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Synthesis and characterization of C2C12-laden gelatin methacryloyl (GelMA) from marine and mammalian sources

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Keywords

Fish skin, Porcine skin, Gelatin, Hydrogels, Skeletal muscle injury.

Abstract

Gelatin methacryloyl (GelMA) is widely used for tissue engineering applications as an extracellular matrix (ECM) mimicking scaffold due to its cost-effectiveness, ease of synthesis, and high biocompatibility. GelMA is widely synthesized from porcine skin gelatin, which labors under clinical, religious, and economical restrictions. In order to overcome these limitations, GelMA can be produced from fish skin gelatin, which is eco-friendly as well. Here, we present a comparative study of the physicochemical (structural, thermal, water uptake, swelling, rheological, and mechanical) and biological (cell viability, proliferation, and spreading) properties of porcine and fish skin GelMA with low and high methacrylation degrees, before and after crosslinking, to check whether fish skin can replace porcine skin as the source of GelMA. Porcine and fish skin GelMA presented similar structural, thermal, and water uptake properties prior to crosslinking. However, subsequent to crosslinking, fish skin GelMA hydrogels exhibited a higher mass swelling ratio and a lower elastic and compressive Young's moduli than porcine skin GelMA hydrogels of similar methacrylation level. Both types of GelMA hydrogels showed great biocompatibility toward encapsulated mouse myoblast cells (C2C12), however, improved cell spreading was observed in fish skin GelMA hydrogels, and cell proliferation was only induced in low methacrylated GelMA. These results suggest that fish skin GelMA is a promising substitute for porcine skin GelMA for biomedical applications and that low methacrylated fish skin GelMA can be used as a potential scaffold for skeletal muscle tissue engineering.

1. Introduction

Hydrogels are crosslinked networks of hydrophilic polymers that are mainly noted for their propensity for water and maintaining a stable structure. For this, hydrogels have been widely used as engineerable extracellular matrix (ECM) mimics for many different biomedical

applications ranging from tissue engineering to drug and gene delivery [1–5]. Gelatin is one of the most popular biopolymers produced via the partial hydrolysis of native collagen, which is a fibrous protein and the principal constituent of animal skin, bone, and connective tissue [6]. Due to its unique functional and technological properties, gelatin has been widely used for tissue engineering [4,7], drug delivery [8,9], food [10], and cosmetic applications [11]. Gelatin contains abundant arginine-glycine-aspartic acid (RGD) sequences that enhance cell attachment [12] and contains target sequences of matrix metalloproteinase (MMP) that promote cell remodeling [13]. Gelatin possesses excellent solubility, biodegradability, biocompatibility, low antigenicity, and a low gelling point, as it is derived from denatured collagen [14]. However, some limitations, such as its low mechanical modulus and its rapid degradation, hinder its use in biomedical applications.

To surpass these disadvantages, gelatin methacryloyl (GelMA) is synthesized through chemical modification of gelatin (**Figure 1A**), through the addition of methacrylate groups to the amine-containing side groups [15]. This methacrylation reaction allows UV-light induced polymerization of gelatin into a hydrogel that is stable at 37 °C, in the presence of a photoinitiator. As it is mechanically stronger than unmodified gelatin hydrogels, GelMA hydrogels have been used to microfabricate complex structures using different micro- and biofabrication techniques, such as photopatterning [16], micromolding [17], self-assembly [18], microfluidics [19], textile techniques [20], and bioprinting techniques [21].

The physicochemical properties of GelMA depend on both the source of gelatin, mammalian or marine, and its level of methacrylation [16,22,23]. Economical, environmental, clinical, and religious restrictions shifted preference of gelatin sources from mammalian to marine. Fish skin is a major waste byproduct of the fish-processing industry [24,25], therefore it is an inexpensive material to acquire, and valorizing its use will decrease pollution. Moreover, when extracting gelatin from mammalian sources, there exists a risk of zoonoses, such as Bovine Spongiform

Encephalopathy [26]. Furthermore, the usage of gelatin derived from mammalian sources, i.e. porcine or bovine skin, is not acceptable for several major religions in the world, including Judaism, Islam, and Hinduism, constituting ~40% of the human population, whereas fish skin gelatin does not pose any religious limitations [6,22]. Therefore, using fish skin GelMA scaffolds could open up tissue engineering applications, for tens of millions of people.

Previously, other studies have compared fish and porcine skin GelMA [22,23]. However, these comparisons were made using fish and porcine GelMA produced with different methacrylation levels. This study offers a more complete overview by comparing fish and porcine GelMA with similar methacrylation levels (low and high). Furthermore, previous studies did not compare the fish and porcine GelMA's structural, thermal, water sorption, and rheological properties, which are fully characterized in this report. Moreover, GelMA from porcine origin has been extensively used for tissue engineering applications, including skeletal muscle tissue engineering [27–30]. Researchers have employed the mouse myoblast cell line (C2C12) in order to assess the suitability of porcine skin GelMA for skeletal muscle tissue engineering applications, showing promising results [27–30]. However, none of the previous studies have assessed the suitability of fish skin GelMA for this application. To the best of our knowledge, this is the first study that investigates the use of fish skin GelMA hydrogels as C2C12 myoblasts-laden scaffolds.

In this report, we compare the physicochemical (structural, thermal, water uptake, swelling, rheological, and mechanical) and biological (cell viability, proliferation, and spreading) properties of porcine and fish skin GelMA. Mouse C2C12 myoblasts were encapsulated in low and high methacrylated porcine skin GelMA hydrogels and, for the first time, in low and high methacrylated fish skin GelMA hydrogels, to determine their biocompatibility, as well as to assess their potential for skeletal muscle tissue engineering scaffolds in future studies.

2. Materials and methods

2.1. Reagents

Gelatin from porcine skin (Type A, 300 bloom), gelatin from cold-water fish skin, methacrylic anhydride (MA), Irgacure 2959 (PI) (2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone), deuterium oxide (99.9 atom % D), 2,4,6-Trinitrobenzene-sulfonic acid solution, and Dulbecco's phosphate-buffered saline (DPBS) were all purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). Dulbecco's Minimum Eagle Media (DMEM), Fetal Bovine Serum (FBS), L-Glutamine, penicillin, streptomycin, LIVE/DEAD Viability/Cytotoxicity Kit, and PrestoBlue Kit were all purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. GelMA synthesis

GelMA was synthesized, from porcine and cold water fish skin, according to the general method first adopted by Van Den Bulcke *et al.* [15]. In brief, gelatin was mixed at 10% (w/v) into DPBS at 60 °C and stirred until fully dissolved. Then, 1.25% (v/v) and 20% (v/v) of MA were added in a dropwise manner to the gelatin solution at 50 °C, at a rate of 0.5 mL/min under stirred conditions and allowed to react for 1 h. After 1 h, the reaction was stopped following 5X dilution with additional warm (40 °C) DPBS. To remove salts and unreacted MA, the mixture was dialyzed for 5 days at 40 °C against distilled water using 12–14 kDa cutoff dialysis tubing. The solution was finally lyophilized, generating a porous white foam that was stored at -20 °C until further use.

2.3. Fourier-transform infrared

Fourier-transform infrared (FTIR) spectra of freeze-dried GelMA were recorded using a Tensor 27 mid-FTIR Bruker spectrometer (Bruker, Karlsruhe, Germany) equipped with a diamond ATR (Attenuated Total Reflectance) module and a DTGS (Deuterated-Triglycine Sulfate) detector between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution. Raw absorbance spectra were smoothed using a nine-points smoothing function. After elastic baseline correction using 200 points, $\text{H}_2\text{O}/\text{CO}_2$ correction was then applied.

2.4. Determination of GelMA degree of substitution

2,4,6-trinitrobenzene-sulfonic acid (TNBS) assay was performed to determine the GelMA's degree of substitution (DS) according to the method developed by Habeeb *et al.* [31]. Briefly, GelMA and gelatin samples were first dissolved in 0.1 M sodium bicarbonate buffer at a concentration of 1.6 mg/mL, then mixed with an equal volume (0.5 mL) 0.01% TNBS solution and incubated for 2 h. To stop the reaction, two solutions of 0.25 mL of 1 M HCl and 0.5 mL of 10% (w/v) sodium dodecyl sulfate were added. The amino group concentration (AC) of each sample was determined from a glycine standard curve after measuring their absorbance at 335 nm. The DS was calculated according to the current formula:

$$\text{DS (\%)} = (1 - \text{AC of GelMA} / \text{AC of gelatin}) \times 100 \quad (1)$$

To verify the DS, proton nuclear magnetic resonance (¹H-NMR) spectra were collected at 40 °C in deuterium oxide at a frequency of 400 MHz using an AVANCE III 400 NMR spectrometer (Bruker, Karlsruhe, Germany). 50 mg of each GelMA sample was dissolved in 1 mL of deuterium oxide at 40 °C. The peak area of aromatic amino acids (7.5 – 7.3 ppm) in the GelMA samples was employed as a reference in each spectrum. Baseline correction was applied before obtaining the areas of the peaks of interest. The peak area (PA) of lysine methylene protons appearing between 3.1 – 2.9 ppm was used for calculation of the DS following the equation:

$$\text{DS (\%)} = (1 - \text{PA of GelMA} / \text{PA of gelatin}) \times 100 \quad (2)$$

2.5. Thermal gravimetric analysis

The thermal resistance of GelMA samples (~ 5 mg) was examined using a Discovery thermal gravimetric analysis (TGA) 5500 (TA instruments, New Castle, DE, USA) in open pans under nitrogen atmosphere at a gas flow rate of 25 mL/min and a heating rate of 20 °C/min from 35 to 600 °C. The degradation temperature (Td) was determined from the onsets of percent change in derivative weight versus temperature.

2.6. Differential scanning calorimetry

The differential scanning calorimetry (DSC) measurement of GelMA samples (~ 5 mg) was carried out using a Discovery DSC250 (TA instruments, New Castle, DE, USA) under a dynamic inert nitrogen atmosphere, with a flow rate of 50 mL/min in a Tzero hermetic aluminum capsule. Cooling was performed to -80 °C at a rate of 20 °C/min, followed by heating to 250 °C at a rate of 10 °C/min for all samples.

2.7. Dynamic vapor sorption (DVS)

Water sorption isotherms were determined gravimetrically using the DVS technique (Surface Measurement Systems, SMS, London, UK). The DVS apparatus monitors the moisture sorption capacity of a sample as a function of relative humidity (RH) of the surrounding air. The changes in sample weight over time at a fixed temperature and the desired RH (between 0 and 70%) were recorded. About 55-60 mg of GelMA were placed on the quartz sample pan. The program was initially set to maintain the humidity at 0% for 6 h (drying phase). The samples were then submitted to successive steps of 10% RH increase, up to 70% RH.

2.8. GelMA hydrogel preparation

GelMA solution was prepared by dissolving 10% (w/v) of the freeze-dried prepolymer into a DPBS solution at 50 °C. Then, 0.5% (w/v) of PI was added and the temperature was increased to 70 °C to reach complete solubilization. GelMA solution was UV crosslinked (360-480 nm) in a specific PDMS mold for 40 s to create hydrogel discs.

2.9. Mass swelling ratio

The mass swelling ratio was evaluated using five cylindrical samples (2 cm diameter, 2 mm height). The samples were kept in DPBS for 24 h. The excessive water was gently removed using a paper tissue and the swollen weight of the samples was measured using a precision

balance. Later, the samples were lyophilized for 3 days and their dry weight was measured. The mass swelling ratio was given by the following formula:

$$\text{Mass swelling ratio} = \text{Swollen weight of the sample} / \text{Dry weight of the sample}$$

(3)

2.10. Rheological testing

Amplitude sweep tests at a frequency of 1 Hz were performed using a Kinexus pro rheometer (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a plane-plane geometry with a diameter of 20 mm. The hydrogel was loaded into a 1 mm gap between the plates and allowed to relax until the normal force was zero. The amplitudes of shear stress were carried out over a pressure range from 0.01 Pa to 250 Pa. The test was done with a constant frequency of 1 Hz at 37 °C and the measuring system was covered with a humid chamber to minimize the evaporation of the water. Three different hydrogel discs are tested for each type of hydrogel with the same experimental parameters.

2.11. Mechanical testing

The mechanical measurements were performed using a universal testing machine (Lloyd-LRX, Lloyd Instruments, Fareham, UK). The samples were prepared in a cylindrical shape (2 cm diameter and 2 mm height). Compression tests were performed at a crosshead speed of 1 mm/min until fracture occurred. Prior to all measurements, the zero-gap was determined. Five samples of each condition were tested. The compressive modulus was determined as the slope of the linear region corresponding to the elastic part (10–20% strain) of the stress-strain curve.

2.12. Cell culture

C2C12 cells were cultured in DMEM containing 4.5 g/L of glucose, supplemented with 10% FBS, 2 mM of L-Glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Cells were maintained at 37 °C, 100% humidity, and 5% CO₂, and were passaged every 2 days.

2.13. Encapsulation of cells in GelMA

Cells were counted and resuspended as 4×10^6 cells/mL. 25 μ L of cell suspension (100 000 cells) were combined with 25 μ L of 20% GelMA and 1% PI so that the final concentration of GelMA would be 10% and 0.5% PI. Hydrogels were crosslinked with UV light (Omniculture S2000, Excelitas Technologies, Salem, MA, USA) (800 mW) for 40 seconds on one side and directly placed in cell culture medium in a well plate. Hydrogels with encapsulated cells were maintained at 37 °C, 100% humidity, and 5% CO₂.

2.14. Viability of cells in GelMA and proliferation assay

The viability of the encapsulated cells was assessed using a standard Live/Dead staining kit and using the metabolic assay PrestoBlue on days 1 and 3. Calcein AM and Ethidium Homodimer-1 comprised the Live/Dead staining kit and were used according to the manufacturer's protocol with an incubation time of 30 minutes. Afterward, fluorescent images were taken using a fluorescence microscope (Axio Observer D1, Carl Zeiss, Weimar, Germany), and ImageJ (v1.52p, NIH) software was used for image analysis. PrestoBlue proliferation assay was performed according to the manufacturer's instructions with an incubation time of 2.5 hours. Cell growth was evaluated using spectrophotometry with absorption measurements at 570 nm and 600 nm.

3. Results and discussion

3.1. Structural properties

Spectrophotometric techniques were first used to characterize the chemical structure of the engineered prepolymers. In addition to the functional group and conformational analyses of chemical reactions, FTIR and ¹H-NMR are widely used for chemical identification. FTIR was used to characterize the presence of specific chemical groups in the GelMA. The results show the spectrum of porcine and fish skin GelMA (**Figure 1B**). Gelatin's characteristic peaks were

clearly noticed through the presence of peptide bonds (N–H stretching), indicated by a peak at 3200 cm^{-1} , and through the presence of amide I, II, and III, indicated by peaks at 1650 , 1550 , and 1400 cm^{-1} respectively [32,33]. The peaks that can be assigned to the methacrylic anhydride (MA) were indicating saturated and unsaturated C-H stretching (2850 , 2950 , 3150 , and 3800 cm^{-1}), and H-C-H wagging (100 cm^{-1}). The spectra broad peak at 3300 cm^{-1} can be associated with the stretching of the hydrogen-bonded hydroxyl groups, derived from the modification of the gelatin with the MA [34].

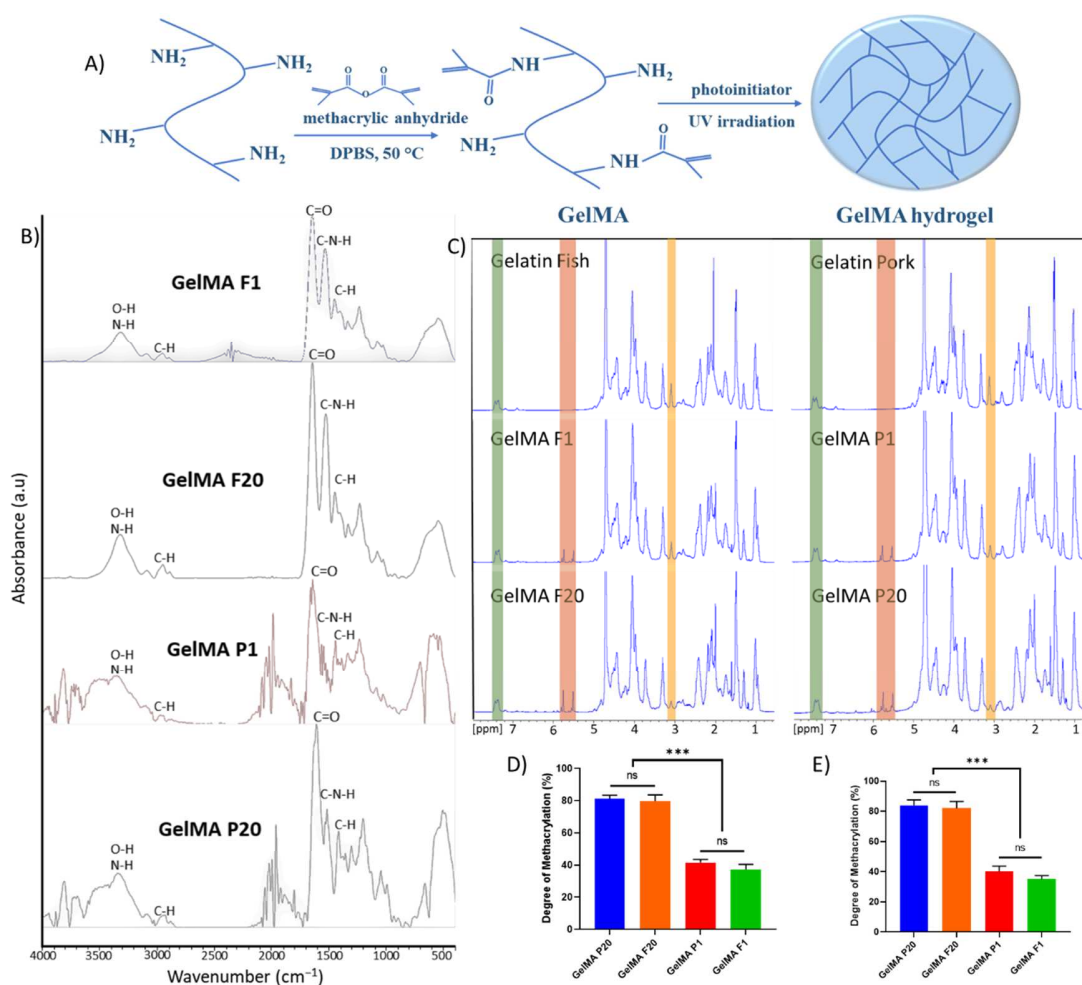


Figure 1. A) Schematic representation of the preparation and the crosslinking of GelMA. B) FTIR spectra of fish (F) and porcine (P) skin GelMA. C) $^1\text{H-NMR}$ verification of F and P skin GelMA. Peaks correspond to acrylic protons (2H) of aromatic amino acids (Green), methacrylamide grafts of lysine groups and hydroxyl lysine groups (Red), and methylene protons (2H) of unreacted lysine groups (Yellow). D) Degree of substitution (DS) of F and P skin GelMA as determined by $^1\text{H-NMR}$. E) DS of F and P skin GelMA as determined by TNBS assay. The reported data are the means of three replicates. Parametric data were analyzed using a

one-way ANOVA followed by Tukey's test. Significance was indicated as *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

Porcine and fish skin GelMA were synthesized using various concentrations of MA to create prepolymers with different degrees of methacrylation. Since reactive amine groups on the polypeptide backbone are the main target of MA [16], $^1\text{H-NMR}$ analysis and TNBS assay were used to quantify the DS of GelMA. Results presented in **Figure 1C-E** confirmed the substitution of free amine groups in GelMA. These results showed that the addition of a high MA volume percentage (20%) produced a high DS for both porcine GelMA (P20) ($81.3 \pm 2.2\%$ $^1\text{H-NMR}$; $84.3 \pm 3.6\%$ TNBS) and fish skin GelMA (F20) ($79.8 \pm 3.8\%$ $^1\text{H-NMR}$; $81.7 \pm 4.6\%$ TNBS). However, the addition of a lower volume percentage of MA (1.25%) produced a low DS for porcine skin GelMA (P1) ($41.6 \pm 1.9\%$ $^1\text{H-NMR}$; $40.4 \pm 3.6\%$ TNBS) and fish skin GelMA (F1) ($37.3 \pm 3.2\%$ $^1\text{H-NMR}$; $35.2 \pm 2.4\%$ TNBS). On the one hand, the methacrylation degree was not significantly different between porcine and fish skin GelMA from the same methacrylation level, but on the other hand, it was found to be significantly different between low and high methacrylated GelMA no matter their initial source. The results presented for the DS of porcine skin and fish skin GelMA were found to be in agreement with previously published results [16,22,23].

3.2. Thermal properties

To explore the stability of GelMA, TGA was conducted (**Figure 2A**). About 15% (wt/wt) weight loss was observed between 30 and 250 °C, where the highest loss occurred below 110 °C. This result was attributed to the loss of moisture, whereas a weight loss of about 65% (wt/wt) was observed between 250 and 600 °C which can be associated with protein degradation [35,36]. The remaining residue was around 20% at 600 °C for all samples.

The Td is the temperature at which the maximum reduction in mass occurs and it is calculated from the first-order derivative of TGA curves [36]. Porcine skin GelMA, P20 (334.8 ± 3.1 °C)

and P1 (336.1 ± 2.0 °C) had Td values close to that of fish skin GelMA, F20 (334.2 ± 4.2 °C) and F1 (336.6 ± 3.2 °C). The obtained Td values are similar to the previously reported Td value of gelatin [37], which suggests that the methacrylation of gelatin did not significantly affect its degradation temperature. The Td values of all GelMA samples were not significantly different (Figure 2a), which suggests that they possess similar thermal stability.

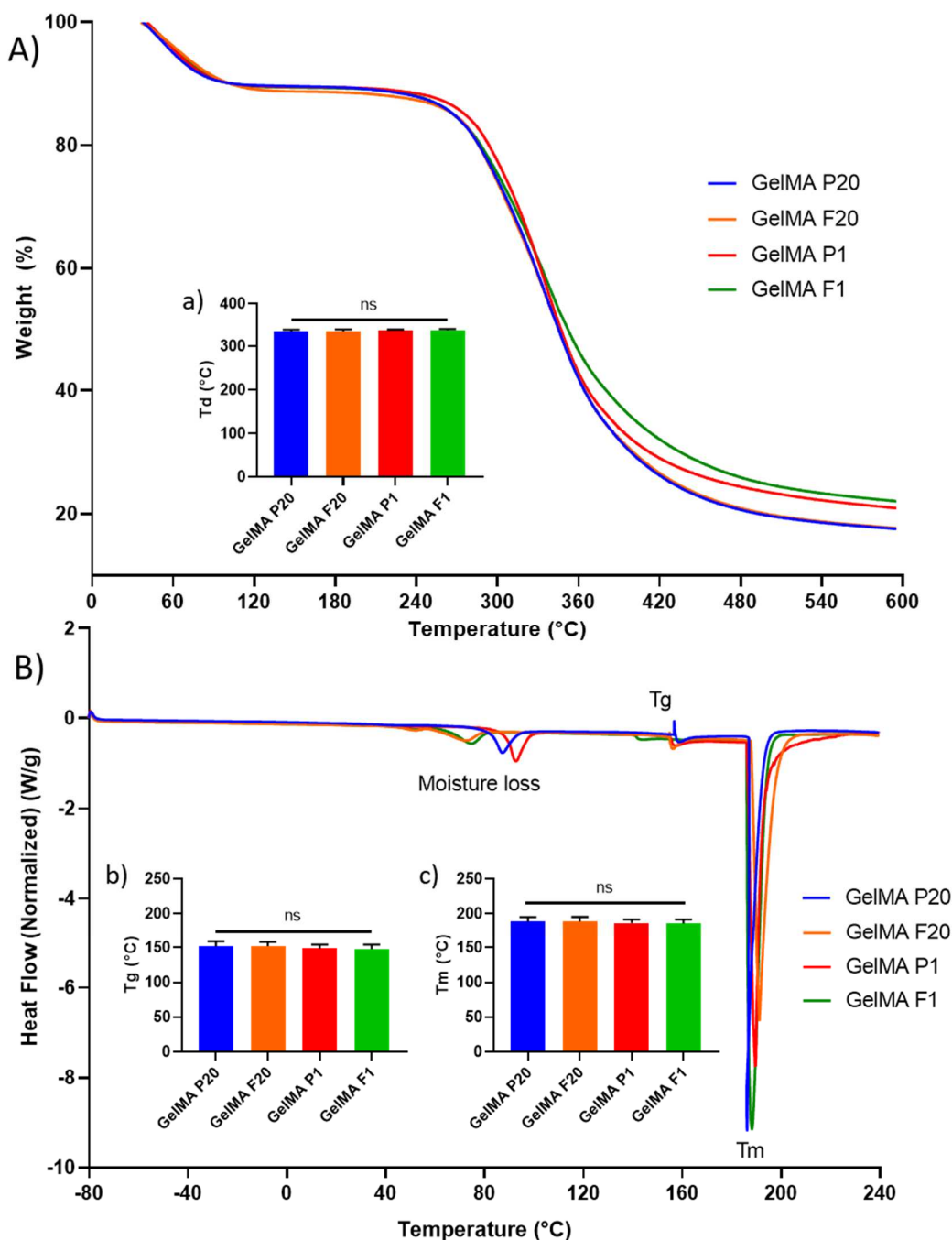


Figure 2. A) Thermal gravimetric analysis (TGA) curves of fish (F) and porcine (P) skin GelMA and their respective (a) degradation temperature T_d ($^{\circ}\text{C}$) values. B) Differential scanning calorimetric (DSC) curves showing the moisture loss, transition temperature (T_g), and melting temperature (T_m) peaks of F and P skin GelMA and their respective (b) T_g ($^{\circ}\text{C}$) and (c) T_m ($^{\circ}\text{C}$) values. The reported data are the means of five replicates. The reported data are the means of three replicates. Parametric data were analyzed using a one-way ANOVA followed by Tukey's test. Significance was indicated as $*(p < 0.05)$, $** (p < 0.01)$ and $*** (p < 0.001)$.

DSC thermograms of GelMA are presented in **Figure 2B**. Generally, three peaks were presented by all prepolymers that could be related to their transition due to moisture loss, transition temperature (T_g), and melting temperature (T_m) [38]. Since all samples were solid at room temperature, it can be assumed that they are in the glassy region. One small endothermic peak in the range of $55\text{--}85\text{ }^{\circ}\text{C}$ for fish skin GelMA and $70\text{--}100\text{ }^{\circ}\text{C}$ for porcine skin GelMA was observed, with the corresponding maximum at $72.7 \pm 1.7\text{ }^{\circ}\text{C}$ for F20, $74.9 \pm 2.1\text{ }^{\circ}\text{C}$ for F1, $86.7 \pm 1.8\text{ }^{\circ}\text{C}$ for P20, and $91.2 \pm 1.8\text{ }^{\circ}\text{C}$ for P1. This small endothermic peak is related to water evaporation together with helix-to-coil transition [39]. Both phenomena are interconnected since water molecules stabilize the helix structure and helix-coil transition is naturally driven by the loss of water.

By increasing the temperature, a thermodynamic change occurs above T_g , due to the increased thermal motion of the sample [40]. T_g values of fish skin GelMA, F1 ($148.1 \pm 6.3\text{ }^{\circ}\text{C}$) and F20 ($152.3 \pm 5.9\text{ }^{\circ}\text{C}$), were similar to that of porcine skin GelMA, P1 ($148.8 \pm 5.4\text{ }^{\circ}\text{C}$) and P20 ($152.0 \pm 7.1\text{ }^{\circ}\text{C}$) with the same methacrylation level. T_g is influenced by factors such as molecular weight and crosslinking. The longer the chain length, the greater the molecular weight, resulting in increased stiffness of the polymer structure. Since the methacrylate groups have a higher molecular weight than amino groups, T_g of GelMA with a high methacrylation degree is higher than that of GelMA with a low methacrylation degree. However, this increase is not significant (**Figure 2b**).

Beyond T_g , the polymer chains move freely, and the material behaved like rubber until it eventually reaches its T_m [40]. T_m is considered a first-order transition and correlated to the melting of crystallites of partially degraded collagen helix into gelatin. Upon comparison of GelMA from the same source, it was shown that the lower DS, P1 (186.9 ± 5.0 °C) and F1 (186.1 ± 5.7 °C), had a lower T_m than those with a high DS, P20 (188.6 ± 6.5 °C) and F20 (188.0 ± 5.0 °C). It can be seen that for GelMA with the same DS, T_m was comparable. However, as for T_g values, T_m values are also not significantly different for all GelMA samples. DSC results suggest that the thermal properties of GelMA samples are analogous regardless of their source or methacrylation level.

3.3. Physical properties

From both scientific and commercial points of view, parameters related to the polymer water vapor sorption are crucial, as they play a big role in the modification of its properties, and thereby its range of applications [41–43]. The hydration behavior of proteins, such as collagen or gelatin, directly affects their chemical and enzymatic reactivities, as well as the formation of their secondary structure [41]. Moreover, the GelMA sorption behavior is an important parameter to study for future incorporation of nanoparticles or bioactive molecules within its matrix [41]. The influence of the methacrylation degree on the water vapor uptake of GelMA was studied at 25 °C. The linearly increasing prepolymer masses during sorption as a function of the relative humidity are presented in **Table 1** and **Figure 3A**. The water vapor uptake was higher for GelMA with low methacrylation degrees compared to high methacrylated GelMA. This can be attributed to the higher porosity of low methacrylated GelMA [16].

Table 1. Effect of methacrylation degree on the water vapor sorption of porcine (P) and fish (F) skin GelMA.

Target RH (%)	Change in Mass (%) - dry			
	GelMA P20	GelMA F20	GelMA P1	GelMA F1

0.0	0.00	0.00	0.00	0.00
7.8	2.80	2.26	1.85	3.27
15.6	4.43	3.74	3.61	4.96
23.3	5.92	5.15	5.25	6.51
31.1	7.29	6.53	6.77	7.91
38.9	8.73	8.06	8.30	9.37
46.7	10.25	9.61	9.96	10.90
54.4	11.90	11.28	11.84	12.50
62.2	13.68	13.05	14.07	14.41
70.0	15.97	15.06	16.64	16.54

The swelling behavior of hydrogels is an essential parameter to study after crosslinking, as it affects the hydrogel's mechanical properties and solute diffusion [16]. This behavior depends on the hydrogels' structural properties, hydrophilicity, crosslinking density, and interaction with the solvent [22,44]. To study this swelling behavior, porcine and fish skin GelMA hydrogels with different methacrylation degrees were fully swelled in DPBS at 37°C for 24 h to obtain their swollen weight and then lyophilized for three days to obtain their dry weight. Using the swollen and dry weight of GelMA, the mass swelling ratio was calculated. The low methacrylated GelMA had a higher mass swelling ratio than the high methacrylated GelMA (**Figure 3B**). Fish skin GelMA had a significantly higher mass swelling ratio than porcine skin GelMA for both methacrylation degrees. This might be due to the presence of fewer hydrophobic amino acids in fish skin than in porcine skin GelMA, which makes fish skin more hydrophilic than porcine skin GelMA [45,46]. These results are in full agreement with previously reported results [16,22,23].

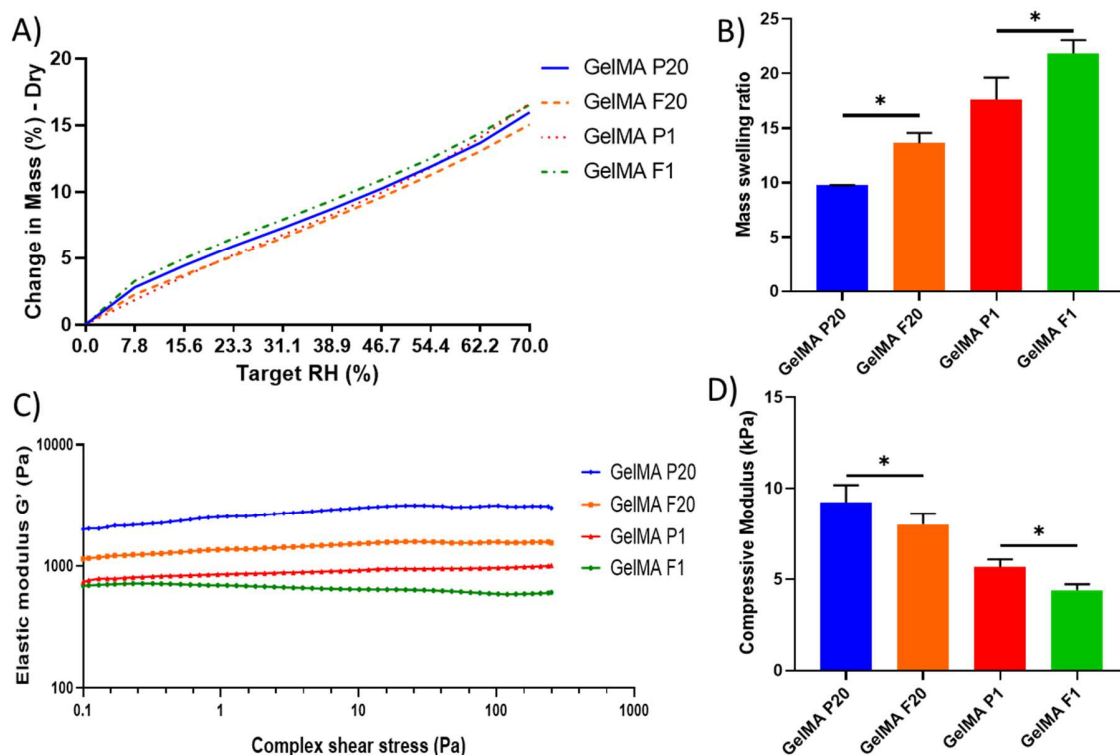


Figure 3. A) The isotherm curves for water sorption in fish (F) and porcine (P) skin GelMA. B) The mass swelling ratios of F and P skin GelMA hydrogels. C) Amplitude sweep data showing elastic modulus (G') as a function of complex shear stress of F and P skin GelMA. D) The compressive modulus of F and P skin GelMA hydrogels. The reported data are the means of five replicates. Parametric data were analyzed using a one-way ANOVA followed by Tukey's test. Significance was indicated as *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

Rheological measurements were carried out to assess the elastic properties of GelMA hydrogels. Results, presented in **Figure 3C**, show that the elastic modulus G' , also known as real modulus or storage modulus, of fish porcine GelMA was lower than the G' of porcine skin GelMA within the same level of methacrylation. Additionally, G' is higher for the high methacrylated GelMA of the same source. This difference in elasticity can be explained by the higher amount of proline and hydroxyproline present in porcine skin GelMA [47]. These two amino acids stabilize the ordered conformation during gel formation. On the other hand, increasing the level of methacrylation increased the elasticity of GelMA. Van Den Bulcke *et al.* suggested that high methacrylated GelMA are composed of more chemical bonds, and thus have a denser network with higher elastic modulus and network properties than low methacrylated GelMA [15].

It has been reported that cell behavior, function, proliferation, and differentiation are significantly affected by the mechanical properties of hydrogels [48–51]. Unconfined compression tests on swollen hydrogels were used to study the effect of the source and the methacrylation level of GelMA hydrogels on its mechanical properties. As shown in **Figure 3D**, the compressive Young's modulus was significantly different between porcine skin GelMA (P20 = 9.23 ± 0.95 kPa and P1 = 5.66 ± 0.43 kPa) and fish skin GelMA (F20 = 8.01 ± 0.6 kPa and F1 = 4.40 ± 0.31 kPa), and between high methacrylated and low methacrylated GelMA as well. Yoon *et al.* previously reported a significant difference between high methacrylated fish and porcine skin GelMA and between fish skin GelMA with different methacrylation degrees [22]. Wang *et al.* reported it between high methacrylated porcine skin and low methacrylated fish skin GelMA [23]. Nichol *et al.* reported this significance between low and high methacrylated porcine skin GelMA [16].

Lysine is a major methacrylation site and is present in good amounts in gelatin from both sources [25,45,52]. Since more polymeric chains are crosslinked in high methacrylated GelMA, their mechanical properties will be stronger than low methacrylated GelMA. Porcine gelatin contains higher amounts of hydrophobic amino acids (methionine, phenylalanine, proline, isoleucine, leucine, valine, and alanine) than fish gelatin, which can form stronger hydrophobic interactions in porcine skin GelMA [23]. Moreover, fish gelatin also contains fewer amounts of imino acids (proline and hydroxyproline) than porcine gelatin, which are responsible for the higher structural stability of porcine skin GelMA [22]. Therefore, both the source of gelatin and the level of methacrylation affect the compressive Young's modulus of GelMA. However, the level of methacrylation of GelMA has a higher effect on the compressive Young's modulus of GelMA.

3.4. Biological properties

In order to assess the suitability of the fish and porcine skin GelMA hydrogel employment for tissue engineering applications, the cell viability of C2C12 cells was evaluated upon 3D

encapsulation. Biocompatibility was assessed using live/dead staining, displayed in **Figure 4A**, and quantified in **Figure 4B**, as well as using a PrestoBlue metabolic assay, shown in **Figure 4C**. The viability of C2C12 cells was not significantly different in the porcine and fish-derived GelMA hydrogels, as they exhibited around 90% viability on day 1 and 80% viability on day 3. Similar viability results were obtained using NIH 3T3 fibroblasts in 3D porcine and fish skin GelMA [22,23] and in fish skin GelMA [53]. The cell viability in all hydrogels was high, indicating a great potential for tissue engineering applications. Viability can be improved by optimizing the UV exposure time for each cell application, as the UV dosage and the number of free radicals formed during photopolymerization affect the cell viability [54]. The non-significant difference in cell viability as a result of methacrylation degree was also found by Zhu *et al.* [55], where human hepatocellular carcinoma cells were encapsulated in porcine GelMA with different degrees of methacrylation.

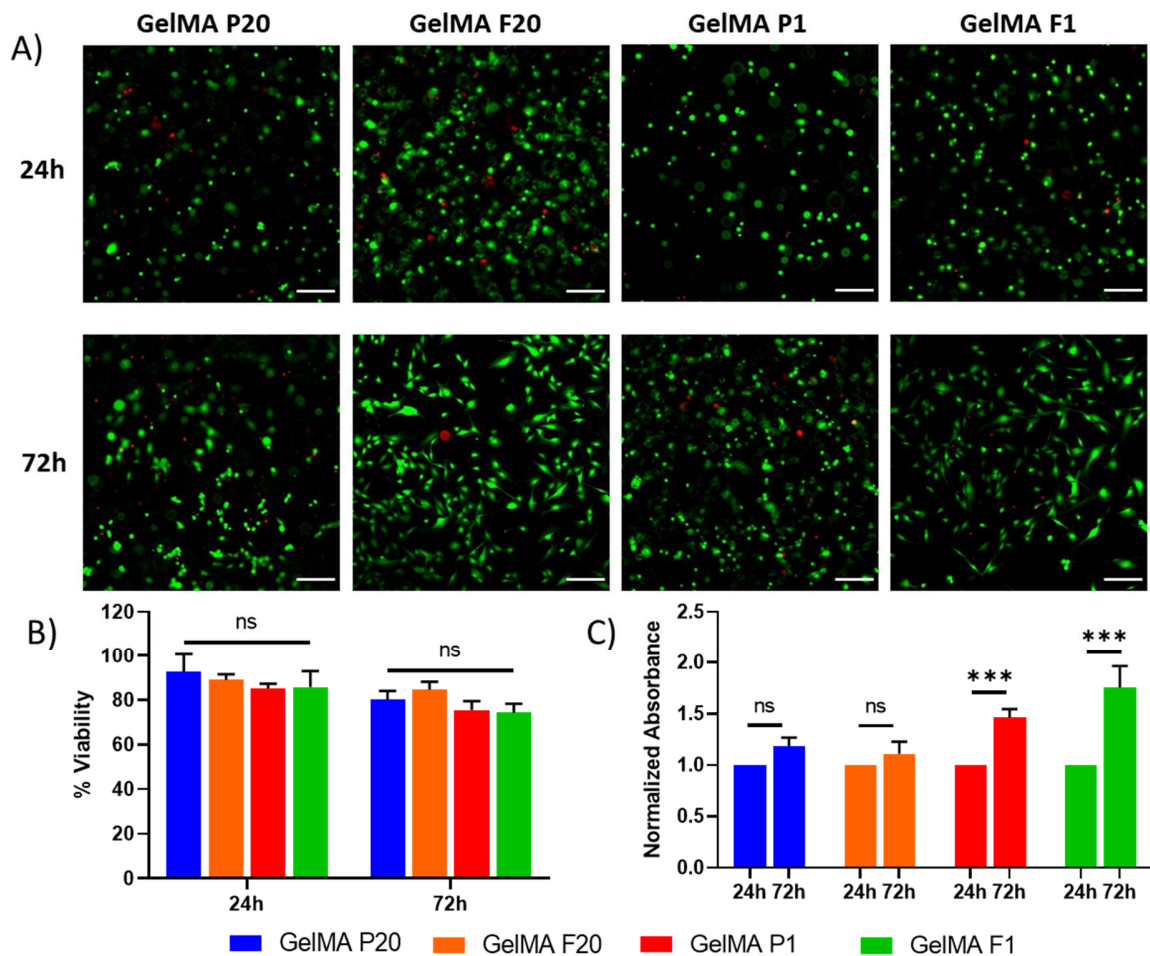


Figure 4. Viability of C2C12 cells in F and P skin GelMA hydrogels. A) Live/dead images of 3D cultured C2C12 cells, where green denotes live cells and red denotes dead cells. Scale bar = 200 μm . B) Quantification of live/dead to % viability on day 1 and day 3. C) Normalized absorbance of PrestoBlue assay on day 1 and day 3 showing the proliferation of C2C12 cells. The reported data are the means of three replicates. Parametric data were analyzed using a two-way ANOVA followed by Holm-Sidak's test. Significance was indicated as *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

Moreover, the cell morphology showed a good spreading of C2C12 cells in the fish-derived GelMA, which was previously observed for NIH 3T3 fibroblasts [22]. The spreading of C2C12 cells in porcine GelMA was observed by Costantini *et al.* [27], as there was more cell spreading for lower stiffness GelMA with an increased swelling ratio. This is likely a result of different cell signaling pathways upon mechanotransducing cues [27,54]. Zaupa *et al.* also showed an increased capacity of cell remodeling in fish skin GelMA over mammalian GelMA (bovine),

despite the similar Young's moduli of the differently derived GelMA [56]. The larger swelling ratio allows for cell-cell contact, which increases proliferation and cell spreading.

Pepelanova *et al.* showed that the DS did not significantly impact the cell spreading of mesenchymal stem cells, however, the higher degree had less cell spreading than the lower degree, corroborating these results [54]. The influence of the level of the methacrylation does not seem to affect the cell viability and cell spreading, however, the proliferation rate is enhanced upon a lower level of methacrylation. This can be a result of the increased swelling ratio, allowing more nutrients to reach the cells as a result of the larger pore sizes [16,57]. Moreover, cell proliferation is improved as it is not hindered by the polymer network, allowing for direct cell-cell contact and signaling [54].

In conclusion, the biocompatibility of porcine GelMA is already largely established, and these results show the feasibility of using fish skin GelMA for tissue engineering applications. Further work is required to indicate how the pore size and material stiffness work together to optimize the cell microenvironment specific to an application. Additionally, as other researchers have shown, fish skin GelMA is more easily degraded by human metalloproteinases and collagenases which could lead to better incorporation and application *in vivo* [56].

4. Conclusion

Throughout this study, the potential of replacing the widely used porcine skin GelMA with fish-derived GelMA was explored. The physicochemical and biological properties of porcine and fish skin GelMA produced with low and high methacrylation degrees were investigated. The DS was found to be independent from the GelMA source, as it was similar between polymers with the same methacrylation degree and significantly different between low and high methacrylated GelMA. The methacrylation level and the source of GelMA did not affect the thermal and water uptake properties. However, fish skin GelMA hydrogels had a significantly higher mass swelling ratio and a lower elastic and compressive Young's moduli than porcine skin GelMA. On the

other hand, with the increase in methacrylation degree, the mass swelling ratio decreased, and the elastic and compressive Young's moduli increased. Even though cell proliferation was only induced in low methacrylated GelMA and a better spreading was only observed in fish skin GelMA, all GelMA hydrogels showed great biocompatibility towards the encapsulated C2C12 myoblasts. Notably, low methacrylated fish skin GelMA showed the best proliferation and spreading of the laden C2C12 myoblasts, which encourage future investigations as a potential scaffold for skeletal muscle tissue engineering. All in all, for clinical, environmental, religious, and economical reasons, GelMA derived from marine sources might be a promising substitute to mammalian sourced GelMA for biomedical applications. Future studies will focus first on the *in vitro* differentiation of laden C2C12 myoblasts into myotubes and then on the biofabrication, using a handheld bioprinter, and the nanofunctionalization, with plant- and marine-derived nanoliposomes, of fish skin GelMA for the *in vivo* treatment of skeletal muscle injuries.

Author contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of interest

The authors declare that they have no known competing for financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

¹H Proton Nuclear Magnetic Resonance
NMR

ANO VA	Analysis of Variance
DME M	Dulbecco's Minimum Eagle Media
DPBS	Dulbecco's Phosphate Buffered Saline
DS	Degree of Substitution
DSC	Differential Scanning Calorimetry
ECM	Extracellular Matrix
F1	Fish skin gelatin with addition of 1.25% methacrylic anhydride
F20	Fish skin gelatin with addition of 20% methacrylic anhydride
FBS	Fetal Bovine Serum
FTIR	Fourier Transform infrared spectroscopy
GelM A	Gelatin Methacryloyl
MA	Methacrylic Anhydride
MMP	Matrix Metalloproteinase
P1	Porcine skin gelatin with addition of 1.25% methacrylic anhydride
P20	Porcine skin gelatin with addition of 20% methacrylic anhydride
PI	Photoinitiator (2-hydroxy-4'-(2-hydroxy ethoxy)-2-methyl propiophenone)
RGD	Arginine-glycine-aspartic acid
RH	Relative humidity
Td	Degradation Temperature
Tg	Transition Temperature
TGA	Thermal Gravimetric Analysis
Tm	Melting Temperature
TNBS	2,4,6-trinitrobenzene-sulfonic acid

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