to reduce cytotoxicity problems.

For our GelMA synthesis and pre-polymer solution preparations, we modified some significant variables, for example; volume of MA, GelMA concentration, UV-light exposure (time and wavelength), final pH adjustment, and centrifugation. To characterize the microstructure of GelMA at different concentrations, we observed extruded freeze-dried GelMA at 5 and 7% (w/v) using SEM (See Figure 7). We found that the pore size significantly decreases when the concentration of GelMA is increased. The final pore sizes were measured with ImageJ. Pore sizes had an average of 21.5 μm^2 (±6.53 standard error) for 5% samples and 8.53 μm^2 (±3.23 standard error) for 7% samples. For this characterization, we used a medium DoM GelMA with a 37% of

amine conversion according to H-NMR results (See Figure 9). The pore sizes after freeze-drying obtained by Puckert et al., 2017 were, 50, 30, and 24 μm for 49.8%, 64.8% and 73.2% of substitution of DoM, respectively. We can observe a decrease of the pore size when the DoM increases even when they do not specify the photo-initiator and the DoM of the GelMA used.

We observed that modifications in the methods for GelMA preparation, either adding different volumes of MA or pH modifications in the solution, directly impact the final DoM of the hydrogel. In turn, the DoM highly impacts the polymer properties. GelMA batches with a higher DoM exhibit a high degree of crosslinking, smaller pore sizes of the gel and less swelling. Likewise, lower DoM results in large pore sizes, low degree of crosslinking and more swelling ratio [61].

Chemical Characterization

Degree of Methacrylation Assay

Different GelMA solutions were dissolved and mixed with the Fluoraldehyde OPA reagent solution to determine the conversion, also called substitution, of the amine groups from the Gelatin.

As previously mentioned, we modified both, variables for synthesis and variables for pre-polymer preparation of GelMA. For the degree of methacrylation assay we changed the MA volume to analyze the absorbance of the methacryloyl groups present in the gelatin polymer chain. We also changed the reaction time of the MA with the gelatin solution and the temperature of the reaction. All the modifications of the variables were made to assess their relation to the final degree of methacrylation. The Table 4 shows the samples with the modifications on the variables. We also included commercial GelMA from Sigma to compare our results.

Table 4. Samples submitted to the degree of methacrylation assay. The variables modified are the volume of MA, reaction time and temperature with M5S* sample, which was synthesized at 30 °C.

Samples	Estimated Conversion of Amine Groups	Preparation Conditions					
		MA Volume (%)	Reaction Time	pH adjustment	Crosslinking Time (min)	Color Variation	Stability at 37 °C
M5S*	14%	5%	3 h (S)	7.4	4.5	✗	✓
M5S*	57%	5%	2 h (S)	7.4	4.5	✗	✓
H10S*	97%	10%	2 h (S)	7.4	4	✗	✓
H10S*	57%	10%	3 h (S)	7.4	4	✗	✓
H10S*	9%	10%	24 h (S)	7.4	4	✗	✓
LCG	78%	-	-	-	5	✓	✓
MCG	97%	-	-	-	2.5	✓	✓
HCG	93%	-	-	-	2.5	✓	✓

The first letter of the samples name nomenclature corresponds to the expected DoM, L= low, M= medium, H=high. The second letter corresponds to the MA volume, 5=5% (v/v) and 10=10%(v/v). The S corresponds to a slow reaction time, the S, refers to a super slow reaction time. The * is related to a final pH adjustment to 7.4.

Gelatin, in general, has many variations on the total composition of amino acids but, a typical composition is reported by Stevens, 2009. The chemical modification of the methacryloyl groups of methacrylic anhydride occurs in the reactive functional groups existing in gelatin, which are the hydroxyl and amino groups of the amino acid residues (See

Figure 8, B). We implemented different methods to estimate the degree of substitution in GelMA samples obtained by different strategies.

Next, we demonstrated the use of the so called fluoraldehyde assay, as a simple method to determinate of methacrylation to assess the extent of substitution of the amine groups. In these fluoraldehyde assays, the amino residues that were not substituted by a methacryloyl group converted into blue fluorescent species (

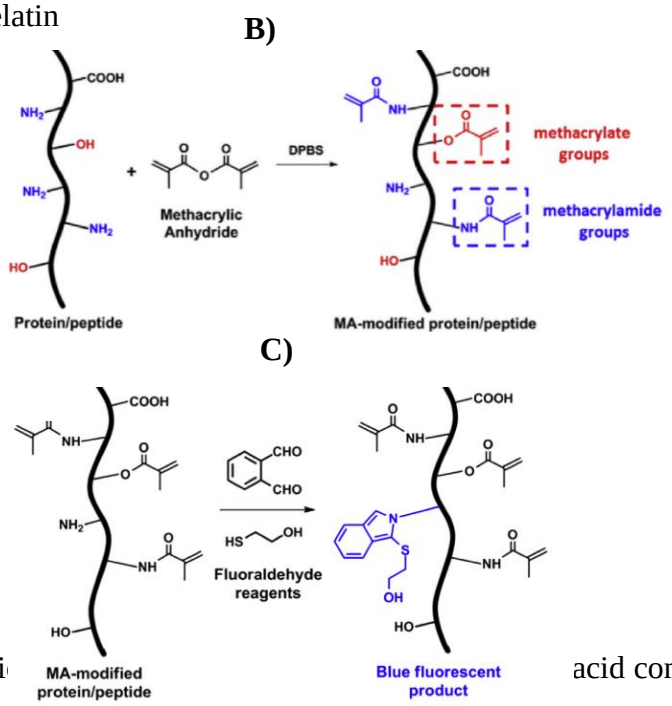
Figure 8, C). With this in mind, we performed our experiments with a control of gelatin which showed the highest fluorescent value on the microplate reader. To obtain more accurate values, we used a blank that consisted of pure DPBS which showed the lowest fluorescence value.

Once we obtained the values of fluorescence in the microplate reader, we used the eq. 1, stated in *Materials and Methods*, to obtain the conversion of amine groups or DoM (See Figure 9).

The results obtained from the samples showed that lower temperatures (30 °C) for MA addition in GelMA synthesis do not promote methacryloyl groups high degrees of substitution into the gelatin chain. For example, the contrast of sample M5S* sample M5S, which were synthetized at 30 °C and 50 °C, respectively, shows a higher DoM in the sample produced at higher temperature. Contrary to our results, Shirahama, *et.al*., found that the final DoM of GelMA samples synthesized at 35 °C and 50 °C showed no significant difference. So those results support that the GelMA reaction can be conducted at 35 °C with equivalent results to the conventional 50 °C (Shirahama, Lee, Tan, & Cho, 2016).

A) Typical Composition of Gelatin

Amino Acid Residue	Content
Ala	9%
Arg	8%
Asp	6%
Glu	10%
Gly	21%
His	1%
HydroLys	1%
HydroPro	12%
Ile	1%
Leu	3%
Lys	4%
Met	1%
Phe	2%
Pro	12%
Ser	4%
Thr	2%
Tyr	1%
Val	2%



] lifi acid composition of gelatin. B) Modification of protein and peptides with MA by chemical substitution in amino and hydroxyl groups which introduces photo-cross linkable methacryloyl groups. C) Reaction between unmodified amines (corresponding mostly to lysine residues) and the Fluoraldehyde reagents generating fluorescent derivatives which show excitation and emission maxima at 340 and 455 nm respectively. Figures obtained and modified from [62], [63].

Other important comparison in our results showed that longer periods of reaction time (after 120 min; i.e., H10S*) decrease the final DoM. This suggests that the exposure of gelatin to very acid pH (caused by the methacrylic acid for extended time periods) are detrimental to obtain a high DoM. Shirahama et.al, demonstrated, through a time dependent DoM of GelMA synthesis experiment, that the highest DoM can be reached after 30 min and before 180 min of reaction. In terms of reaction time, our highest DoM was reached at 2h reaction time.

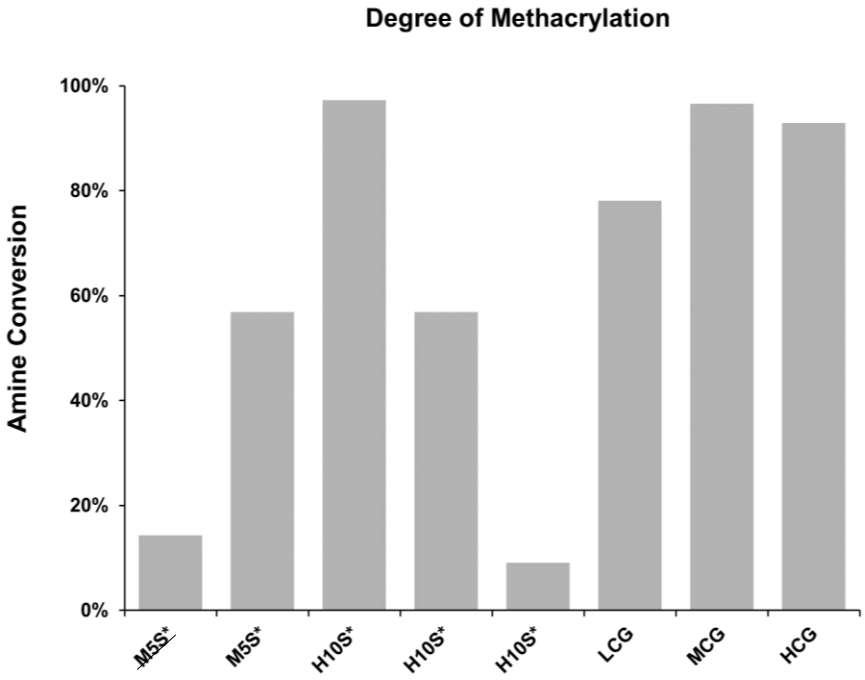


Figure 9. Diagram of the degree of methacrylation (DoM) of GelMA samples corresponding to the percentage of amine conversion obtained by the Fluoraldehyde OPA reagent solution.

Finally, the resulting conversion of amine substitution between the commercial samples (LCG, MCG, HGC) showed irregular results than expected. The LCG exhibited a 78% conversion of amine groups which corresponds to a high methacrylated GelMA. Likewise, MCG and HGC displayed inconsistent degree of methacrylation. According to our results, the medium methacrylated GelMA reflects 4% more amine substitution than the high methacrylated GelMA.

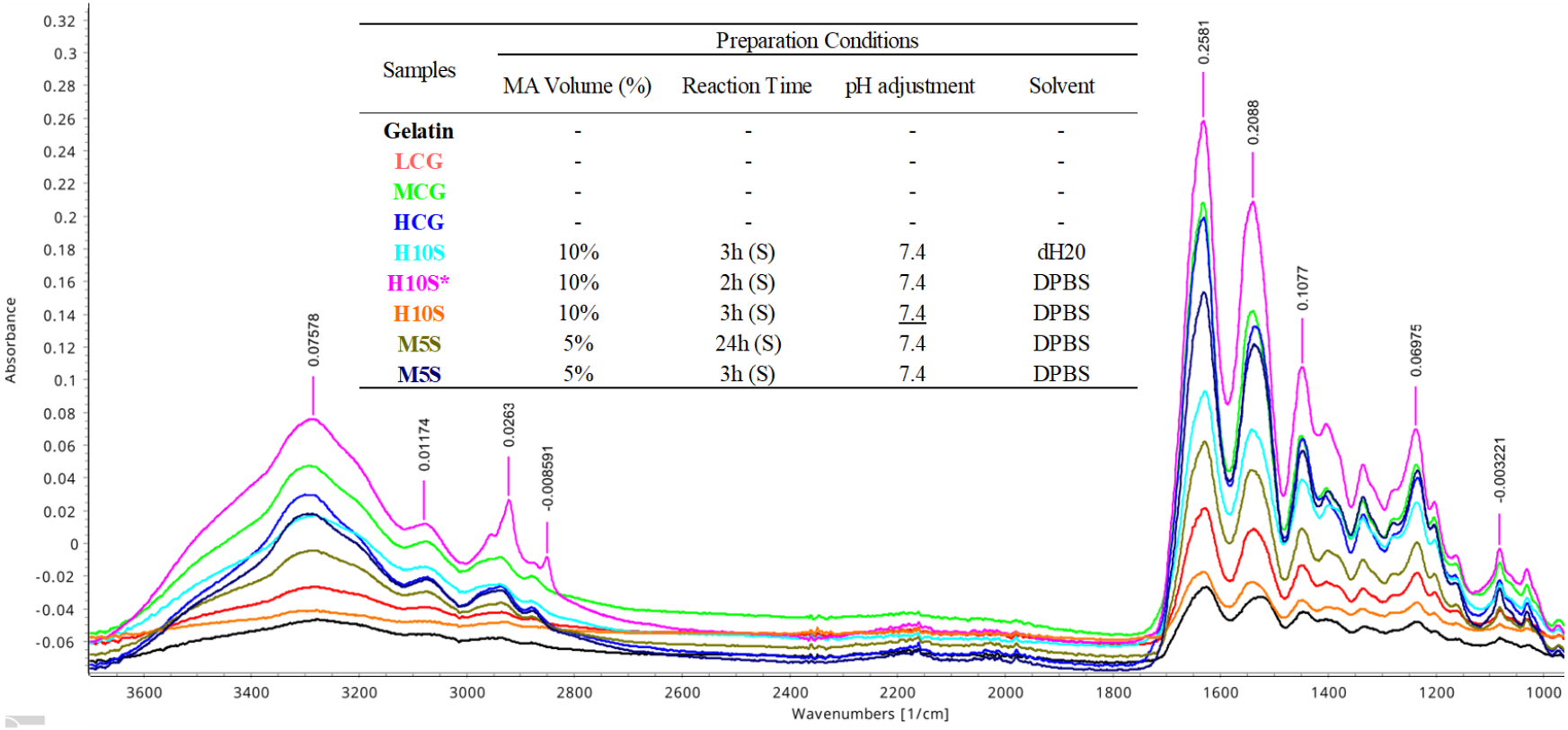
FTIR

Fourier-Transform Infrared Spectroscopy or FTIR is a spectroscopic method that obtains the molar absorption and transmission peaks from vibration frequencies between the bounds of

atoms in a material. FTIR is mainly used to characterize the presence of specific chemical groups in hydrogels and to study interaction between composite polymers[64].

In the same way than the DoM assay, the FTIR characterization was performed to compare peaks shown in the spectra of different GelMA samples synthesized at different conditions. For this analysis, the modifications of the samples focused mainly on the use of H_2O as solvent for Gelatin solution, volume of MA added and reaction time. The spectra in Figure 10 presents a set of FTIR peaks corresponding to the synthesized GelMA. The nomenclature used for this sample is like the previous one. The first letter, corresponds to the expected degree of methacrylation (L= low, M= medium, H= high), the following number refers to the volume of MA used (5 and 10%), the last letter resembles to the reaction time (S*= 2h, S=3h, **S**=24h)**.**

In the FTIR spectra we identified 4 main peaks corresponding to the functional groups from the methacryloyl substituent added to the polymer chain. In the 3287, 1653, 1539 and in the 1236 wavenumber [1/cm], we identified a N-H bond (from amines and amides), a C=O bond (from amides), C=O bond (2nd from amides) and C-N(from amines and amides) respectively (See Figure 10). Other reports also identify the 3287 and1663 (wavenumber/cm) as important peaks[65], however they mention that the methacrylate substituent groups incorporated to the gelatin were not detectable by FTIR spectra, and may only reflect the substitution of less than 5% of the amino acid residues[4], [66], [67]. We demonstrated that lower methacrylated samples show lower absorbance on the peaks mentioned. The lowest methacrylated spectra represents the Gelatin sample that we used to analyze the difference between other spectra. The highest values of the peaks belong to our *home-made* GelMA synthesized by using 10% (v/v) of MA and a 2h reaction time.



Samples	Preparation Conditions			
	MA Volume (%)	Reaction Time	pH adjustment	Solvent
Gelatin	-	-	-	-
LCG	-	-	-	-
MCG	-	-	-	-
HCG	-	-	-	-
H10S	10%	3h (S)	7.4	dH20
H10S*	10%	2h (S)	7.4	DPBS
H10S	10%	3h (S)	7.4	DPBS
M5S	5%	24h (S)	7.4	DPBS
M5S	5%	3h (S)	7.4	DPBS

Figure 10. FTIR spectra of synthesized GelMAs at different conditions and parameters. LCG, MCG, HCG corresponds to commercial GelMA. The rest of the samples corresponds to *home-made* GelMA varying some conditions of synthesis and pre-polymer solution preparation. The Y axis displays absorbance and the X axis displays wavelength**.**

Since FTIR is a quantitative analysis tool samples were sorted from the one with higher amine substitution to the one with no substitution to observe the relation between them:

Table 5. Samples sorted from the relatively highest to the lowest methacrylated sample.

Peak at 1653 from C=O from amides	
Sample	Relative DoM
H10S*	+
MCG	
HCG	
M5S	
H10S	
M5S	
LCG	
H10S	
Gelatin	-

In view of the spectra results, a relation between the DoM obtained from the Fluoraldehyde OPA reagent was found. The most and the less substituted samples were the same spots in both characterizations. However, a significant difference in the LCG (Low Commercial GelMA) sample was found. In the DoM assay the LCG sample located close to the high methacrylated samples, although in the FTIR samples it was found to be less substituted. The FTIR characterization was performed successfully to characterize the chemical composition of the different samples before and after their functionalization with methacryloyl groups.

H-NMR

NMR spectroscopy is a technique used to study atomic nuclei with an odd number of protons or neutrons (or both). NMR spectrometry is another form of absorption spectrometry like IR or UV spectrometry. Under the appropriate conditions a sample exposed to a magnetic field can absorb electromagnetic radiation in the radiofrequency region from the radiofrequencies governed by the sample. Since all nuclei carry a native charge, in some nuclei, the charge spins generating a magnetic dipole along the axis [68]. For our purpose, the methacrylation degree of the gelatin was powered by H-NMR spectroscopy. The Degree of Methacrylation (DoM) was

defined as the percentage of ε-amino groups of the gelatin (See Figure 11, A)(lysine and hydroxylysine) that are modified or substituted in the final GelMA product (See Figure 11, B)[46].

We compared the spectra of the unmodified gelatin with the spectra of the modified derivatives. We found differences in three different peaks, 5.3 ppm ≥ δ ≥ 6 ppm, 2.8 ppm ≥δ ≥ 3 ppm and δ = 2 ppm (δ chemical shift) (See Figure 11, C). Those peaks were identified to show a 2H signal from the acrylate group substituent, a 2H signal corresponding to the lysine methylene and a 3H signal of the methyl group from methacryloyl group respectively. We compared peak signals from reports previously described in the literature [9], [69], [70].

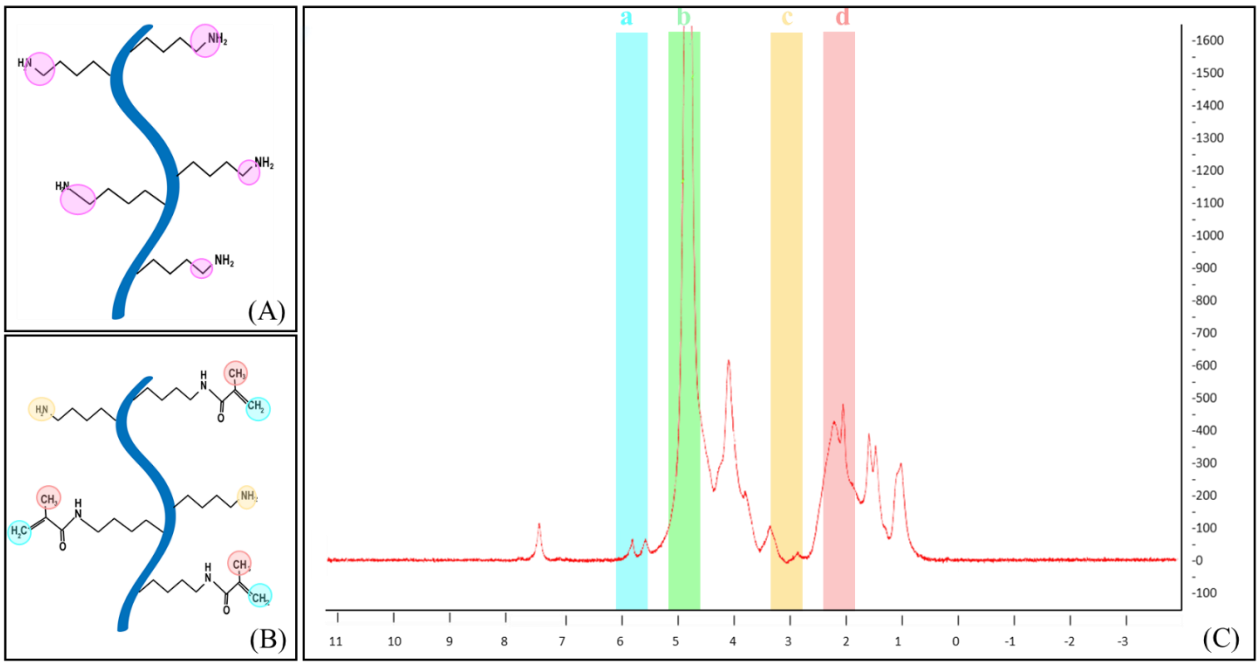


Figure 11. Chemical structure of (A) unmodified gelatin and (B) methacrylated Gelatin or GelMA. (C) H-NMR spectra of GelMA in deuterium oxide (D2O). a. Shows 2H signal from acrylate groups. b. Indicates de residue proton in D2O. c. Indicates the presence of lysine methylene (2H). d. Denotes the 3H signal of the methyl group from methacryloyl.

The binding of the methacryloyl groups to the amino acid residues was confirmed by the observation on the proton peaks of the lysine methylene. It can be inferred that the other peaks belonging to the methacryloyl substituent (5.3 ppm ≥ δ ≥ 6 ppm, and δ = 2 ppm) do not reflect an

accurate approximation to determine the DoM via HNMR since they can still be part of methacrylic acid formed in the Gelatin + MA reaction and that wasn´t completely removed after purification process. Therefore we decided to obtain the DoM from the lysine methylene peaks since they don´t represent a fact of noise residues.

Afterwards, we obtained the area by integrating the lysine methylene signals peaks (2.8 ppm $\geq\delta\geq$ 3 ppm) from the pure gelatin and GelMA samples and calculated the DoM using eq.2

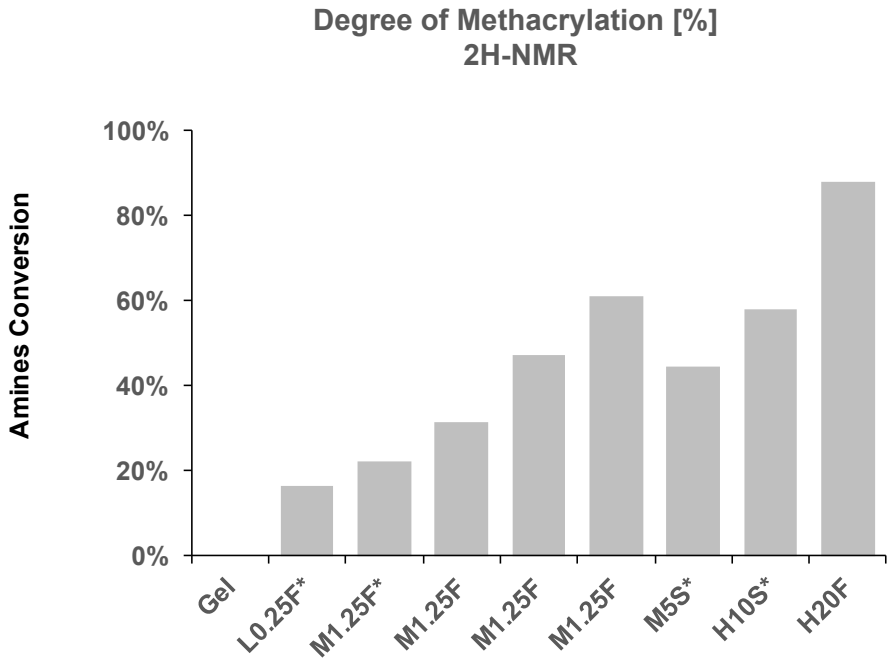


Figure 12. Degree of methacrylation [%] using NMR. Different sets of GelMA synthesized at different conditions and using D2O as solvent. Y axis represents the total percentage of substituted lysine residues in the gelatin, x shows nomenclature of the analyzed sets.

The DoM was calculated to range from 16% to 88%, which was created by varying the amount of MA added to the synthesis reaction. In Figure 12 the order of the different analyzed GelMA in the x axis, from left to right, represents the order that we were expecting to get before NMR analysis. However, the samples M5S* and H10S* that were centrifuged by following recommendations from [37] showed lower DoM compared to M1.25F samples in which less volume of MA was added to the gelatin solution. We attribute that reason to the centrifugation

process, in which the pellet is discarded. In this step a large part of the methacrylated gelatin is lost since this compound, as it is a polymer substituted, represents the compound of greater weight in the GelMA solution. By implementing centrifugation in our production, we observed a significant decrease in the purification time and water waste (4 days and 50 l), nevertheless the DoM wasn´t the expected.

For the NMR analysis we used the GelMA samples shown below in Table 6. For this test the volume of the MA was modified according to [13] except for the M5S* and H10S* which were prepared following the materials and methods section in this report. The volumes that we used were 0.25%, 1.25% and 20% (v/v) of MA to obtain a low, medium and high DoM respectively. The reaction time of the solutions was completed up to 1h after complete MA addition at a rate of (0.5 ml/min). The sample M1.25F with 61% of DoM was previously synthesized by Dr. Ali Tamayol´s Lab and no data is shown about the reaction time. We conducted this experiment in the University of Nebraska Lincoln in collaboration with LIMB Lab.

Table 6. Samples submitted to the degree of H-NMR analysis. The variables modified are the volume of MA, reaction time and centrifugation. The first letter of the samples name nomenclature corresponds to the expected DoM, L= low, M= medium, H=high. The second letter corresponds to the MA volume, 0.25=0.25% (v/v), 1.25=1.25%(v/v), 5=5%(v/v) 10=10%(v/v) and 20=20% (v/v). The S corresponds to a slow reaction time and the F, refers to a fast reaction time. The * is related to a centrifugation prior to purification.

Samples	Estimated Conversion of Amine Groups	Preparation Conditions			
		MA Anhydride	Reaction Time	Centrifugation	pH adjustment
Gel	0%	0%	-	No	No
L0.25F*	16%	0.25%	1 h (F)	Yes	7.4
M1.25F*	22%	1.25%	1 h (F)	Yes	7.4
M1.25F	31%	1.25%	1 h (F)	No	No
M1.25F	47%	1.25%	1 h (F)	No	No
M1.25F	61%	1.25%	LIMB*	No	No
M5S*	44%	5%	3 h (S)	Yes	7.4
H10S*	58%	10%	3 h (S)	Yes	7.4
H20F	88%	20%	1 h (F)	No	No

The results show that we were able to accomplish high reproducibility when using the protocol suggested by [13]. Our L0.25F* batch showed a 16% of DoM, compared to the reported which showed a 19.7%, our M1.25F batch has a range between 31 and 61% while the reported has a 53.9% DoM, finally our H20F batch showed an 88% of total amines conversion compared to 81.4%. We also found that centrifugation affects negatively the final percentage of amines conversion due to the loss of GelMA in the pellet formation.

We successfully verified through H-NMR the extent of conversion of free amino groups of different GelMA samples to find a relation between the modified variables of the GelMA synthesis and pre-polymer solutions preparations.

Photo-crosslinking Assay

Photo-polymerization is the process used for solidification of the hydrogel from a pre-gel precursors, which allows for encapsulation of cells at a near physiological conditions, and various light sources are used [71].When exposed to specific wavelengths of light, the photo-initiator absorbs the energy and forms radicals that can subsequently convert liquid macromere solutions to a polymerized network. Different methods for GelMA crosslinking have been used to create gelatin hydrogels that maintain stability at physiological temperatures (37 ºC), more resistant to degradation by proteolytic enzymes such as gelatinase and collagenase [72] and mechanically robust [73], [74]. In this experiment we also verified GelMA stability at 37 ºC as a result of proper polymerization.

Polymer hydrogels can be polymerized by the effect of free radicals created by photo-initiators. [55], [59], [75]. We conducted photo-crosslinking assay by simply exposing our samples to UV-light in this way, photo-initiators can be characterized by the wavelength at which they most strongly absorb[57], [58]. As previously mentioned, LAP and Irgacure are a UV-activated photo-initiators with biocompatible amenabilities. For this experiment, we used Irgacure 2959 and a 365nm JowBeam UV-light.

Since we started synthesizing GelMA at different conditions, many batches didn´t result as expected. Some of them didn´t crosslink properly so we decided to discard those samples from the subsequent characterization. This because free radicals are generated from photo-initiators, which initiate the chain polymerization of the methacryloyl substituents. Propagation can occur between the same or different chains [4]. We infer that batches did not crosslink because the low free radicals did not push chain elongations.

We determined that the increase in the crosslinking time is a result of the low degree of substitution in the gelatin chains. The homemade samples submitted to the photo-crosslinking assay showed lower absorbance for methacryloyl groups than the commercial samples. Our home made GelMA showed a higher crosslinking time compared to the commercial samples. When we exposed the samples to UV-light we monitored the color variation and we observed that the commercial GelMA changed to an opaque tone. We put media on the samples and checked their stability in an incubator at 37 ºC. The home-made samples remained transparent and clear, but the commercial turned the media into orange color. It is important to mention that the degree of substitution, GelMA concentration, initiator concentration, and UV-light exposure time are the mayor parameters that allow tuning of the physical properties of the resulting GelMA hydrogels[8].

Table 7. GelMA photo-crosslinking assay. Different home-made samples were compared with commercial ones in terms of cross-linking time.

Samples	Estimated Conversion of Amine Groups	Preparation Conditions			Crosslinking Time (min)	Color Variation	Stability at 37 °C
		MA Volume (%)	Reaction Time	pH adjustment			
M5S*	14%	5%	3 h (S)	7.4	4.5	✗	✓
M5S*	57%	5%	2 h (S)	7.4	4.5	✗	✓
H10S*	97%	10%	2 h (S)	7.4	4	✗	✓
H10S*	57%	10%	3 h (S)	7.4	4	✗	✓
H10**S***	9%	10%	24 h (S)	7.4	4	✗	✓
LCG	78%	-	-	-	5	✓	✓
MCG	97%	-	-	-	2.5	✓	✓
HCG	93%	-	-	-	2.5	✓	✓

Different conditions were modified, and subsequent color variation and temperature stability was analyzed.

Mechanical Characterization

High biocompatibility of GelMA hydrogel makes it a high promising material to seal, repair and support tissue regeneration. Mechanical properties of GelMA can be easily tuned and represent a high point of interest depending on the final application of the hydrogel. Mechanical strengths can be tuned by changing the DoM, the concentration of the GelMA, or the photo-initiator used. Under those conditions, GelMA at a higher DoM and higher concentration results in an increased mechanical strength compared to low DoM and low concentration samples [76].

The mechanical characterization of GelMA consisted of three main tests: adhesion, shear and compression tests. For the first two characterizations we compared 31% DoM GelMA at 5 and 7% (w/v) for the third characterization we used a the a nano-composite GelMA at 5% (w/v) with 31% DoM. Mechanical characterization was carried out in University of Nebraska Lincoln. The nano-composite characterization will be further discussed.

We conducted the adhesion test as seen in Figure 13. We placed GelMA at 5 and 7 % (w/v between an adhesion interface. The ultimate adhesion stress for the 5% samples resulted in 22.26 ±3.75 while the 7% samples showed a 30.50 kPa ± 1.94. The means of the two groups showed statically significant different by conducting one-way-ANOVA with Tukey post-hoc tests.

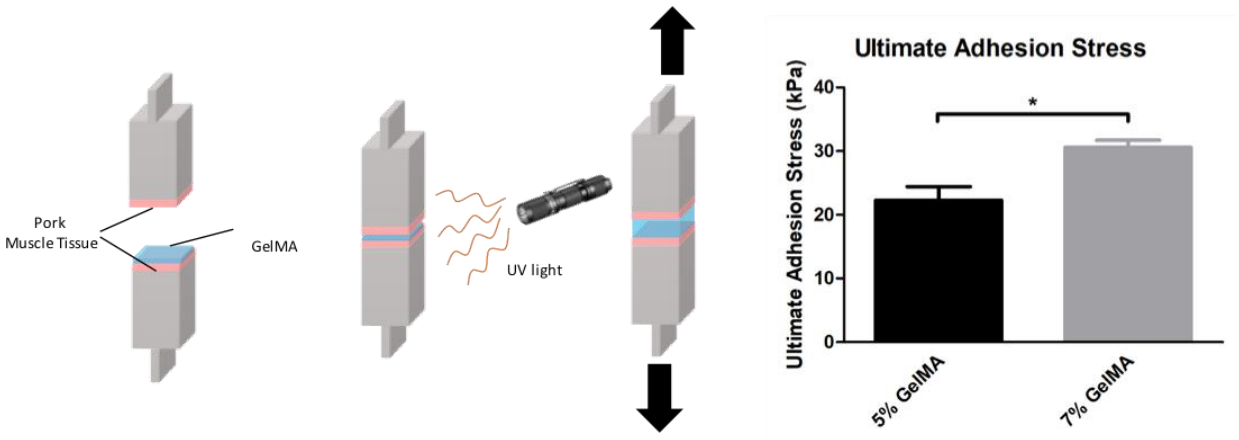


Figure 13. Schematic for the wound closure test based on ASTM F2458-05. This method can be used to compare adhesives strengths. The sample is placed between an interface of two

grips. GelMA is then-crosslinked with UV-light. Afterwards, the sample is loaded to the equipment and the experiment runs to failure at a constant speed. The maximum stress is monitored and registered.

Assmann et. al., 2017, examined several sets of GelMA hydrogels to study their behavior as sealants. They engineered different GelMA concentrations (10, 15, 20 and 25% (w/v)) and used a concentration of 8%(v/v) of MA (DoM not specified). The results in the adhesion test show and approximate adhesive strength of 7%, 20%, 35% and 50% for the 10, 15, 20 and 25% (w/v) GelMA samples analyzed. The values that we found in our samples show similarity between the ultimate adhesion stress for 15% (w/v) GelMA sample and our 5%(w/v) GelMA sample. The ultimate adhesive strength of our 7% (w/v) GelMA sample is similar to the 25%(w/v) reported sample. In is worth mentioning that we used 1.25 % (w/v) MA to synthesize GelMA while they mentioned to use 4X times more than in our protocol. Now, as we used 120 s to crosslink our samples, the comparison point is the 25% (w/v) set (crosslinked for 120 s) which resulted to show a 42 kPa adhesive strength (10 kPa more than our 7% (w/v) GelMA). [76]

We ran the shear test using the same type of GelMA than used in the adhesion test. The samples were placed in an interface between two glass slides which had a pork muscle tissue glued to it (See Figure 14). The control samples (pork tissue & pork tissue), the 5 and 7% (W/V) GelMA and the 5 and 7% (W/V) GelMA & TMSPMA showed a 53.03 kPa, next, a 32.14 kPa and 34.88 kPa, finally a 59.34kPa and 57.37kPA of ultimate strength, respectively.

Our results show a low ultimate shear stress compared to Assmann, et.al., 2017 who demonstrated a minimum adhesive strength 3x times higher than our highest value. No further reports were found to compare our final shear stress values to compare GelMA adhesive properties.

We were able to report the suitability of GelMA in certain mechanical applications following ASTM standard test to obtain an ultimate stress. We also observed a direct tendency to increase the ultimate stress of the simple by simply increasing the ratio of mass/volume on pre-polymer solution preparation. GelMA can support different tissue types and serve as adhesive or sealant if a certain ultimate stress is reached.

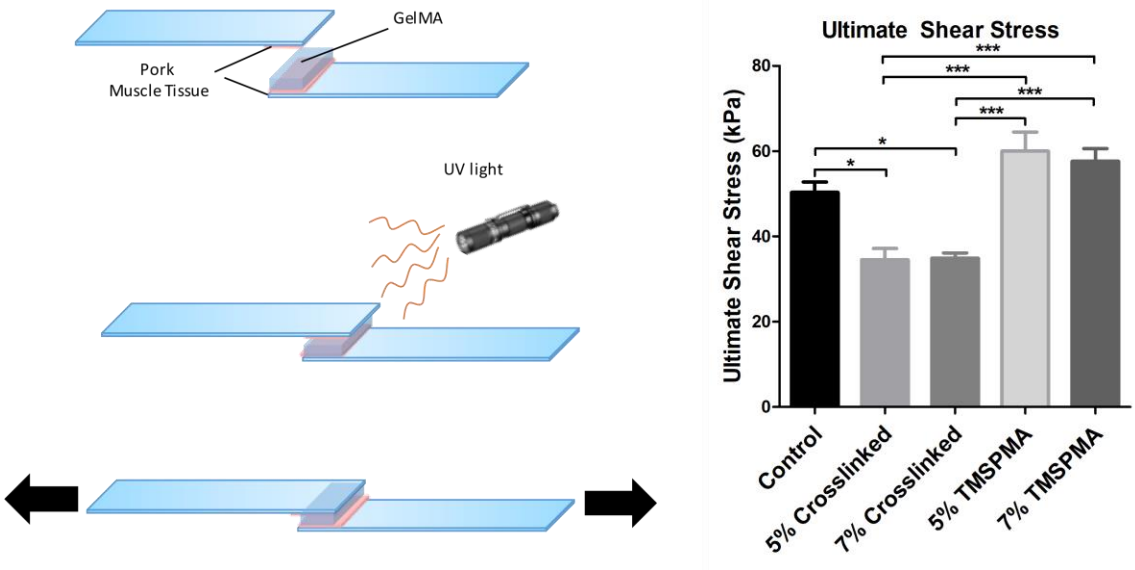


Figure 14. Schematic for the shear test based on ASTM F2255-05. This method can be used to compare adhesives. The sample is placed between an interface of glass slides. GelMA is then-crosslinked with UV-light. Afterwards, the sample is loaded to the equipment and the experiment runs to failure at a constant speed. The maximum stress is monitored and registered.

Biological Characterization

It has been shown that different cells adhere to and grow in GelMA matrixes and that can be encapsulated within the GelMA hydrogel with excellent viability[13], [77].We carried out cell growth tests in 2d and 3D in order to check the biocompatibility and the stability of our GelMA constructs in cell proliferation, moreover to verify that the quality of the final product did not represent a reason for cellular cytotoxicity. In regenerative medicine the growth, proliferation and differentiation of human skeletal myoblasts into myofibers are critical factors to take in mind to study the dynamics of muscle injuries or congenital defects [78] . Most of the myoblast studies have focused on the widely used C2C12 myoblast line which is used for ease of culture, differentiation potential and accessibility [79, p. 12] .

Hydrogels have been widely used to study cell-matrix and cell-cell interactions, cellular proliferation, migration [11]and differentiation [12]. GelMA has a potential advantage over other

synthetic polymers such as biocompatibility, low immunoresponse and bioactive motifs in the chemical structure. In contrast to gelatin, modification with methacryloyl side groups allows the GelMA molecule to undergo rapid polymerization in the presence of UV-light and a photoinitiator, resulting in covalent crosslinking through the creation of a methacryloyl backbone [80]. This feature gives GelMA stability at physiological temperature and allows fine-tuning of mechanical properties. Moreover, the resulting material is transparent, which facilitates microscopic analysis [81]

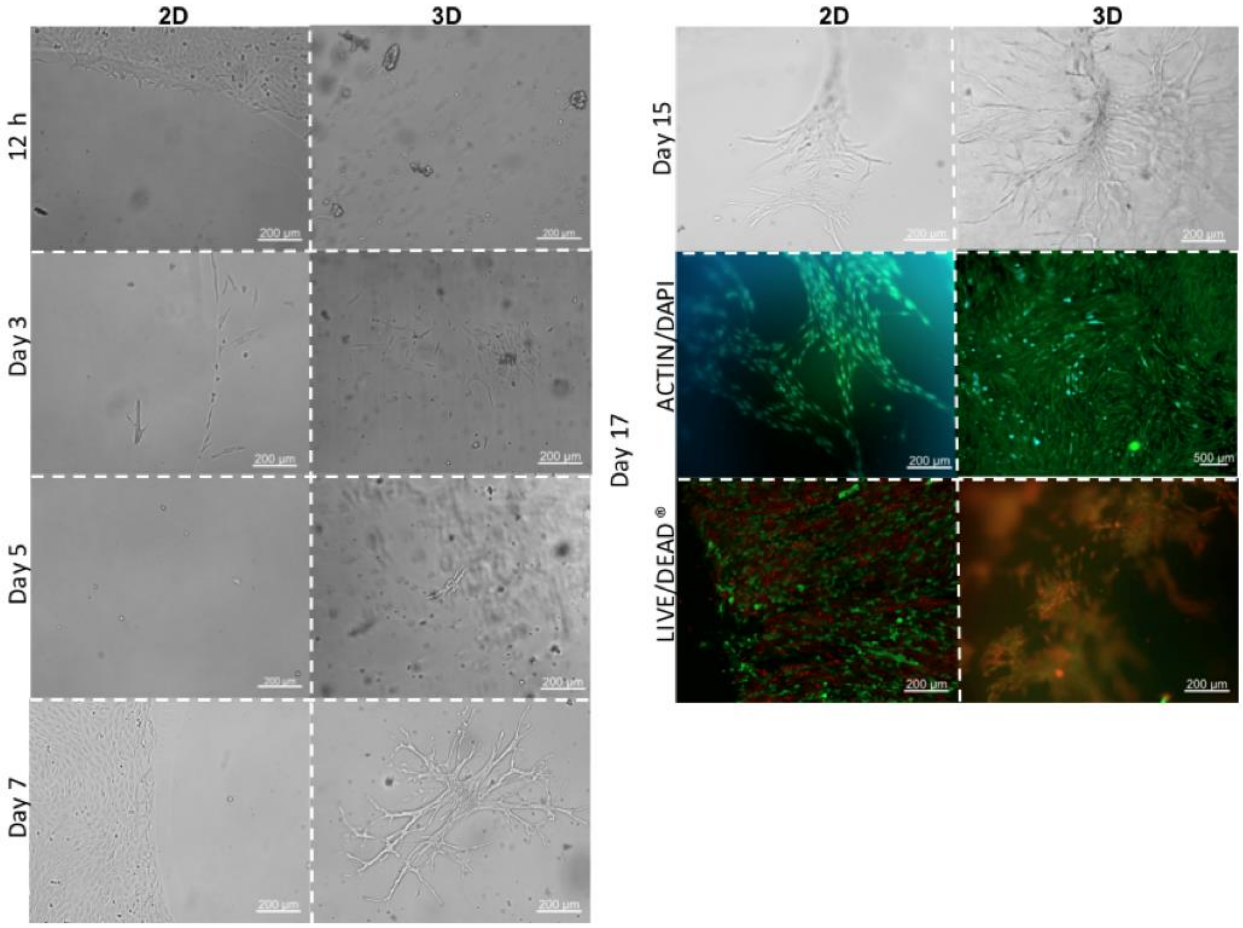


Figure 15. 2D and 3D cell proliferation tracking. C2C12 proliferation tracking in GelMA through 17 days. Bright field micrographs are shown from 12h to day 15. Fluorescent assays are shown at day 17.

The cell growth monitoring was carried out successfully. It was observed that the C2C12 myoblasts proliferated in the wells in which they were cultured, either in 2D or 3D encapsulated. Similarly, elongation and intercellular interaction were observed throughout the follow-up, which reinforces the claim that our GelMA synthesized at home is viable for cell proliferation. In the 2D tests we noticed that the cells tended to proliferate first at the bottom of the plate rather than on the surface of the GelMA. However, when the cell population completely covered the surface of the well, the cells began to proliferate on the surface of the GelMA. In the same way, it was observed that they began to proliferate in the three-dimensional matrix of the constructs. The 3D constructs proliferated more slowly than the 2D constructs, however it was possible to identify intercellular agglomerations that allowed their correct signaling and proliferation within the GelMA matrix. We decided to stop the experiment on day 17 because the cells that were proliferating in a single phase (2D) entered confluence required more regular medium change as well as more space to promote cell proliferation and differentiation.

Even though our monitoring of cell proliferation was more qualitative than quantitative, the observation in the increase of cell number is very evident. Giving this, we demonstrated that our home-made GelMA favorably provides a three- and two-dimensional support for cellular growth and tissue formation. is It is clear that later, it would be possible to carry out a more precise and precise follow-up in terms of the number of cells, however our analysis was sufficient when observing proliferation of the myoblasts by microscopy. Finally, Actin / Dapi and LIVE / DEAD stains were performed to better observe the final estate of the cells. The live/dead staining was performed to evaluate a possible toxic effect of side-products generation and possible damage from UV-light curing. As a final remark, the versatility of our GelMA hydrogel addressees to the specific tissue engineering demands, in terms of cell proliferation, and to produce in vitro tissues towards applications in clinical and biomedical research.

Nano-particle Loaded GelMA Characterization

Photo-crosslinking Assay

The photo-crosslinking assay showed different crosslinking times. The samples prepared with Irgacure were fully crosslinked at 240 s relative to those prepared with LAP which were fully crosslinked at 25 s. No noticeable difference was observed between the samples added with nanoparticles and those traditionally prepared. In this sense, the nanocomposites hydrogels did not undergo any modification since it is inferred that the nanoparticles do not interact at the molecular level with the GelMA matrix. This photo-crosslinking test served to determine that the LAP as a photoinitiator would serve better for cell encapsulation since it requires less time to make cross-linking and therefore the cells are exposed less time to UV light.

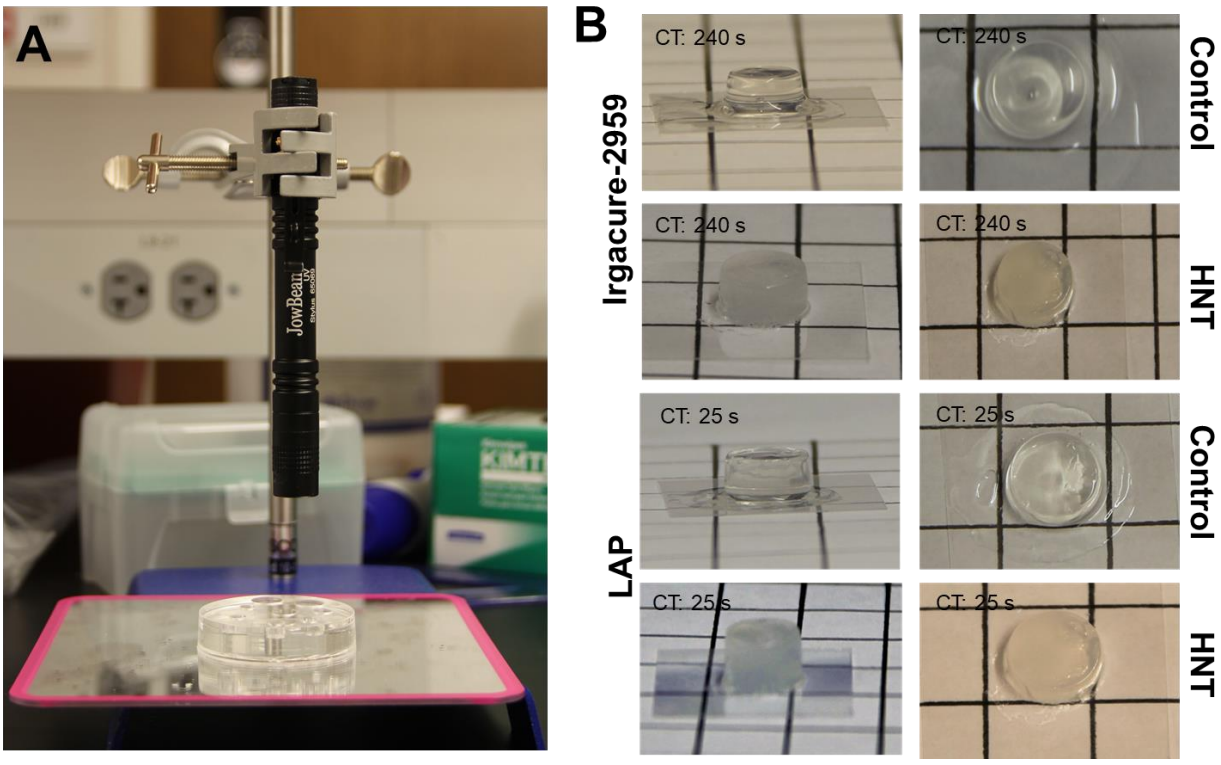


Figure 16. Nano-particle loaded hydrogels photo-crosslinking assay. The samples were divided depending on the type of photoinitiator to be used. A control with pure GelMA and a sample with 1mg / ml of HNT was used.

Mechanical Characterization

Halloysite nanotubes (HNTs), a kind of aluminosilicate clay, have been used successfully as a nanosized filler to enhance the mechanical properties of polymers due to their high aspect ratio and tough structure [82]The only mechanical test that was performed on nano-particle loaded hydrogel samples were compression tests on a Uniaxial Testing Machine. The compression tests were carried out preparing 155 µl cylindrical constructs. In the mechanical tests, the maximum compressive strength of pure GelMA was compared with 1mg/ml of HNT. According to the results obtained, pure GelMA showed a maximum compressive modulus of 153kPa (±45) and GelMA loaded with HNT which had a 280 kPa (±72). The compression modulus for the nano-particle loaded hydrogel was twice than of the pure GelMA.

Some authors have reported the compressive modulus of GelMA hydrogels: Dan Wei reported a compression modulus of 5 kPa for a 73% DoM GelMA, Zhou et al., 2019 reported 0.5 KPa for 10% (w/v) GelMA and Yoon et a., 2016 reported 10 kPa for 10% (w/v) high GelMA[83][67][84]. Some other reports also demonstrate the proportional increase of the compression modulus with the mass/volume fraction of the GelMA. The compressive modulus was also directly proportional to the GelMA mass/volume fraction, i.e. Nichol et al estimated compressive modulus values of 2.0, 10.0, and 22.0 kPa, respectively, for 5,10, and 15% w/v GelMA (with a DoM of 53.8%) The corresponding values for GelMA with a higher degree of substitution (81.4%) were higher; 3.3,16.0 and 33 kPa for 3, 10 and 15% (w/v) GelMA[12] Chen et al., 2012 et al observed that the compressive modulus of a GelMA hydrogel was directly proportional to the degree of methacryloyl substitution (2.0 ± 0.18kPa (49.8%), 3.2 ± 0.18 kPa(63.8%) and 4.5 ±0.33 kPa(73.2%) [85]. Another approximation of the mechanical properties is reported by Ji et al., 2017, they added HNT to gelatin and obtained that composite scaffolds were enhanced significantly by HNTs to 300%, comparing to those of gelatin scaffold. [86].

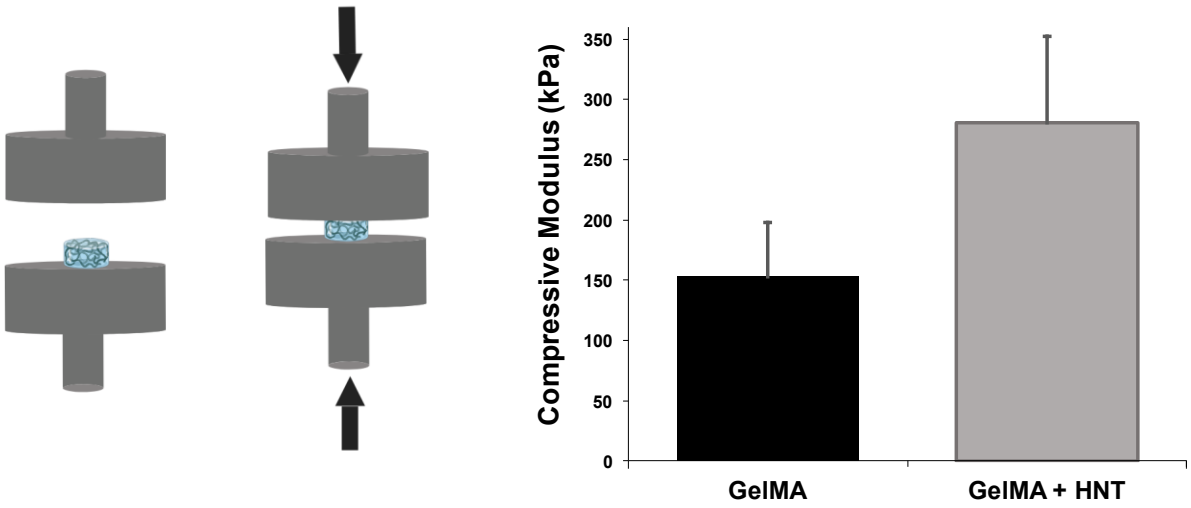


Figure 17. Schematic for the compressive test. This method was used to compare compression strength between pure GelMA and nanoparticle-loaded GelMA. First, GelMA is crosslinked with UV-light. The sample is placed between two compression clamps. Afterwards, the experiment runs to complete compression.

After analyzing and comparing the data obtained in the mechanical compression tests, the values obtained are approximately 10 times higher than those reported in the literature. This might be due to different factors, the over-crosslinking of the constructs (60 s of UV-light exposure) or a possible failure in the sensitivity of the load cell of the uniaxial machine. A fact that is certain, is that the compression modulus of the GelMA samples added with nanoparticles exhibit a compression modulus of almost double that of the pure samples. In summary, we have successfully prepared HNT/GelMA scaffolds. The HNTs were dispersed well in the GelMA matrix, thus significantly improved the mechanical properties of the composite scaffolds. The GelMA loaded with Halloysite nanotubes showed a potential application in tissue engineering to improve the mechanical properties.

Bio-Ink Extrusion Evaluation

Bioprinting is a manufacturing technique in which cells or other biological materials are automatically deposited in a predetermined pattern. The main objective of this technique is to

manufacture constructs with cells without damaging them to resemble tissues that can mimic the native tissue[87]. GelMA hydrogels were found to be promising cell-laden bioink with excellent cell viability. [88] We aimed to use our home made GelMA as a bioink in initial experiments to create scaffolds towards the fabrication of complex tissues or organs.

The bioink extrusion tests were carried out in a BioX Bioprinter from Cellink. For this test, different combinations of GelMA were explored. The rheological properties of GelMA did not allow us to maintain a stable hydrogel to be extrudable in the form of bioink. We explored different combinations of bioink by varying degrees of methacrylation and GelMA and HNT concentrations (See Figure 18). It is important to mention that the room temperature of our laboratory in that time of the year was slightly below the gelatin melting point (32 ºC), this is why we decided to increase the viscosity of our bioink. As a widely used biomaterial, GelMA has been printed alone but normally with concentrations higher than 10%.[53], [89], [90] The strategy used in this study to enhance the extrusion ability of low-concentration GelMA bioinks was mixing GelMA with gelatin. We modified the composition of our polymer by adding an extra 8% (w/v) of dissolved porcine gelatin in the pre-polymer solution. By adding gelatin into GelMA we adapted a bioinks a two-step crosslinking process reported by Yin et.al., 2018.In addition to the viscosity increasing by extra gelatin addition, we also decreased the temperature of the bioinks inside the bioprinter cartridges prior to be used. We prepared a water bath at 15 ºC and cooled down the cartridges for 30 min to promote a physical crosslinking of the GelMA-Gelatin-HNT bioink.

We tried to bioprint different bioinks by extrusion. Initially we started using 5% (w/v) GelMA solutions of medium and high DoM. These bioinks were used with their variants of nanoparticle concentrations and with an extra 8% of gelatin. The results were not favorable for these bioinks since they were not sufficiently solid to maintain a stable construct. As can be seen in Figure 18, the variants marked inside a gray box extruded irregular constructs. On the other hand, the best bioprinting results were observed with the bioinks in which we implemented a concentration of 7% (w/v) of GelMA and its variants of extra gelatin and HNT´s. We consider that bioinks were printable when we observed in the microscope that its structure remained stable after been printed.

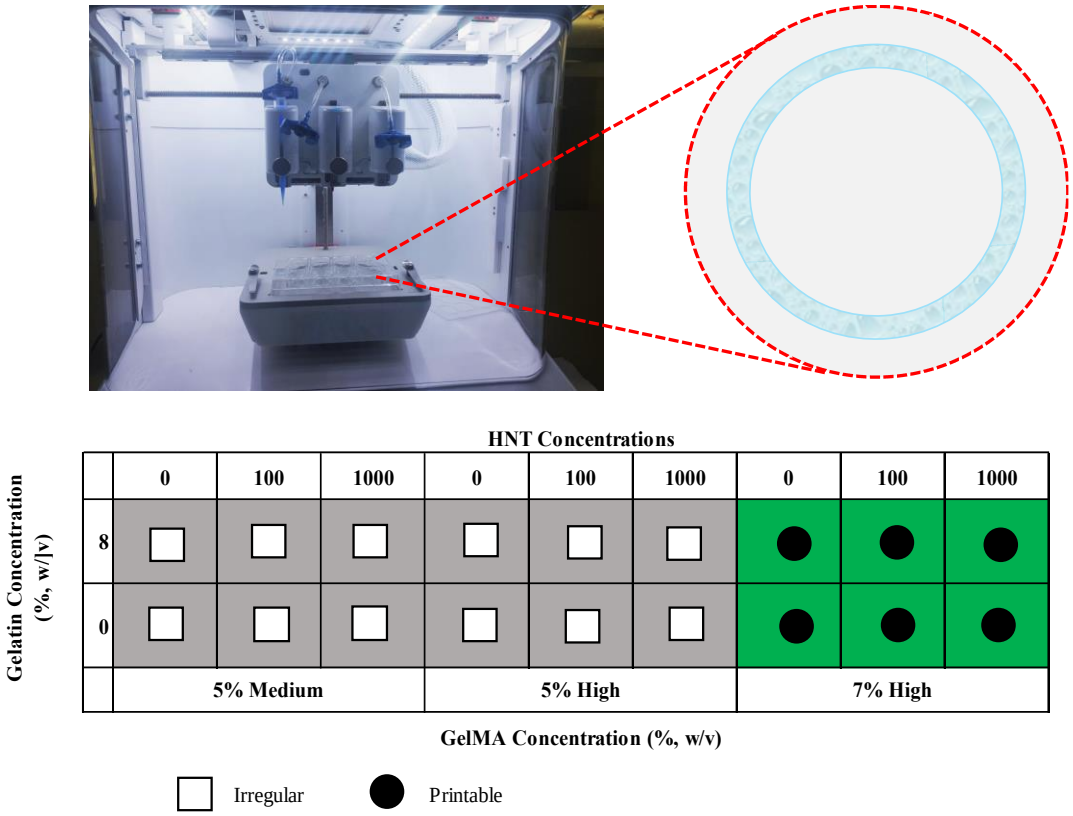


Figure 18. Different formulations for extrusion bioprinting. Schematic of the 3D printed o-ring utilizing BioX by implementing different combinations of GelMA, HNT´s and Gelatin used to enhance bioink rheology.

We considered that our bioink was extrudable or bioprintable when it maintained a semi-solid state and able to hold the o-ring shape. The bioinks made of 5% GelMA were considered not-extrudable due to the shape of the toroid was not constant. Moreover, it deformed immediately and had discontinuities on the final stroke. An example of an effectively briprinted ink can be seen in Figure 19 . The shape of the stroke is continuous, clean, it does not present imperfections and also maintains its three-dimensional topography before being exposed to UV-light.

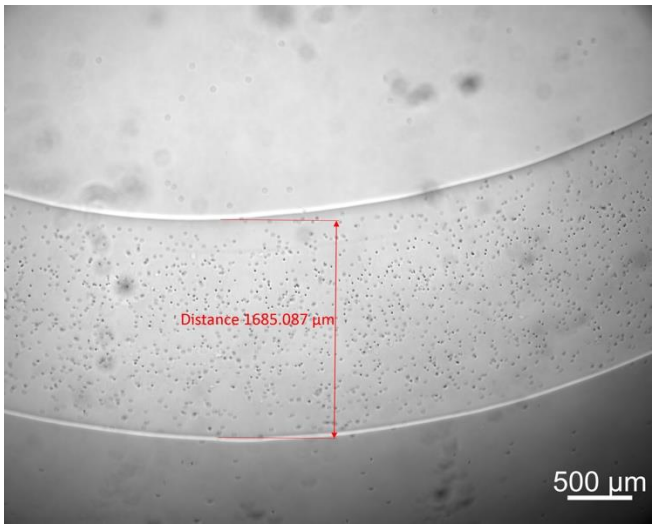


Figure 19. Bioprinted o-ring. Nano-composite o-ring construct bioprinted with BioX.

Biofabrication of hydrogels involves two basic requirements when talking about biomaterials features: processability for building high-resolution tissue-like structures and cytocompatibility to facilitate migration, proliferation, and differentiation of the embedded and endogenous cells[5]Our biggest problem when using our home made GelMA as a bioink was its low rheological properties to maintain its volumetric structure. The high temperatures of the city of Monterrey caused our bioink to remain in a liquid state most of the time, which is not convenient for bioprinting applications. One of the ways to attack this problem without modifying the properties and composition of our ink was the use of a hydrophobic base to promote the formation of three-dimensional structures. We successfully deposited our GelMA with cells based on electrospun-zein-nanofibers (fabricated in collaboration with University of Nebraska Lincoln) to promote hydrophobic interaction with GelMA bioink (See Figure 20). It was possible to form spherical structures that maintained their stability for up to 10 days. The advantages of implementing this type of nanostructured materials are; biocompatibility, stability physiological conditions for long periods of time and thus avoiding the modification of GelMA properties that are not favorable for cell growth. The disadvantage of using this method of deposition is that the structure of the constructs is limited to the hydrophobic interaction of the base with the bioink, as in this case, we only achieve the formation of spheres. Cell growth was not reported because it was only a proof of concept that avoids modifying the properties of GelMA.

This experiment helped us to realize that the bioprinting of biocompatible inks made of GelMA is not an easy thing. To bioprint constructs it is necessary to take into account many factors of great importance among which are, the viscosity of the ink, the temperature, printing speed, extrusion pressure, sterility of the experiment, cell viability, among others. Currently, there is no consensus for which rheological modifier works best for GelMA bioprinting however good results have been obtained with hydrogel materials and inorganic nanoparticles.

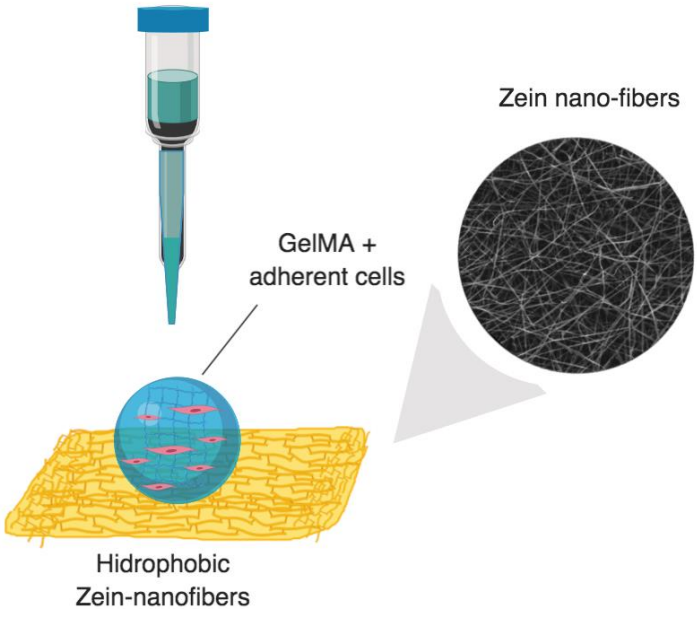


Figure 20. GelMA extrusion bioprinting on zein-nanofibers. The deposition of GelMA over zein-nanofibers promotes the formation of spheres by hydrophobic interaction.

Biological Characterization

Nanocomposite hydrogels are “nanomaterial-filled” polymeric networks that exhibit grater physical features relative to traditionally made hydrogels. The addition and interaction of nanoparticles controls a range of physical, chemical and biological properties (1W). The combination of organic polymer (in this case GelMA) and inorganic (Halloysite nanotubes) nanoparticles[91].

The capability of patterning soft biopolymer materials to three dimensional (3D) structures as scaffolds is critical for emerging tissue engineering technology. [92]. We conducted biological characterization based on the bioprinted constructs that were mixed with 8% (w/v) gelatin. The constructs that were not properly extruded were excluded from the biological characterization. We cultured cells using bioinks with concentration of 5% (w/v) of medium and high DoM GelMA, however, as the constructs were not stable, they were not considered for the biological characterization. On the other hand, encapsulated cells in the bioinks prepared with 7% (w/v) GelMA with a high degree of methacrylation as well as gelatin and nanoparticles. It is important to mention that Halloysite nanotubes have attracted great interest from scientists in preparing scaffolds for tissue engineering because their excellent biocompatibility has been assessed suitable for both cell culture and animal tissue, and their tubular structure can load various kinds of drugs[93]–[95] Cell growth was monitored for 15 days. Some previous reports demonstrated the biological properties of the composite scaffold that were investigated by culturing MG53 cells on them. Moreover they mention that the excellent mechanical properties of the HNT`s/gelatin scaffolds could be a promising bone material[86].

We 3D printed O ring-shaped constructs on 12-well plates. As shown in Figure 21 the cultured cells showed less proliferation in the constructs with 0 μg/ml of HNT. We observed that in those constructs the cells did not elongate over time. It was determined that the cell viability was very low since the cells did not elongate, however the culture media showed change in tonality after every 3 days, which indicated cell-metabolic activity within the GelMA matrixes. On the other hand, the cells seeded in constructs with nanoparticles showed a more considerable cell growth than the control without nanoparticles. The interaction of HNT and our hydrogel can be explained in previous reports by a FTIR characterization which suggest the presence of hydrogen bond interactions between HNT`s and the H ions of the GelMA[96].We observed the greatest difference in the constructs with 1000 0 μg/ml of HNT, in which we observed evident cell elongation from the third day. We also observed a greater cellular number over time in comparison with the other two concentrations. It is important to mention that the staining of the constructs with 0 0 μg/ml of HNT was not possible to observe the positive effect of the actin/dapi and LIVE/DEAD® staining. This is attributed to the fact that the bioink was too rigid to promote cell elongation and the permeability of the construct. On the other hand, the evident cell growth in the

nanoparticle constructs is attributed to the positive properties that inorganic nanoparticles add to the hydrogels, such as conductivity, allowing the cells to have a better proliferative environment.

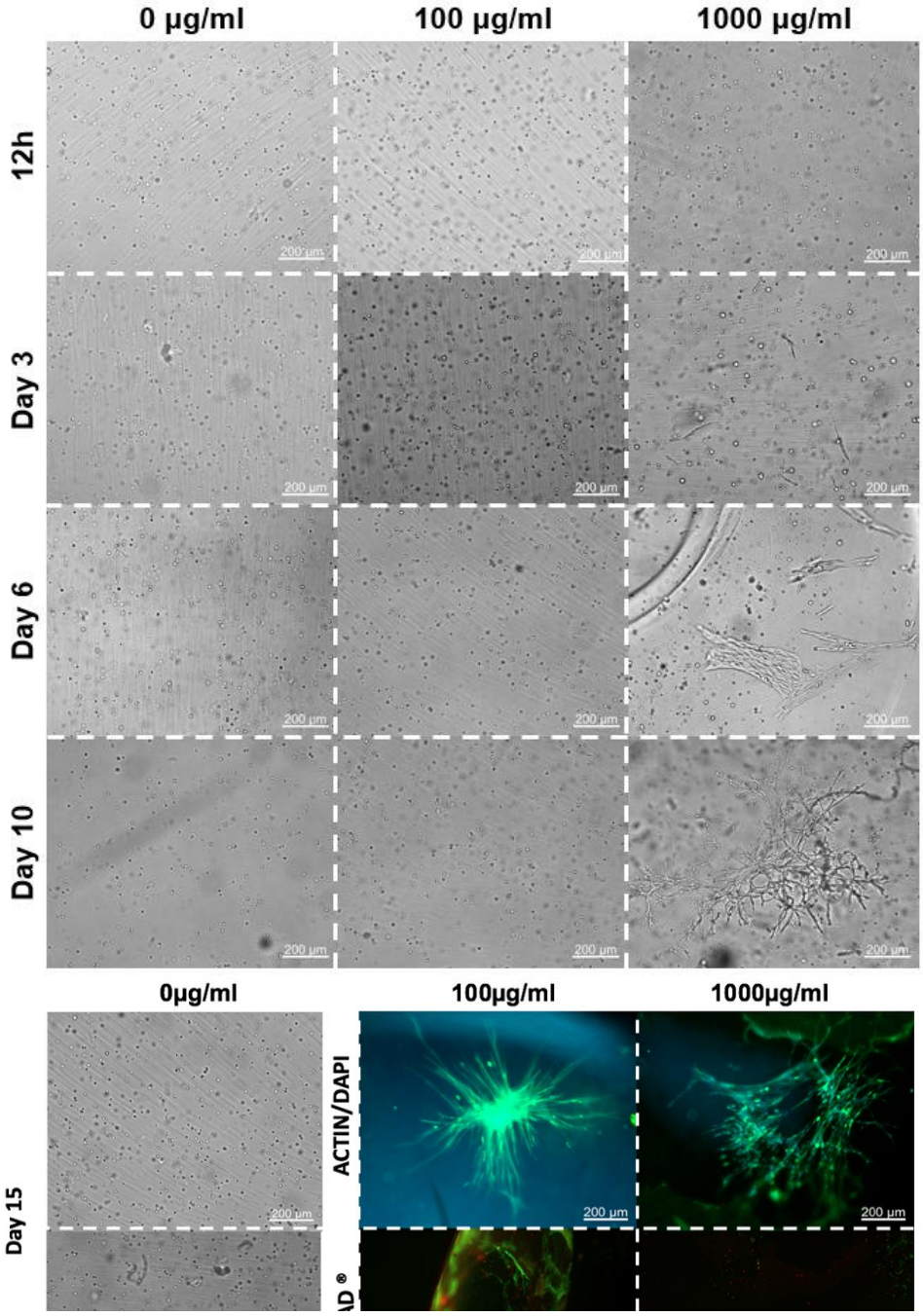


Figure 21. 3D cell tracking of GelMA bioprinted constructs. C2C12 proliferation tracking in nano-particle loaded GelMA/gelatin bioink through 15 days. Bright field micrographs are shown from 12h to day 15. Fluorescent assays are shown at day 15.

Conclusions and Perspectives

Conclusions

In conclusion, we successfully improved the synthesis process of GelMA by standardizing an accurate methodology. This methodology was optimized mainly by reducing the total production time. Reported protocols take around 10 days to produce 5 grams of GelMA. We were able to simultaneously manufacture 12 g in 5 days. We also were able to fabricate a GelMA-based bioink with loaded nanoparticles that allowed to create a viable microenvironment for adherent cells culture. that it allows the viability of the cells towards is applications as bioink in microtissue scaffolds.

Throughout this work, the process of synthesizing GelMA was improved, going from a manually traditional to a semi-automated process. To attain this, the synthesis process was improved by monitoring MA addition. On the other hand, the exchange of water for a continuous exchange by means of the peristaltic pump was semi-automatized with the purpose of reducing the purification times. We suggest a GelMA synthesis by: using DPBS as solvent, selecting a MA addition rate of 0.5ml/ min, setting up a reaction time of 1 hour, avoiding pH control along process, using LAP photoinitiator and avoiding a centrifugation to reduce purification time.

The products mechanical, chemical and biological properties were assessed by different tests. Chemical tests were done such as FTIR, NMR, and Fluoraldehyde-OPA to verify the degree of substitution of the amines of GelMA. Photocrosslinking tests were carried out to check that the product was functional. In addition, tensile, shear and compressive tests were managed to compare their mechanical performance. Finally, we performed biological test to successfully demonstrate biocompatibility of our home-made GelMA.

With all this, a hybrid GelMA was formulated with halloysite nanoparticles. This resulted in the improvement of a bioink-like hydrogel to promote cell proliferation enhance bioprinting amenabilities.

All in all, we decently improved the process of GelMA synthesis by implementation of a semi-automated process, which led us to carry out a series of characterizations for product quality verification, and the development of a GelMA bioink with halloysite nanotubes, which resulted in an enhancement of the biological and mechanical features of our bioink.

Perspectives

The development of new and better biomaterials represents a challenge for the future of tissue engineering. We successfully improved and optimized the GelMA synthesis and manufacturing process. In addition to that, we also made use of nanotechnology to improve its structural and biological properties. The future of this project addresses to use GelMA as main resource for the development of new biomaterials. Those biomaterials will allow researchers to design micro and nano-structures to encapsulate cells to generate complex and functional tissues. Much research is still needed about new nanoparticles that have been synthesized in the world recently. It takes exhaustive time to investigate the effect of the combination with biomaterials, especially with hydrogels. Similarly, the development of bioinks with a high rate of cell proliferation represents a very large area of opportunity. In this sense, developing GEL-based bio-ink that can be used to print different types of tissues represents an area of opportunity. Tests with more cell types are needed, as well as complete screenings of genetic expression to determine what type of GelMA- nanoparticle composite is the most viable for a certain type of cell.

Appendix

Appendix A
Abbreviations and acronyms

Table 8. Abbreviations

	Description
GelMA	Gelatin Methacryloyl
dH_2O	Distilled water
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium bicarbonate
CO_2	Carbon dioxide
NH_2	Amine
D_2O	Deuterium oxide
N-H	Amine bond
C=O	Carbonyl
H	Hydrogen
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
MA	Methacrylic Anhydride
pH	Power of hydrogen
NP	Nanoparticle
CNT	Carbon nanotube
PAM	Polyacrylamide
DoM	Degree of Methacrylation
Ph.D.	Postgraduate doctoral degree
BSc.	Bachelor of science
Et.al.	And others
n.d.	Not defined
i.e.	That is
. stl	Standard Triangle Language

Table 9. Acronyms

	Description
HNT	Halloysite Nanotubes
HNP	Halloysite Nanoparticles
NP	Nanoparticle
DPBS	Dulbecco´s Phosphate Buffer Saline
SEM	Scanning Electron Microscopy
2D	Two Dimensional
3D	Three Dimensional
GO	Graphene Oxide
HA	Hyaluronic Acid
C2C12	Mouse Myoblasts
ECM	Extra Cellular Matrix
PDMS	Polydimethylsiloxane
DMEM	Dulbecco's Modified Eagle´s Medium
FBS	Bovine Fetal Serum
PEG	Poly (Ethylene Glycol)
PHEMA	Poly (2-Hydroxyethyl Methacrylate)
PG	Proteoglycans
FN	Fibronectin
RGD	Arginine-Glycine-Aspartic Acid
UV-light	Ultra-Violet Light
GAG	Glycosaminoglycan
CD4	CD4 Hyaluronan Receptors
FN	Fibronectin
FTIR	Fourier-Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
OPA	*o*-phthaldialdehyde
TMSPMA	3-(Trimethoxysilyl)propyl methacrylate
ASTM	American Society for Testing and Materials
ITESM	Instituto Tecnológico y de Estudios Superiores de Monterrey
CONACYT	Consejo Nacional de Ciencia y Tecnología
LIMB	Laboratory for Innovative Microtechnologies and Biomechanics
UNL	University of Nebraska Lincoln

Appendix B
Variables and symbols

Table 10. Description and units of the variables used.

Variable	Description	Units
M	Concentration	Mol/L
RPM	Rotational Speed	Revolutions/min
mm	Distance	millimeter
cm	Distance	centimeter
mm^2	Area	Square millimeter
cm^2	Area	Square centimeter
mm^3	Volume	Cubic millimeter
cm^3	Volume	Cubic centimeter
δ	Chemical shift	Parts per million
kDa	Mass (molecular scale)	Kilo Daltons
µl	Volume	microliters
ml	Volume	milliliter
L	Volume	Liter
s	Time	seconds
min	Time	Minutes
h	Time	hour
^oC	Temperature	Celsius
Pa	Pressure	N/m^2
mg	mass	milligram
W	power	watt
nm	wavelength	nanometers

Appendix C
Conference and poster presentation

Congreso de Investigación y Desarrollo-2018 celebrated in Monterrey, Nuevo León.

Oral Presentation: “3d bioprinting platform based on chaotic flows.”

Víctor Hugo Sánchez Rodríguez, Ricardo García Ramírez, Mohamadmahdi Samandari, Grissel Trujillo-de Santiago

Actual bioprinting methods are not able to rapidly draw complex and high-resolution structures. Chaotic flows create structure by the repeated orientation and deformation of a fluid that promotes stretching and folding repeatedly. This 3D bioprinting platform is based on the use of a Journal Bearing which involves a mechanism of an inner and outer cylinder to draw high-resolution 3D structures. Proof of concept experiments involve the use of fluorescent inks immersed in not Newtonian liquids. The high viscosity o the material and low speeds preclude a modellable and predictable structure. The development of a platform that allows the creation of a large amount of interface between materials at an exponential speed will allow the improvement of the current manufacturing strategies, because these strategies are based on print heads that require a large amount of time to make structures in high resolution, in addition to limiting the maximum resolution that can be aspired in this kind of manufacturing. In the following report the chaotic impression in 3D is exposed, in which fluids are used to draw complex structures and in high resolution. A bioink (i.e. cells, fluorescent particles) is injected into the "bio paper" (i.e. PDMS, GelMa), a Newtonian viscous liquid, and a chaotic flow is induced with a constant folding and stretching action that generates a laminar structure defined. The computational modeling of this type of chaotic flow is also presented, since said chaotic flows that are printed are deterministic and the process of particle convection and structure generation can be modeled. Due to the above, it is then possible to generate defined, predictable and high-resolution structures. Chaotic printing could be a facilitator in multiple processes that need a high resolution and would allow the rapid generation of interface due to the exponential speed in which it is unfolded the ink in the polymer. Several possible applications of this platform are defined, such as the development of tissue-like structures with applications in biomedical engineering.

International Conference on Nanotechnology Tec.Nano 2018

Oral Presentation: “GelMA Semi-Automated Process: Halloysite Nanotubes Composite Scaffolds for 3D Cell Culture”

Víctor Hugo Sánchez Rodríguez, Sara Cristina Pedroza González, Jesús Valencia Gallegos, Elda Graciela López, Mario Moisés Álvarez, Grissel Trujillo de Santiago

Gelatin methacryloyl (GelMA) is a semisynthetic biomaterial that conserves the advantages of native collagen such as the presence of cell-binding domains with protease-cleavage sites. GelMA-based hydrogels are currently used as biomaterials to develop cell-laden systems for several biomedical applications. This biomaterial provides the necessary physiological microenvironment and biocompatibility for tissue engineering studies. However, currently used methods to produce GelMA do not consider a strict control of the key parameters of the reaction process (i.e., mixing, location and rate of addition of methacrylic anhydrate, and pH) which leads to batch to batch inconsistencies and low yields. In this work, we present a semi-automated method for the synthesis and purification of a GelMA-based hydrogel with the aim of standardizing the process to enhance yield and quality of the final product. Our protocol includes the use of custom-made jacketed reactor with temperature, agitation, and pH control. The addition of methacrylic anhydride, the key reagent of the synthesis, is controlled by a syringe pump. A continuous dialysis stage follows reaction, effectively shortening the time needed for methacrylic acid removal. Automated equipment and pH control reduce synthesis and purification time and enhance reactivity leading to higher degree of substitution. Using this synthesis and purification strategies, we conducted multiple sets of GelMA production and correlated reaction conditions to final yields and qualities of the product. Moreover, we present information on the rates of methacryloyl functionalization during the reaction stage, and the rate of methacrylic acid removal during the purification stage. While conventional protocols require a 2 weeks preparation time and result in inconsistent quality of the final product, the methodologies presented here yield consistent quality GelMA in 7-8 days. Our final product characterization includes crosslinking test, degree of functionalization (DoF), spectroscopic characterization in FT-IR and NMR and 3D cell culture using halloysite nanotube (HNT). HNT are incorporated into our GelMA to improve cell-attachment of the hydrogel scaffolds. We compared adherent-like cell proliferation, cell viability, scaffold stability in both; hybrid and native hydrogel. Hybrid GelMA-HNT hydrogel exhibits great potential for applications in tissue engineering.

International Annual Meeting of American Institute of Chemical Engineers (AIChE)- Pittsburgh, PA.

Poster: “Semi-Automated Process to Synthesize Gelatin Methacryloyl (GelMA) Hydrogel”

Víctor Hugo Sánchez Rodríguez, Sara Cristina Pedroza González, Jesús Valencia Gallegos, Elda Graciela López, Mario Moisés Álvarez, Grissel Trujillo de Santiago

Gelatin methacryloyl (GelMA) is a semisynthetic biomaterial that conserves the advantages of native collagen such as the presence of cell-binding domains with protease-cleavage sites. GelMA-based hydrogels are currently used as biomaterials to develop cell-laden systems for several biomedical applications. This biomaterial provides the necessary physiological microenvironment and biocompatibility for tissue engineering studies. However, currently used methods to produce GelMA do not consider a strict control of the key parameters of the reaction process (i.e., mixing, location and rate of addition of methacrylic anhydrate, and pH) which leads to batch to batch inconsistencies and low yields. In this work, we present a semi-automated method for the synthesis and purification of a GelMA-based hydrogel with the aim of standardizing the process to enhance yield and quality of the final product. Our protocol includes the use of custom-made jacketed reactor with temperature, agitation, and pH control. The addition of methacrylic anhydride, the key reagent of the synthesis, is controlled by a syringe pump. A continuous dialysis stage follows reaction, effectively shortening the time needed for methacrylic acid removal. Automated equipment and pH control reduce synthesis and purification time and enhance reactivity leading to higher degree of substitution. Using this synthesis and purification strategies, we conducted multiple sets of GelMA production and correlated reaction conditions to final yields and qualities of the product. Moreover, we present information on the rates of methacryloyl functionalization during the reaction stage, and the rate of methacrylic acid removal during the purification stage. While conventional protocols require a 2 weeks preparation time and result in inconsistent quality of the final product, the methodologies presented here yield consistent quality GelMA in 7-8 days.

Conference Journey to Innovation, Monterrey, Nuevo León 2019.

Poster: “GelMA, Semi-Automated Process: Halloysite Nanotubes Composite Scaffolds for 3D Cell Culture”

Víctor Hugo Sánchez Rodríguez, Sara Cristina Pedroza González, Jesús Valencia Gallegos, Elda Graciela López, Ali Tamayol, Mario Moisés Álvarez, Grissel Trujillo de Santiago

A wide range of nanoparticles, including carbon-based, metallic, ceramic, polymeric, among others, can be laden to hydrogels to enhance different properties (i.e.: mechanical, rheological, biological, etc.). In this project, the fabrication of nano-composite hydrogels using GelMA and halloysite nanotubes aims to improve its biological and physical properties inspired in a biological tissue which can be helpful in several biomedical applications. We developed a proof-of-principle strategy to use our hydrogel as a nano-composite bio-ink for 3D printing. To fulfill the goal of proper rheology for GelMA extrusion, a variety of bioinks were prepared by mixing GelMA, gelatin, and halloysite-nanoparticles (HNT). The concentration of GelMA, gelatin, and nanoparticles was varied to create an executable 3D ink at room temperature taking advantage of a two-step thermal- / photo-crosslinking strategy. This approach demonstrated to obtain reproducible cell-laden scaffolds by using extrusion as a tool for 3D printing. All in all, hydrogel characterization and nanoparticle-functionalization results will bring up a big study opportunity towards its application and feasibility as bio-ink for cell-laden fibers and microtissue scaffolds

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