VA-086 Methacrylate Gelatine Photopolymerizable Hydrogels: a Parametric Study for Highly Biocompatible 3D Cell Embedding

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Abstract

The ability to replicate *in vitro* the native extracellular matrix (ECM) features and to control the three-dimensional (3D) cell organization plays a fundamental role in obtaining functional engineered bio-constructs. In tissue engineering (TE) applications, hydrogels have been successfully implied as biomatrices for 3D cell embedding, exhibiting high similarities to the natural ECM and holding easily tunable mechanical properties. In the present study, we characterized a promising photo-crosslinking process to generate cell-laden methacrylate gelatin (GelMA) hydrogels in the presence of VA-086 photoinitiator using a ultraviolet LED source. We investigated the influence of pre-polymer concentration and light irradiance on mechanical and biomimetic properties of resulting hydrogels. In details, the increasing of gelatin concentration resulted in enhanced rheological properties and shorter polymerization time. We then defined and validated a reliable photopolymerization protocol for cell embedding (1.5% VA-086, LED 2mW/cm^2) within GelMA hydrogels, which demonstrated to support bone marrow stromal cells viability when cultured up to seven days. Moreover, we showed how different mechanical properties, derived from different crosslinking parameters, strongly influence cell behaviour. In conclusion, this protocol can be considered a versatile tool to obtain biocompatible cell-laden hydrogels with properties easily adaptable for different TE applications.

Keywords: GelMA, Cell-laden hydrogels Photopolymerization, 3D Cell Culture, Biomaterials, Tissue Engineering

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[1] **Abbreviations**
TE – Tissue engineering
ECM – Extracellular matrix
GelMA – Methacrylate gelatine
PI – Photoinitiator
DPBS - Dulbecco's phosphate buffer saline
MA - Methacrylic anhydride
VA086 - 2,2-Azobis (2-methyl-*N*-(2-hydroxyethyl)propionamide
BMSCs - Bone marrow stromal cells

1. Introduction

In the field of tissue engineering (TE), the ability of replicating *in vitro* the complexity of the native microenvironment is crucial to obtain functional bio-constructs. In the last two decades, 2D standard culture methods have been recognized to be poorly representative of such *in vivo* complexity, e.g. exposing cells to non-physiological substrates in terms of mechanical stiffness [1]. Recently, a shift in paradigm has thus been observed towards the development of 3D cell culture models, attempting at better mimicking the native extracellular matrix (ECM). Among other materials, hydrogels exhibit high similarities to the natural ECM, which is characterized by an intricate network of proteins and polysaccharides [2]. First, their high water content contributes at creating highly swollen and interconnected network structures, which allow for maximizing nutrient and gas transfers [3]. Moreover, their mechanical properties, strongly dependent on their macromolecular structure as well as on the method and degree of crosslinking [4,5], can be easily tuned to the biomechanical characteristics of different soft tissues according to the specific application [6]. Furthermore, the presence of functional groups allows to chemically modify the hydrogel structures and to add specific biofunctions attempting to resemble the variety of cues found in the native ECM [7]. Hydrogels are thus highly versatile materials in many TE applications.

A variety of synthetic [5,8-10] and natural [11-14] macromolecules have been used so far to fabricate hydrogel-based engineered environments [3], either as substrates for cellular seeding [13] or as cell-laden 3D matrices [2]. In particular, the fabrication of cell-laden hydrogels is tightly correlated to the biocompatibility of the crosslinking approach. Most chemical methods are not compatible with 3D encapsulation of viable cells, usually relying on cytotoxic chemicals or

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HUVECs GFP - human umbilical vein endothelial cells expressing green fluorescent protein

enzymatic agents to trigger the polymerization [11]. Thermal reticulation has been widely investigated as a biocompatible process for creating cell-laden hydrogels [15-17]; however, the presence of living cells poses an upper temperature threshold to 37°C, generally resulting in relatively long crosslinking times. For this reason, additional rotating systems may be required to thermally crosslink cell-laden hydrogels in order to prevent cell tendency to settle down and thus achieve a uniform 3D cell distribution within the matrix [18]. As an alternative, photopolymerization has been successfully investigated in several studies to obtain 3D cellular matrices within tens of seconds, by exposing a precursor polymer solution to a light source in the presence of a photoinitiator (PI)[19,20]. Although promising, the suitability of the photopolymerization process for embedding viable cells relies on the definition of optimal and biocompatible combinations of polymeric precursors, light source and PI. Among others, methacrylate gelatin (GelMA) has been widely investigated in the last few years in combinations with different PI molecules and lights sources [21]. GelMA is indeed a cheap and easy to handle derivative of collagen, which presents both natural cell binding motifs, such as RGD and MMP-sensitive degradation sites, and different amino acid side-chain functionalities (carboxylic acid, amines, hydroxyl) which allow for further covalent modifications [11]. Irgacure 2959 is widely considered the golden standard for GelMA hydrogel cross-linking [11,21-23]; however, with an adsorption peak around 320nm and a not negligible toxicity to cells, its use had to be accurately optimized for its limited biocompatibility properties [24]. Recently, the use of a lower cytotoxic PI featuring a more conservative activation UV band (peak at 375nm) - namely VA-086 - was demonstrated for alginate-based hydrogels photopolymerization . Subsequently, we showed the possibility to successfully generate cell-laden GelMA hydrogels in the presence of VA-086 through an inexpensive ultraviolet LED source (emitting peak at 385nm) [25]. Considering the increasing interest around VA086-GelMA hydrogels for TE

applications [26], a characterization of this promising photopolymerization process is thus required.

In the present study, we systematically investigated different parameters to assess how the choice of degree of functionalization (methacrylic anhydride), pre-polymer concentration (GelMA) , and light irradiance influences VA086-GelMA hydrogels crosslinking, mechanical and biological properties. The final aim is thus to define a repeatable and reliable protocol for obtaining cell-laden VA086-GelMA hydrogels exhibiting controlled and versatile properties, easily tunable depending on the required application.

2. Materials and methods

2.1. Gelatin methacrylate synthesis and ^{1}H NMR characterization

Methacrylated gelatin (GelMA) was synthesized following a previously reported protocol [21] (Fig.1a). Briefly, type A porcine skin gelatin (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) was dissolved at 10% (w/v) in Dulbecco's phosphate buffer saline (DPBS, GIBCO) solution (pH=7.5) at 50°C. Methacrylic anhydride (acrylate agent, MA, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) was then added to the gelatin solution at a rate of 0.1 ml/min until reaching the desired volume and allowed to react while vigorously stirring. After three hours, the reaction was quenched by adding pre-heated DPBS and the obtained solution was dialyzed for ten days against distilled water at 50°C throughout 12-14KDa cutoff dialysis tubing (Sigma-Aldrich Corporation, St. Louis, Missouri, USA). The solution was finally filtered, freeze-dried for 66 hours and stored at -80°C until further use. In the present study, two reactions were established by adding different amount of MA. In details, two batches were obtained by adding either 10% or 15% (v/v) MA to the gelatin solution, yielding low MA and high MA GelMA, respectively.

The efficacy of the methacrylation reaction was determined by ^{1}H-NMR analyzing the signals coming from the methacrylic moiety. In details, 30 mg of low MA, high MA and plain gelatin were dissolved in 0.6 mL of D_2O; the sealed NMR tubes were heated to 70°C till complete dissolution. The spectra were recorded at 400 MHz on a Varian Mercury instrument.

2.2. Hydrogel photopolymerization

GelMA hydrogels were obtained by radical cross-linking of methacrylamide modified gelatin in the presence of a photoinitiator (PI) activated by a 1.8 W LED (385 nm; LZ4-00UA00, LED Engine, Inc.) light source (Fig. 1b). In details, the PI molecule 2,2-Azobis (2-methyl-*N*-(2-hydroxyethyl)propionamide) (VA-086, Wako Chemicals GmbH, Germany) was dissolved in DPBS at room temperature at 1.5% (w/v). Subsequently, GelMA was added to the PI solution and mixed at room temperature until complete dissolution. Through a standard micropipette, 20µl of this prepolymer solution were placed inside a Petri dish into a silicone cylindrical well (diameter= 6mm and height= 0.5mm) obtained by means of a biopsy puncher. The sample was thus irradiated using the LED source until complete polymerization, and the required time was recorded. A sample was considered fully polymerized whether it satisfied the following criteria: (i) it maintained a 3D shape without releasing macroscopic debris upon silicone template removal and (ii) it did not dissolved when immersed in PBS and incubated at 37°C up to 1 hours. The described photopolymerization protocol was fully characterized for both the previously synthesized materials (lowMA and highMA), by assessing the influence of different parameters on the cross-linking time. In particular, for each methacrylation degree, the considered parameters were (i) the irradiance of the light source (1.5-14 mW/cm^2), and (ii) the GelMA concentration (5%, 7.5% and 10% w/v) in the pre-polymer solution (see Table 1). Furthermore, the effect of PI concentration (0.5%, 1%, 1.5% w/v) was evaluated on a GelMA concentration of 10% (w/v), for both methacrylation degree. Each condition was tested in triplicates and the polymerization times were recorded.

2.3. Rheological characterization

Rheological measurements were carried out with an AR 1500ex rheometer (TA Instruments, USA) using a cone-plate geometry (diameter= 2cm, truncation= 32um, working gap= 32µm). Dynamic shear oscillation tests were performed to evaluate viscoelastic properties of both low and high MA GelMA at 5%, 7.5% and 10% w/v in the prepolymer solution. Mechanical spectra were recorded at both 25°C and 37°C in a constant strain mode, with a deformation of 0.3% and incremental frequencies ranging from 0.01 to 10 Hz. Samples were obtained photo-polymerizing 500 µl of GelMA pre-polymer (1.5% of VA-086) inside a 24-wells plate. The data represent the average of the storage (G') and loss (G'') modulus with standard deviation.

2.4. Cell sorting and expansion

Bone marrow stromal cells (BMSC) were isolated from bone marrow aspirates obtained from donors undergoing total hip replacement, after written consent. Bone marrow was centrifuged and cells were plated at a density of 1 x 10^5 cells/cm^2 and cultured overnight. Suspended cells were then removed and adherent cells were expanded. BMSC culture was carried out in complete medium consisting of α-modified Eagle's medium, 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 292 µg ml^{-1} L-glutamine (all GIBCO®) supplemented with 5 ng ml^{-1} fibroblast growth factor-2 (Peprotech). When 70-80% confluence was reached, cells were harvested and frozen. At need, cells were then thawed, seeded at lower density (3 x 10^3 cells cm^{-2}) and expanded again. Medium refresh was performed every four days.

Primary human umbilical vein endothelial cells constitutively expressing green fluorescent protein (HUVEC GFP; Children's Hospital, Boston, MA) were expanded in endothelial cells growth medium EGM^{-2} supplemented with the bullet kit which includes: 2% FBS, ascorbic acid, heparin, hydrocortisone and human growth factors (fibroblast, R3-insulin) including

vascular endothelial growth factor (VEGF) (all LONZA®). Cells were passaged when 70-80% of confluence was reached and the medium exchange was performed every four days.

A standard cell culture incubator, maintaining 5% of CO_2 level and 37°C, was employed for cell culture.

2.5. Cell embedding and 3D culture

Biological validations were carried out to assess the influence of methacrylation degree and concentration of GelMA on 3D cellular behaviour. BMSCs were thus embedded within both low and high MA GelMA hydrogels at different concentrations (5%, 7.5% and 10% w/v), and cultured up to seven days. In details, the methacrylated gelatin was completely dissolved in a filtered 1.5% (w/v) PI solution, subsequently used to resuspend the previously trypsinized BMSCs at a final concentration of 2 x 10^6 cells/ml. The photopolymerization reaction was thus carried out as previously described maintaining sterile conditions, by irradiating the sample for 5 minutes with a LED irradiance of 2 mW/cm^2. Samples containing the same cell numbers were produced and frozen to be subsequently used as DNA references. The obtained cell-laden samples were incubated under standard culture conditions and cultured up to seven days, while refreshing media every 3 days. Cell metabolic activity and proliferation inside 3D hydrogels were then evaluated after 3 and 7 days in culture. In details, at the defined time points, each sample was washed in DPBS, placed in a new well, and incubated at 37°C in 10% v/v AlamarBlue solution (Invitrogen Corporation, Isbad, CA, USA). After 4 hours, the absorbance of the resulting solution at 570 nm was measured using a spectrophotometer (Victor X3, PerkinElmer, Waltham, MA, USA). The DNA in each sample was then evaluated by means of the CyQUANT cell proliferation assay (Invitrogen Corporation, Isbad, CA, USA). Samples were frozen at -80°C overnight and digested for 16 hours at 60°C with 250 µL of ProteinaseK (Sigma-Aldrich Corporation, St. Louis, Missouri, USA). After digestion, 5 µl of the sample and 195 µl of the working solution, previously prepared according to the manufacturer's

protocols, were placed into a 96 black multiwall plate. Sample fluorescence intensity was detected with a spectrophotometer at 485/538 nm and subsequently related to cell DNA content through a calibration curve. The reference samples were used to normalize DNA content in each sample. Finally, for each sample the metabolic activity value was normalized to the DNA content to assess the specific cellular metabolic activity.

2.6. BMSC-HUVEC 3D co-culture

To evaluate cell elongation and interaction within gels with different mechanical properties, co-cultures of BMSCs and HUVECs GFP were established with high MA for all GelMA concentrations (5%, 7.5% and 10% w/v). BMSCs were made fluorescent through a 10 minutes incubation in culture medium enriched with 5µl/ml of Vibrant (Invitrogen Corporation, Isbad, CA, USA). Cells were resuspended at a final concentration of 3 x 10^6 cells/ml in the pre-polymer solution, maintaining a BMSC/HUVEC ratio of 1:1. The polymerization was performed as previously described and samples with either only BMSCs or HUVECs were established as controls. All the cell-laden samples were cultured in HUVEC culture medium and maintained in standard culture conditions for up to seven days, while medium change was performed every 3 days. Fluorescence images at different time points were collected.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation, and statistical analyses (one-way ANOVA) were performed using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA).

3. Results

3.1. Methacrylation assessment

As previously described for MA-gelatin[11], the presence of the methacrylic moiety before cross-linking was assessed by ^{1}H-NMR, since the resonances from the methacrylamide methylene protons occur in a region free from other signals due to the protein (5.2-5.7 ppm). The degree

of methacrylation was quantified for both materials using aliphatic signals as references as reported in Van Vlierberghe[27], resulting equal to 6% and 11% for low and high MA GelMA, respectively.

3.2. GelMA Photopolymerization

To establish an optimal polymerization protocol for both previously synthesized materials (low and high MA), GelMA cross-linking time was assessed as a function of the LED irradiance at 385nm, varying different pre-polymer parameters. First, the influence of the gelatin concentration was evaluated, maintaining VA-086 concentration at 1.5% (w/v). Figure 2a shows a comparison between polymerization times of samples obtained for GelMA concentrations of 5, 7.5 and 10% (w/v) for different irradiance conditions. Time values higher than 360s were considered too long for cell embedding procedure and, coherently, not reported. As expected, the polymerization time decreased increasing the irradiance for all the tested conditions. Maintaining constant the GelMA concentration, high MA GelMA resulted in shorter cross-linking time as compared to low MA GelMA. In details, for the 7.5% (w/v) GelMA, the polymerization time ranged from 67 sec for the high MA to 97 sec for the low MA, considering an irradiance of 14 mW/cm^2. Furthermore, increasing the GelMA concentration the polymerization time decreased for both high and low MA GelMA. In particular, for low MA pre-polymers, an increase of GelMA concentration from 7.5% to 10% (w/v) led to a reduction in cross-linking times from 250sec to 160sec and from 100sec to 50sec, when samples were irradiated with 1.5mW/cm^2 and with 14mW/cm^2, respectively. Cross-linking data of low MA GelMA at 5% (w/v) were discarded, being longer than 360sec. Likewise, for high MA GelMA, polymerization times decreased from 320sec to 140sec at 1.5mW/cm^2 and from 140sec to 35sec at 14mW/cm^2, when GelMA concentration was increased from 5% to 10% (w/v).

To assess the influence of PI concentration on polymerization times, both high and low MA gelatins were tested, fixing GelMA concentration at 10% (w/v) and varying VA-086 concentration from 0.5% to 1.5% (w/v). For both types of GelMA, increasing the VA-086 concentration resulted in a drop of the cross-linking time among all irradiance intensities (Fig. 2b). In details, the increase of VA-086 content in low MA pre-polymer (from 0.5% to 1.5%, w/v) caused the cross-linking time to drop from 300sec to 160sec and from 75sec to 50sec for irradiances of 1.5mW/cm^2 and 14mW/cm^2, respectively. Analogous behavior was found for high MA GelMA, where reaction times dropped from 250sec to 140sec and from 45sec or 30sec at 1.5mW/cm^2 and 14mW/cm^2, respectively. Once again, considering samples obtained with the same PI concentration and polymerized with the same irradiance, higher MA GelMA samples were characterized by shorter cross-linking times.

3.3. Mechanical properties

Dynamic shear oscillation tests were performed on samples with low and high methacrylation degree at GelMA concentration of 5%, 7.5% and 10% (w/v), in order to assess the effect of GelMA functionalization and concentration on hydrogel mechanical properties. The elastic or storage modulus (G') and the viscous or loss modulus (G'') were measured at 37°C for radial frequencies ranging from 0.1-10 Hz to provide quantitative information on viscoelastic properties of samples at standard culture conditions. In general, storage moduli resulted higher than loss moduli for both low and high GelMA methacrylation at all concentrations tested, as shown in figure 3. Increasing GelMA concentration from 5% to 10% (w/v), while maintaining a constant methacrylation degree, resulted in an increase of both G' and G'', suggesting an enhancement of gel stiffness. Considering the storage modulus, GelMA samples at equal concentration showed comparable G' values, which was confirmed for both gelatin functionalization degrees (see supplementary data, Figure SI1). Moreover, temperature was found not to significantly affect hydrogel mechanical properties, as demonstrated by

comparable results achieved at 25°C and 37°C for all the tested conditions (see supplementary data, Figure SI2).

3.4. Cell embedding and 3D culture

BMSCs were embedded in both low and high MA GelMA featuring high, medium and low stiffness (5%, 7.5% and 10% (w/v), respectively), to assess the influence of GelMA properties on cellular behavior. Specific metabolic activity was evaluated after 3 and 7 days of culture, as shown in figure 4a. After 3 days in culture, BMSCs showed similar metabolic activity for all the conditions tested. After 7 days in culture, however, cells within high MA GelMA showed a higher cell metabolic activity as compared to the previous time point. In particular, metabolic activity of both 5% and 7.5% (w/v) GelMA samples had a statistically significant enhancement compared to corresponding samples at day 3. Conversely, cells cultured up to 7 days within low MA GelMA samples showed lower metabolic activity at all tested concentrations, compared to high MA GelMA samples. Furthermore cells within low MA GelMA at 10% (w/v) showed a statistically significant decrease in the metabolic activity from day3 to day7. Data relative to the lowest concentration (5% w/v) for low MA GelMA were not included due to premature degradation of samples. Concerning cell morphology, after 7 days of culture within high MA GelMA, BMSCs appeared more elongated in GelMA at 5% (w/v), while almost rounded in both the 7.5% and 10% (w/v) ones (Fig. 4b).

3.5. BMSC-HUVEC 3D co-culture

To evaluate the influence of hydrogel mechanical properties on cellular behavior, BMSCs and HUVECs GFP were encapsulated and co-cultured up to 7 days within high methacrylated GelMA featuring high, medium and low stiffness (5%, 7.5% and 10% (w/v), respectively). After 3 days in culture, HUVECs started to elongate within the 5% (w/v) GelMA (Fig. 5d), while maintaining a more round-shape in both 7.5% and 10% (w/v) GelMA samples (Fig. 5e and 5f). The maximum cell spreading was reached after 7 days of culture in 5% GelMA

samples (Fig. 5g), in which the formation of a fully developed HUVECs network was detected. In the remaining gels (7.5% and 10% w/v), HUVECs appeared sparsely connected and did not form any network-like structure and BMSCs remained almost rounded for the whole culture period (Fig. 5h and 5i). Culturing BMSCs and HUVECs apart, on the contrary, resulted in isolated and round-shaped cells within all three GelMA concentrations tested and for all the seven days of culture (see supplementary data, Figure SI3).

4. Discussion

In the last few decades, several hydrogel formulations have been widely implied in TE as biomimetic materials for establishing *in vitro* 3D cell culture models [28]. Their structural similarity to natural ECM together with the ease of tuning their biochemical and biomechanical properties make hydrogels suitable candidates for effectively replicating the complexity of tissues found *in vivo* [2-6]. In this regard, considering the wide range of polymeric precursors and crosslinking methods used to fabricate biomimetic hydrogels, systematic studies aiming at elucidating the influence of all involved parameters on hydrogel properties are still required to fully exploit their potential in tissues replication. To date, several polymerization strategies have been successfully investigated [11,15-18], either for fabricating 3D scaffolds for further cell seeding [29] or directly for 3D cells embedding within matrices [7]. Among these strategies, photopolymerization has been widely investigated and demonstrated to be a promising approach to obtain highly viable 3D cellular matrices [19,20]. However, the definition of optimal combinations of polymeric precursors, light sources and photoinitiator molecules remains an open challenge, in terms of both biocompatibility and control over biomechanical and biochemical properties of hydrogels.

In this paper, a commercially available UVA LED (emitting peak at 385nm), exhibiting negligible cytotoxicity effects [25], was employed as controlled light source to photopolymerize methacrylate gelatin hydrogels in combination with the photoinitiator (PI) molecule VA-086, highly activated at this wavelength [30]. The VA-086 has previously been demonstrated to minimally affect cell viability both in its radical and inactive forms [25], making it a promising alternative to the widely used Irgacure 2959, highly activated at lower light wavelengths (adsorption peak around 320nm) [24]. Here, the photo-crosslinking process for fabricating cell-laden VA-086 GelMA-based hydrogels was deeply characterized, showing the influence of gelatin functionalization degree, polymer concentration and light irradiance directly on both mechanical and biomimetic properties of resulting matrices. Based on this characterization, we defined a reproducible protocol to homogeneously embed and culture primary cells within 3D GelMA hydrogels.

The presented photopolymerization protocol was demonstrated to be highly effective for both the synthesized GelMAs allowing to obtain fully cross-linked samples within hundreds of seconds. In particular, in the perspective of cell embedding, we limited the investigation at 360 sec. These short cross-linking times, indeed, were considered compatible with cell embedding procedure, avoiding cells from settling down. All the combinations of methacrylation degrees, polymer concentrations, VA-086 contents and photo-crosslinking times, resulted below the limiting time of 360s, except for those involving 5% lowMA GelMA. Moreover, while increasing LED irradiance resulted in lower cross-linking times, it was also showed to enhance the hydrogel porosity by means of N_2 gas release as a result of UV radical induction [26] (see supplementary data, Figure SI4). Starting from this characterization, an optimal photopolymerization protocol was defined, fixing the LED irradiance at $2mW/cm^2$ and the PI concentration at 1.5% w/v, fulfilling both the biocompatibility and the matrix homogeneity requirements for all conditions tested. In details, samples were polymerized for 5 minutes

subjecting cells to a total irradiation dose of about 600 mJ/cm^2, which results much lower than the dosage previously demonstrated as not toxic to cells, i.e. 1800 mJ/cm^2 [26,30]. As a remarkable advantage, the entire process was easily performed under sterile conditions by placing the samples within standard Petri dishes, thus taking advantage from the optical transparency of polystyrene to UVA.

The use of gelatin-based biomaterials has become widespread thanks to their natural origin, low production cost and biocompatibility. However their suitability as 3D matrices for cell embedding and culturing also depends on their mechanical properties [31], which are influenced by the crosslinking parameters. For this purpose, a rheological characterization of GelMA hydrogels was carried out, showing how the increase of gelatin concentration directly increases sample stiffness, as a consequence of the availability of more cross-links per unit volume, in agreement with previously reported data [21]. The correlation between crosslinking parameters and mechanical behaviour suggested the potentiality of the presented protocol to easily tune the stiffness of the cell-laden matrix according to the required application. In addition, the temperature was found not to significantly affect samples properties, revealing an insoluble network characterized by stable chemical bonds. This behavior, characteristic for a viscous solid with a well-developed network [21], was confirmed by the fact that storage moduli resulted higher than loss moduli for all conditions tested.

It is then worth noting that the lower MA concentration used in this study, i.e. 10% (v/v), corresponds to the amount commonly adopted to achieve highly functionalized GelMA [32,33]. Indeed, further increase in MA content (up to 15% (v/v) in this work) did not yield any significant variations neither in terms of chemical nor mechanical properties. Nonetheless, highMA GelMA resulted in an overall more repeatable photopolymerization protocol particularly at the lowest concentration tested (5% (w/v)), being this also eliciting from cell viability experiments. Such difference could be possibly related to a more homogeneous

functionalization obtained for the highMA GelMA during the reaction, although a more specific study should be carried out.The photo-crosslinking method was exploited to generate GelMA hydrogels and study their behavior as biomimetic matrices for 3D embedding and culturing of both human BMSC and HUVEC GFP cells. Successful BMSCs embedding within both low and high MA GelMA samples revealed the potentiality of our protocol to obtain and culture biocompatible cell-laden hydrogels up to seven days supporting cell proliferation and spreading depending on mechanical properties. Cell behavior and viability results, after seven days in culture, showed how a higher gelatin functionalization resulted in more suitable environments for long term 3D cells culture, creating more stable hydrogel-networks. Moreover, specific cell metabolic activity and viability varied inversely with gel concentration: 5% w/v GelMA samples indeed resulted more viable and metabolically active BMSCs, which also appeared more stretched after seven days in culture as compared to 7.5% and 10% w/v. This finding suggests that mechanical properties characterizing the lower hydrogel concentration better allowed these cell types to remodel and degrade the matrix.

Finally, the presented combination of methacrylated gelatin, VA-086 photoinitiator molecule and UVA LED source was also investigated for generating multi-cell types models, co-culturing BMSCs and HUVECs in 3D. Results obtained within the softer GelMA hydrogels showed to enhance the interactions between BMSCs and HUVECs when co-cultured, promoting the formation of well-established HUVECs network-like structures. In the same conditions, neither HUVECs nor BMSCs cultured alone were able to remodel the surrounding matrix at comparable levels, suggesting that the presented material could be considered a promising co-culturing model for further studies on these two cell populations.

5. Conclusion

In the present study, we fully characterized a highly biocompatible photo-crosslinking process to obtain uniform 3D cell-laden methacrylated gelatin (GelMA) hydrogels. We systematically

reported how methacrylation degree, pre-polymer concentration and UVA irradiance influence mechanical and biological properties of final hydrogels. We introduced a photopolymerization protocol which could in perspective be considered a versatile tool for generating cell-laden hydrogel with easy tunable mechanical and biomimetic properties, depending on parameters setting during photopolymerization process.

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Figure

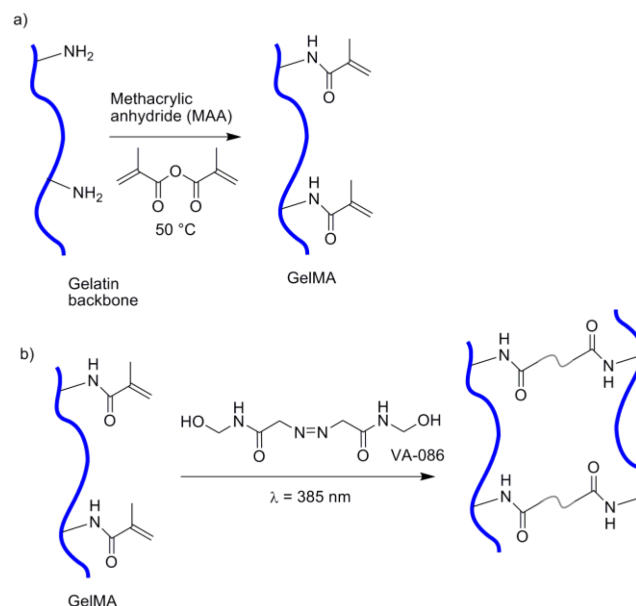
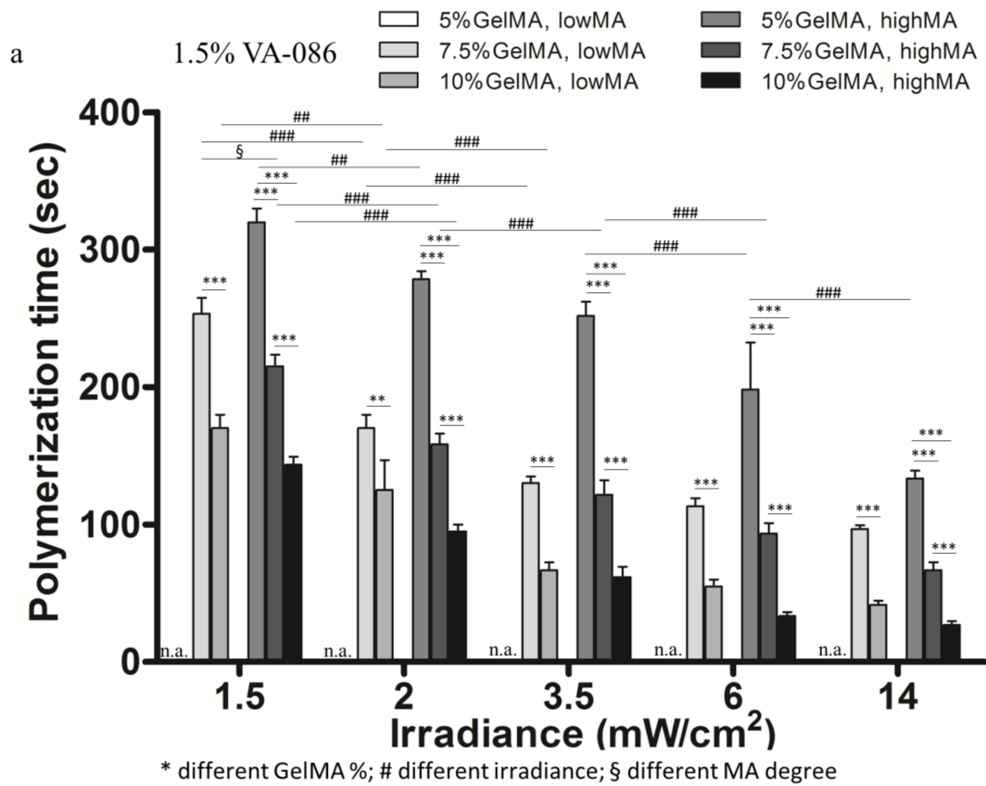
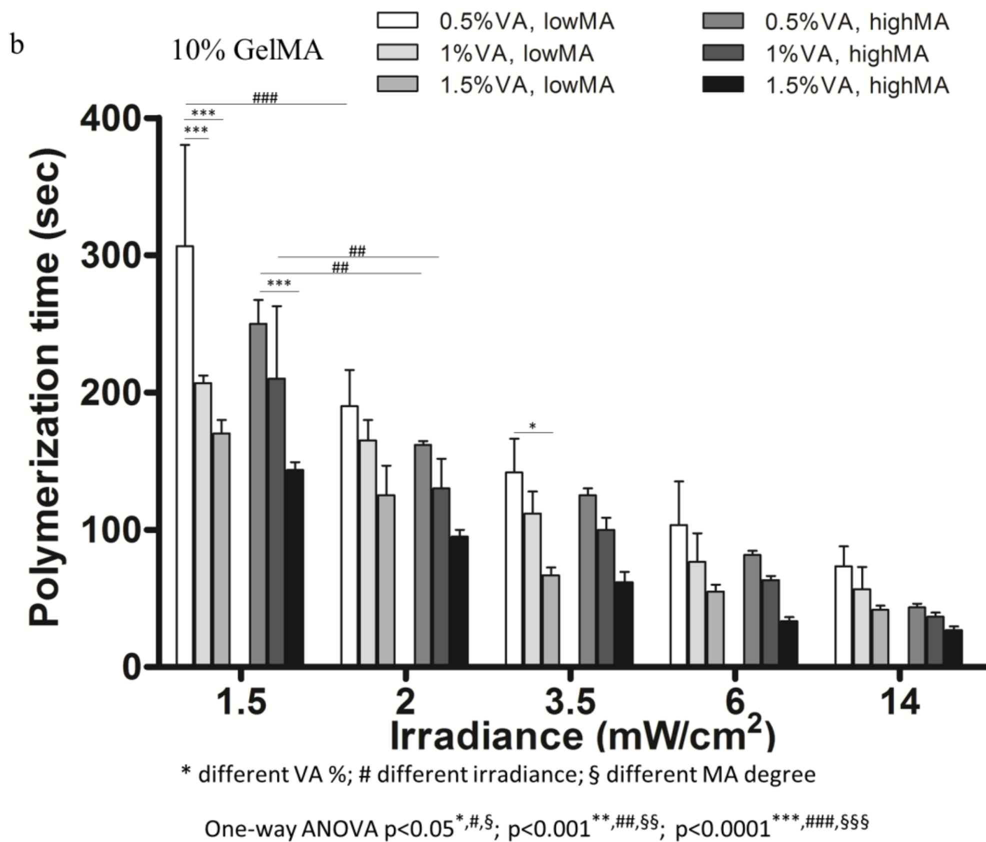


Figure 1. Methacrylated gelatin (GelMA) synthesis and cross-linking reaction. Gelatin monomers were reacted with methacrylic anhydride (MA) to replace lysine groups with methacrylate pendant groups (a). Hydrogel network was then created cross-linking GelMA using a LED light source in the presence of a photoinitiator (b).





One-way ANOVA p<0.05*,#,§; p<0.001**,##,§§; p<0.0001***,###,§§§

Figure 2. GelMA cross-linking time in function of LED irradiance**.** Comparison between polymerization times of GelMA samples obtained varying gelatin (a) or VA-086 (b) concentration for both low and high gelatin methacrylation degree. Error bars represent positive standard deviations.

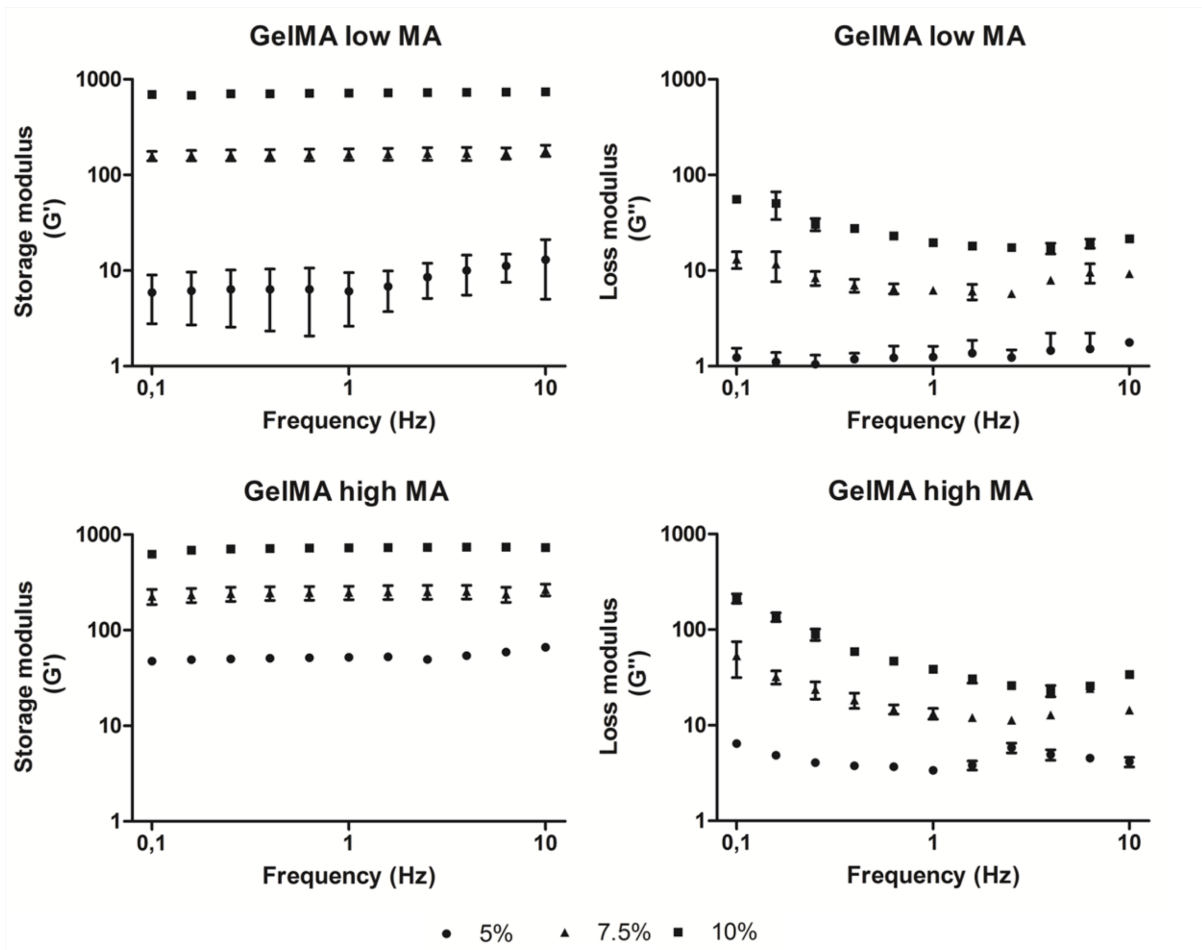


Figure 3. Storage (G') and loss (G'') modulus of cross-linked GelMA in function of frequencies at 37°C. Mechanical properties of low (a) and high (b) methacrylated GelMA. Sample photopolymerization was performed varying gelatin concentration (5%, 7.5%, 10% w/v) while maintaining 1.5% of VA-086 and using a 2 mW/cm^2 irradiance. Axes are in Log scale and error bars identify both positive and negative standard deviation.

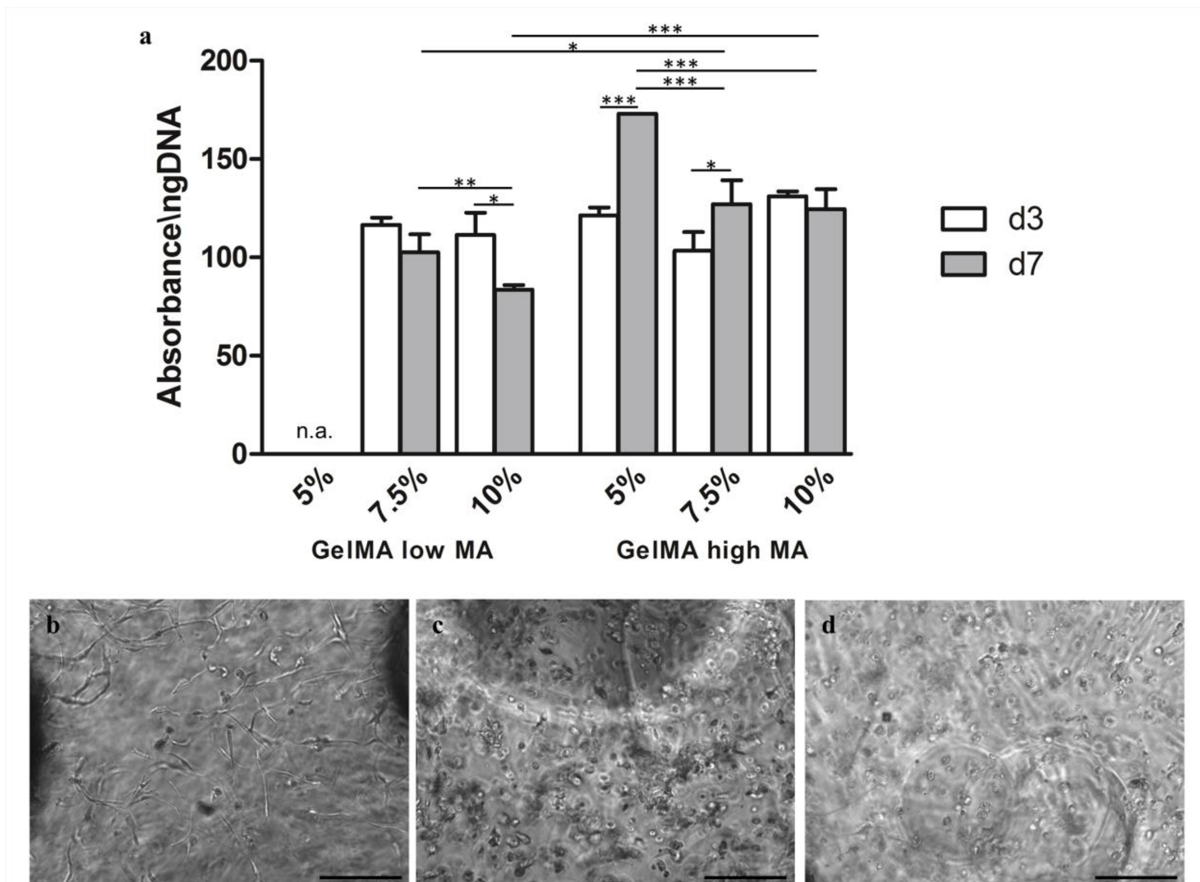


Figure 4. Specific metabolic activity of BMSCs cultured up to 7 days within both low and high methacrylated GelMA at different gelatin concentrations (a). Cell laden GelMA samples were cross-linked with 2 mW/cm^2 irradiance in the presence of 1.5% VA-086. Cell morphology after 7 days of culture was also assessed. BMSCS embedded within high methacrylated 5% GelMA appeared more elongated (b), while 7.5% and 10% gelatin sample showed rounded cells (c, d) (Image scale bar= 200µm).

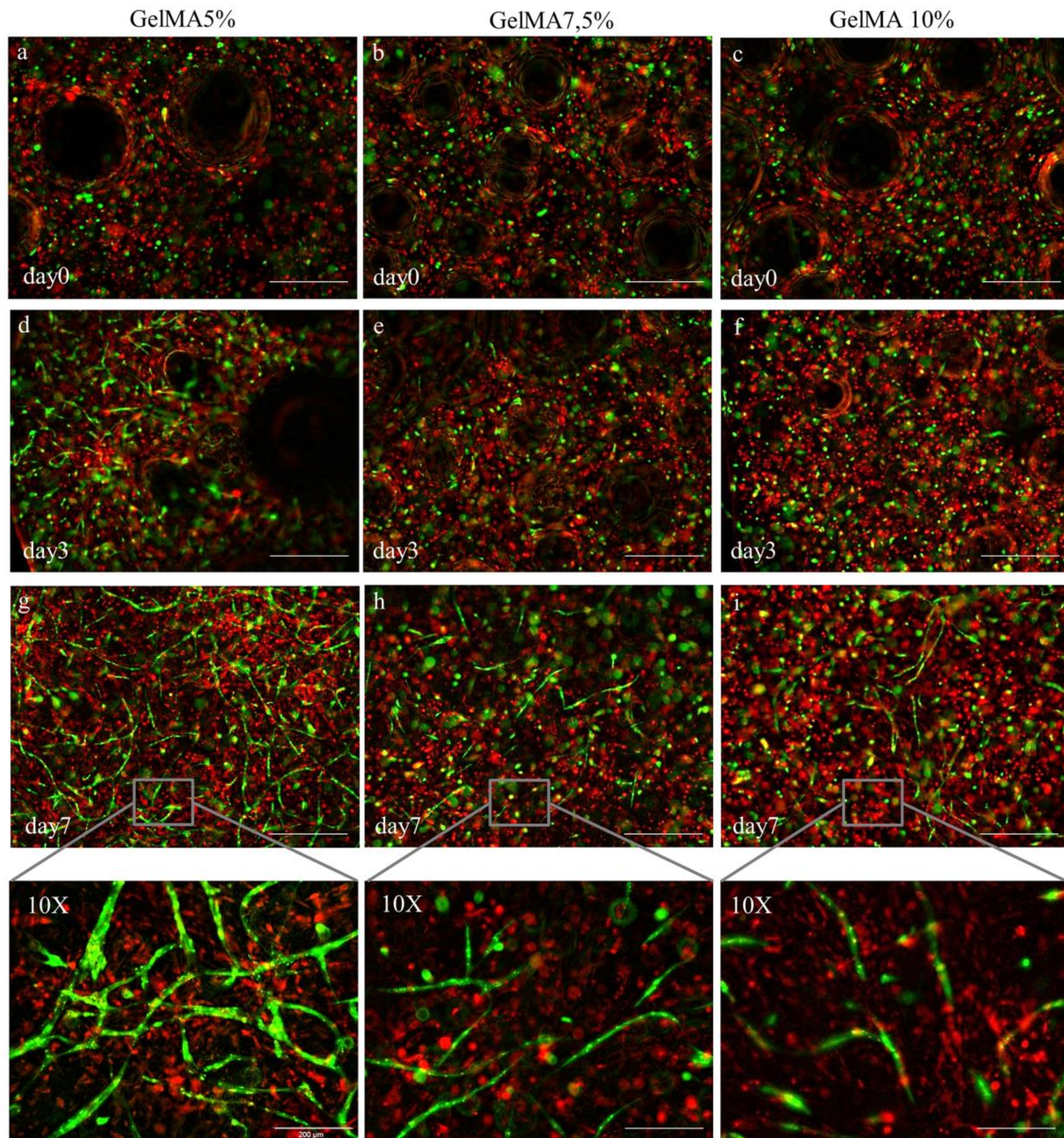


Figure 5. Cell behaviour when co-cultured within high methacrylated GelMA hydrogel. HUVECs GFP and BMSCs (red) were co-cultured up to seven days within 5%, 7.5% and 10% (w/v) GelMA samples. Immediately after embedding, cells appeared rounded in all samples (a-b). After 3 days of culture HUVECs started to interact within 5% (w/v) gel (d) while maintained a rounded-shape in 7.5% and 10% (w/v) hydrogel samples (e-f). After 7 days, within 5% (w/v) GelMA samples, HUVECs developed a network-like structure (g), whereas in 7.5% and 10% (w/v) GelMA, HUVECs appeared sparsely connected and BMSCs remained rounded (h-i). Black and cell free regions resulted from N_2 gas release during photo-polymerization (Image scale bar= 500 µm (a-i) and 200µm for 10X magnifications).