Embedded 3D printing for the development of perfusable *in vitro* 3D model of soft tissue

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Abstract

Hydrogels are widely investigated to develop 3D *in vitro* models for mimicking tissue microenvironment since their properties well match soft tissues ones. One concern is the lack of an adequate channel network, mimicking the tissue vascularization. Optimizing a vascularization strategy still remains challenging. We optimized and characterized a GelMA/Pluronic combination by using embedded 3D printing for the realization of a channel network in a GelMA scaffold.

Keywords: Embedded 3D printing; GelMA; 3D *in vitro* model; soft tissue; vascularization

1. Introduction

3D hydrogel-based models recently gained attention to reproduce *in vitro* extracellular matrix (ECM) [1], to mimic cell-cell and cell-ECM interactions, and to screen and test new drugs. Nevertheless, lack of vascularization in the model remains the main limitation. Embedded 3D printing (E3DP) is arising as a novel technique, involving a supportive bath that can maintain the position of the embedded printed structures allowing the needle movement [2]. This approach is largely exploited by printing inks within a sacrificial hydrogel bath, made of gelatin (Gel) or Carbopol [3], or by printing a fugitive ink in a permanent supportive bath [4]. A permanent supportive bath which exploits adequate rheological properties to ensure E3DP and mimics soft tissues properties for an appropriate 3D *in vitro* model still remains unreported in literature. We

optimized the printing of a channel using Pluronic F127 as fugitive ink, in a bath of methacrylated gelatin (GelMA) to prove the possible combination of the two materials for the realization of hollow channels mimicking a simple vascularization in the scaffold, having physic-mechanical properties similar to soft tissue (e.g., adipose tissue).

2. Materials and Methods

2.1. Materials synthesis

All materials were purchased from Sigma-Aldrich, unless differently specified. GelMA was synthetized [5], dissolved in DPBS (10% w/V) at 37°C, adding 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) as photoinitiator (0.05% v/V), poured in PDMS molds (Ø = 15 mm, h = 4 mm) and pre-crosslinked by UV light (TissueStart™ UV light, λ = 405 nm) for 120 s, obtaining a supportive bath for E3DP of the fugitive ink, Pluronic F127 (23% w/V).

2.2. Rheological characterization

Rheological properties were investigated by using a rheometer (Anton Paar GmbH, Austria), using parallel plates and cone-plate (Ø = 25 mm) geometry, respectively for GelMA and Pluronic F127. Strain (0.01-100%), temperature (4-50°C), and shear rate (0.1-1000 s^{-1}) sweep tests were performed for both materials (SM-1). Thixotropy tests were performed on GelMA (0.1 s^{-1} for 120 s, 100 s^{-1} for 100 s, 0.1 s^{-1} for 300 s). Rotational properties of GelMA were modeled by Eq.1 (Herschel-Bulkley model):

$$\sigma = \sigma_0 + K\dot{\gamma}^n \quad \text{(Eq.1)}$$

where σ = total stress, σ_0 = yield stress, $\dot{\gamma}$ = shear rate, K and n = fitting parameters [6].

2.3. 3D perfusable scaffold characterization

The channel network template was designed (SolidWorks 2020) and printed (TissueStart™, TissueLab, CH) in the supportive bath (**Table 1**) after printing parameters optimization (SM-2). GelMA/Pluronic samples were UV photocrosslinked (OSRAM Ultra Vitalux) for 150 s, and immersed in deionized water (24 h, 37°C) to obtain hollow channels, after Pluronic leaching.

Samples with channels (GelMA_ch) were characterized and compared to bulk GelMA samples (GelMA).

Table 1. Optimized parameters for the E3DP of Pluronic F127.

Printing parameter	Value	Unit of measurement
Printing speed	6	[mm/s]
Extrusion multiplier	4	[-]
Flow	400	[%]
Needle gauge	25 (250 μm)	[G]
Extruder temperature	25	[°C]

3D scaffold morphology was analyzed with a stereomicroscope (Wild M8) by acquiring images before and after manually perfusing the samples (n = 3) with colorant solution. Weight variation ($\Delta W\%$) tests were performed in distilled water at 37°C up to 4 weeks (n = 3) and $\Delta W\%$ was evaluated (Eq.2) by comparing the sample weight (w_t) at different timepoints to the anhydrous sample one (w_0):

$$\Delta W\% = \frac{w_t - w_0}{w_0} * 100 \quad \text{(Eq.2)}$$

Compressive mechanical properties were tested (n = 3) by Dynamic Mechanical Analyzer (DMA Q800, TA Instruments), at 37°C. A load run was applied at 2.5% min^{-1} rate down to -30% strain, followed by an unload run at 5% min^{-1} rate up to 1%.

2.4. In vitro cell response

Cytocompatibility tests were performed embedding 3T3-L1 preadipocytes in GelMA solution (1 x 10^6 cells mL^{-1}) prior to the pre-crosslinking (SM-3). AlamarBlue assay was performed up to 21 days to evaluate cells metabolic activity (n = 3 samples), measuring fluorescence (RFU) with a spectrophotometer (Eq.3):

$$RFU\ (Relative\ Fluorescence\ Unit) = f_{sample} - f_{control} \quad \text{(Eq.3)}$$

where $f_{control}$ = control sample fluorescence and f_{sample} = cell-laden sample fluorescence.

Phalloidin/DAPI staining (SM-4) was performed to qualitatively observe cells distribution in GelMA and GelMA_ch samples. 3T3-L1 metabolic functionality was evaluated by differentiation

into adipocytes (SM-5). Preadipocytes encapsulated into GelMA matrix and EA.hy 926 endothelial cells perfused in the channel (SM-3) were co-cultured up to 7 days and stained (LIVE/DEAD, SM-6).

2.5. Statistical analysis

Data are expressed as mean ± standard deviation. Once verified normality via Shapiro-Wilk test, one-way ANOVA test and unpaired t-test were performed (GraphPad Prism, $p < 0.05$).

3. Results and discussion

3.1. Rheological characterization

Pluronic F127 exhibited a solid-like behavior (i.e., G′ > G′′) at shear strain lower than 1% (**Figure 1.A.i**), and the transition temperature was detected at 23°C (**Figure 1.A.ii**). The printing temperature was set at 25°C.

Key challenge in optimizing E3DP is the development of a supportive bath matrix which can maintain the position of the printed structure during printing. The matrix must possess a yield-stress behavior, exploiting an elastic solid behavior in rest condition, and acting as a liquid-like material when a sufficient shear stress (i.e., yield stress) is applied (Eq.1) [4,7]. Supportive bath thixotropic behavior is a key indicator of speed recovery of the material after exposure to shear rate [7]. GelMA (**Figure 1.B**) recovered approximately 60% of its initial viscosity (8.0×10^4 Pa s). Although GelMA bath exploited a higher recovery time (38.70 s) than supportive bath in agarose (0.89 s) and Carbopol (1.42 s) [7], its ability in retaining embedded printed structures morphology is enforced by post-printing photocrosslinking. Overall, the printing procedure did not affect the shape of the samples, and the supportive material restored its initial shape.

Pluronic F127 showed a shear-thinning response (**Figure 1.C**), that represents the fundamental behavior for a material printable by extrusion-based 3DP [8]. The bath must exhibit a low yield stress to accommodate nozzle movement, a viscosity comparable to the fugitive ink one, and the possibility to be chemically crosslinked after printing [4]. Furthermore, GelMA precrosslinking

(120 s) allowed to obtain a viscosity similar to the fugitive ink during the printing process (η = 103 Pa s). The yield stress value for supportive bath (σ_0 = 0.29 Pa) fitted the range reported in literature for E3DP applications (0.16-8.8 Pa) [8]. Low yield stress resulted in a rapid recovery of the GelMA supportive matrix during the extrusion, and the obtained channel template can be effectively embedded [8].

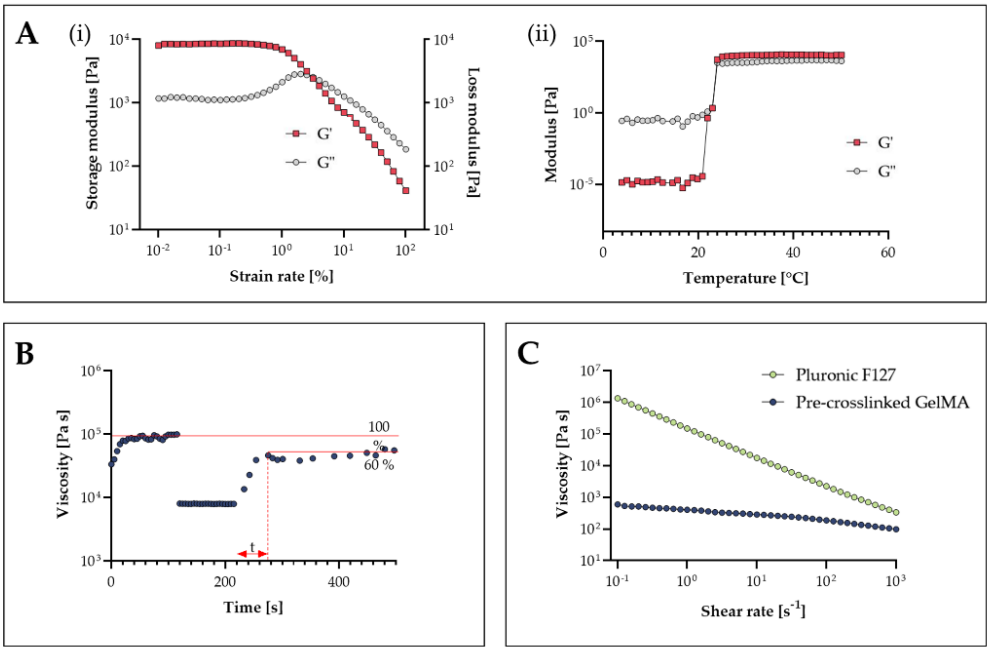

Figure 1. Rheological characterization. (**A**) Pluronic strain (i) and temperature (ii) sweep tests. (**B**) Precrosslinked GelMA thixotropy test. (**C**) Shear rate sweep tests.

3.2. 3D perfusable scaffold characterization

The channel maintained the shape before and after its perfusion (**Figure 2.A.i**), demonstrating the adequacy of the optimized printing parameters and precrosslinked GelMA time. Cross-section of the sample (**Figure 2.A.ii**) showed the obtainment of the channel lumen, and its patency was confirmed by perfusion tests (**Figure 2.A.iii**). Channel diameter (400-900 μm) was consistent with values reported in literature [5,9] for Pluronic F127-based vascular-like structures printed by E3DP in Pluronic F127-DA photocrosslinkable bath [4]. Obtained channel dimension was comparable with human arteries (0.1-10 mm) and veins (1-100 μm) diameters, favorable for mimicking vascular network in the AT 3D model.

The 3D scaffolds were stable in a physiological-like environment up to 4 weeks (**Figure 2.B.i**). A weight loss in the first 24 hours (ΔW% = -25% and -45%, for GelMA and GelMA_ch, **Figure 2.B.ii**) was due to the release of uncrosslinked Gel macromolecules. A higher ($p < 0.05$) weight loss for GelMA_ch was also caused by fugitive ink dissolution.

Stress-strain curves showed a typical viscoelastic behavior (**Figure 2.C.i**). Elastic modulus (8-10 kPa, **Figure 2.C.ii**) was comparable to the range of soft tissues (0.8-18.30 kPa) [10]. E3DP did not influence GelMA_ch mechanical properties, comparing elastic modulus and stiffness values to bulk GelMA ($p > 0.05$, **Figure 2.C.ii**).

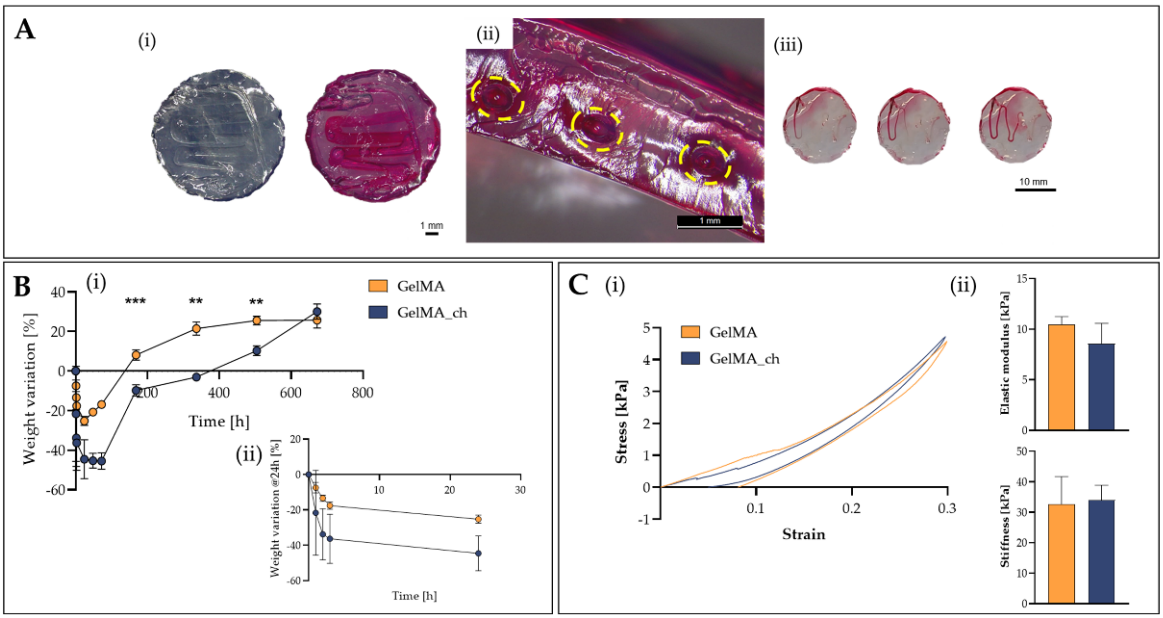

Figure 2. 3D *in vitro* model characterization. (**A**) Stereomicroscopy images: (i) after immersion in water for 24 h (left) and after perfusion (right), (ii) cross-section (channels in yellow), and (iii) after dynamic perfusion. (B) Weight variation tests up to 672 (i) and 24 h (ii). (C) Compressive mechanical properties: (i) σ-ε curves, (ii) elastic modulus and stiffness. ***:p<0.001, **:p<0.01.

3.3. In vitro cell response

Cells metabolic activity increased ($p < 0.05$) from day 1 to day 21, when cells were embedded into GelMA and GelMA_ch. The presence of the channel allowed for better gas/nutrients exchanges resulting in higher RFU values at each time point ($p < 0.05$) for preadipocytes encapsulated in GelMA_ch (**Figure 3.A**). By phalloidin-DAPI staining after 3 days, the presence of the channel that allowed nutrients and oxygen exchange qualitatively promoted a denser concentration of preadipocytes close to the external wall of the channel (**Figure 3.B**). 3T3-L1 homogeneously

colonized GelMA_ch samples after 14 days of culture, qualitatively comparable to 3T3-L1 embedded in GelMA bulk samples (SM-4). Differentiated preadipocytes accumulated a higher amount of intracellular lipidic droplets than undifferentiated ones (**Figure 3.C**), successfully verifying GelMA_ch ability in stimulating their metabolism (SM-5). EA.hy 926 endothelial cells colonized the inner channel surface (**Figure 3.D**) and exhibited an elongated morphology after 7 days of co-culture (SM-6).

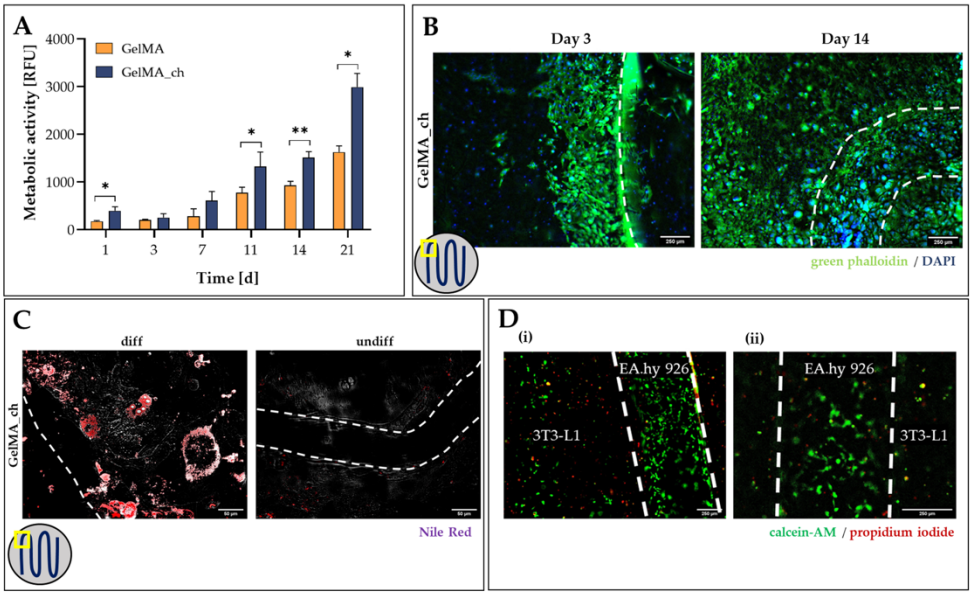

Figure 3. *In vitro* cell response. (**A**) AlamarBlue assay performed on encapsulated 3T3-L1. (B) 3T3-L1 phalloidin/DAPI staining. (**C**) 3T3-L1 Nile Red staining. (D) Co-culture 3T3-L1/EA.hy 926 LIVE/DEAD staining (i) and (ii) (channels walls = white dashed lines).

4. Conclusions

A 3D scaffold embedding a hollow channel was developed exploiting the innovative E3DP technique. For the first time, GelMA bath/Pluronic F127 fugitive ink combination was rheological optimized, and biological tests confirmed scaffold ability in promoting metabolic functionality of encapsulated preadipocytes. The 3D perfusable scaffold can be used to mimic soft tissue properties for the development of an *in vitro* 3D model.

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Figure captions

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