ADMAIORA

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RE	Restricted to a group specified by the consortium (including the Commission Service)	
CO	Confidential, only for members of the consortium (including the Commission Service)	

Document History

Version	Date	Author	Summary of Main Changes
1	13/02/2020	Irene Bernardeschi, SSSA	First version of the template for project Deliverables
2	17/02/2020	Lorenzo Vannozzi, SSSA	Second draft (this document version was shared also with REGENTIS representatives)
3	19/02/2020	Irene Bernardeschi, SSSA	Revision of the second draft and preparation of the third draft
4	26/02/2020	Leonardo Ricotti, SSSA	Revision of the third draft
5	28/02/2020	Irene Bernardeschi, Lorenzo Vannozzi, SSSA	Revision of the third draft and preparation of the fourth draft (also this document version was shared with REGENTIS representatives)
6	28/02/2020	Leonardo Ricotti, SSSA	Revision of the fourth draft
7	29/02/2020	Irene Bernardeschi, SSSA	Final version of the Deliverable and submission

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1 Executive summary

In this Deliverable, the main features of the Pluronic-fibrinogen based formulations developed by REGENTIS are described.

In particular, in the Introduction of section 2 the main specifications identified for the hydrogels are reported, also with a description of the three types of materials developed and the methods and techniques used to optimize the basal hydrogel formulations, based on the specifications. In section 2.1, the Pluronic-fibrinogen based hydrogel formulation optimization is described, together with material characterization results, in terms of mechanical and rheological properties. Diffusivity of proteins in the material and cell viability on human chondrocytes are reported in section 2.2.

The material tended to shrink when incubated at 37°C in a saline solution after being cross-linked with UV light. No marked gradients of fluorescent proteins were observed, indicating a good diffusivity of nutrients into the material. However, such a material formulation did not guarantee a high level of cell viability, probably due to material chemistry. For this reason, the Pluronic-fibrinogen based hydrogel did not result in a good candidate for the project purposes.

2 Introduction

The specifications for the nanocomposite hydrogel have been refined from the project proposal according to Deliverable D2.1, defining a list of priorities. Table 1 reports the list of nanocomposite hydrogel properties, ordered based on their priority for the Consortium.

Table 1: Summary of the specifications for the final nanocomposite hydrogel to be developed in the project.

Property	Specification	Priority level	Notes
Cell viability	90% of ASC viability respect to a 3D biocompatible control hydrogel, 1 day after printing and after UV irradiation, and 95% after 1 week	1	Biological specification derived from D2.2
Printability	Viscosity between 10^2 and 10^5 mPa·s	2	
Stickiness	Fast (less than 1 min) primer+hydrogel adhesion to the cartilage tissue. Failure stress at the interface higher than 10 kPa	2	Newly added property, important to guarantee for a successful clinical procedure: this specification will guarantee a stable localization of the delivered material in the target regions
Piezoelectric properties	Peak piezoelectric coefficient (d_{33}): at least 40 pC/N	2	
Degradation rate	3-12 months	3	
Mechanical properties	Young's modulus ranging between 1 and 1000 kPa, resilience against in vivo-mimicking inputs	4	Targeting the natural cartilage mechanical properties (100 kPa - 100 MPa) is important when dealing with materials to be used as cartilage substitutes, less when cartilage regeneration is targeted
Lubrication properties	Friction coefficient: < 0.05	5	Desirable, but not crucial to achieve the project objectives
Permeability	N.A.	6	This specification has been removed, since the first one (cell viability) will take into account, among different factors, a suitable permeability of the hydrogel. Permeability is related to protein/nutrients diffusion within the hydrogel

As summarized in the table, the priority is cell viability, an essential prerequisite to achieve the ambitious goals of the ADMAIORA project.

The workflow for matching such specifications has been further refined after the call meeting of SSSA with IOR and REGENTIS on June 13, 2019. Since it is of primary importance for the project to find a hydrogel in which cells remain viable for the whole hydrogel thickness (target: 7 mm, compatible with the clinical objective firstly defined in Deliverable D2.1), and based on some preliminary results of biological tests performed by IOR described in Deliverable 3.1 section 2.1, it has been decided that:

- REGENTIS had to send different material formulations to SSSA. The selection had to take into account the preliminary IOR experiments
- SSSA had to test first the permeability/diffusivity of these gels, by using fluorescently marked molecules, used as models of nutrients in the culture medium (see section 2.2). The hydrogels not allowing a good diffusion of nutrients had to be excluded, as theywould hardly allow cell viability. Then, hydrogels had to be tested with human chondrocytes. Only the hydrogels showing good results in this series of tests could be further tested with ASCs.

The target hydrogel thickness was further revised to 2 mm in the project course, following the clinicians' opinion (in the Project meeting held in Tel-Aviv on September 18-19, 2019).

Three different biosynthetic formulations of PEG- and Pluronic-based materials have been processed and tested for cell encapsulation:

1. Material 1: A bio-synthetic finely tuned combination of PEG-DA and denatured human fibrinogen, crosslinkable through UV light. A CE-Marked product for the repair of focal cartilage lesions is based on this composition and commercialized by REGENTIS (GelrinC). GelrinC is as an acellular product for focal cartilage defects, which is cleared in Europe as a class III medical device. This liquid formulation may be advantageous to encapsulate cells even if it is more challenging for bio-printing. A patent deposited by REGENTIS guided the development of such hydrogel formulation[1]. This material had been already tested to support chondrogenesis in 2D culture[2];
2. Material 2: Injectable and curable PEG-fibrinogen/Pluronic paste, which is based on PEG-fibrinogen conjugate, ~ 23% of Pluronic F127 polymer and a photo-initiator. Patents owned by REGENTIS and granted in USA, Europe, China and Hong Kong protect the composition of such material.
3. Material 3: A thermosensitive Pluronic-F127-fibrinogen hydrogel. The Pluronic F127-fibrinogen already showed high compatibility with mesenchymal stem cells.[3] Its composition is protected by patents owned by REGENTIS and granted in USA, Europe, China and Hong Kong.[4].

All the proposed hydrogel formulations are based on covalent conjugation of denatured fibrinogen to synthetic, biodegradable and biocompatible polymers. All the compositions harness the natural regenerative properties of fibrinogen, a key component in tissue regeneration, together with a synthetic polymer that allows tailoring the hydrogel physical properties.

The methods and techniques to optimize the basal hydrogel formulations are summarized below:

1. Evaluation of the material components:
   1.1 PEGylated fibrinogen was measured using the Kjeldahl method;
   1.2 Pluronic and Pluronic-DA and photo-initiator were weighed;
2. Evaluation of the crosslinking:
   2.1 Mechanical strength (Shear storage modulus, G`) was measured using a UV-coupled and temperature-controlled (Peltier plate temperature-controlled) rheometer.
   2.2 The thermal gelation transition temperature was determined by performing temperature swift rheology on the uncured paste. The temperature at which the

[1] D. Seliktar and Y. Shachaf. U.S. Patent, No. US 8,846,020 B2 (2014)
[2] Goldshmid, Revital, et al. "Steric interference of adhesion supports in-vitro chondrogenesis of mesenchymal stem cells on hydrogels for cartilage repair." Scientific reports 5 (2015): 12607.
[3] Goldshmid, Revital, et al. "A method for preparation of hydrogel microcapsules for stem cell bioprocessing and stem cell therapy." Methods 84 (2015): 35-43.
[4] Y. Shachaf, and A. Wechsler, U.S. Patent, No. 14/901,216 (2016)

G` shifted from liquid to paste was considered the thermal gelation transition temperature.

During the optimization of the hydrogel formulations, a relevant work concerned the adjustment of polymerization time. The polymerization time must be kept "clinically acceptable": the material should be crosslinkable in a time range compatible with the surgical procedure needed to implant it by using the handheld bioprinter, approximately 10 min per defect as reported in Deliverable D2.2. In the case of materials with thermosensitive properties (Material 2 and 3), the hydrogel can hold itself (gelate) in the defect until final UV curing.

The Consortium agreed to set 5 mW/cm^2 as safe UV intensity to be applied in the presence of cells. The radiation intensity was then carefully controlled through a UV dosimeter.
All hydrogel formulations proposed by REGENTIS contained Irgacure 2959 as photoinitiator, at a concentration of 0.01 % wt. This photoinitiator shows a maximal absorption at 270 nm. However, since at this wavelength, possible damage to DNA may occur, most users apply UV at 320-365 nm to work with Irgacure 2959**,** which is safer for cells.[5] Thus, a UV source with a wavelength of 365 nm was used for the experiments.

The final target is to fabricate a material allowing to obtain 90% viability of encapsulated cells at 24 h and 95% at 1 week, after UV irradiation. The first day after crosslinking, we may expect some deleterious short-term effects of curing and radicals on viability that should be recovered gradually, thus achieving a higher cell viability in the following days.

[5] Rouillard, A. D., Berglund, C. M., Lee, J. Y., Polacheck, W. J., Tsui, Y., Bonassar, L. J., & Kirby, B. J. (2011). Methods for photocrosslinking alginate hydrogel scaffolds with high cell viability. Tissue Engineering Part C: Methods, 17(2), 173-179.

2.1 Pluronic-fibrinogen based hydrogel formulation: material optimization

In this Deliverable the third candidate material proposed in the ADMAIORA project is described. It is based on a Pluronic-fibrinogen weak thermosensitive gel (commercially named GelrinV).
Such a hydrogel is highly biocompatible with stem cells.[6] It forms a rather weak gel at a relatively high temperature (> 25 °C). This compound could be mixed with the cells and used in a co-axial printing. The use of Pluronic-based hydrogels has been demonstrated suitable for cartilage tissue engineering applications, also in its photoresponsive form.[7,8]

The Pluronic-F127 based hydrogels formulations were tuned by changing the amount of either or both Pluronic F127-OH and Pluronic F127-di-acrylate. This also led to a change in the thermo-responsive properties of each formulation.
Two batches were prepared, namely FF0038 and FF0039. All materials were prepared from sterile solutions in aseptic environment and considered sterile and suitable for cell culture.

The formulations were evaluated by performing rheology tests using a AR-G2 rheometer equipped with a Peltier plate for controlled temperature measurements and a UV curing platform for real-time monitoring of the curing progression.
The results of the mechanical properties as a function of the UV curing time is reported in Figure 1.

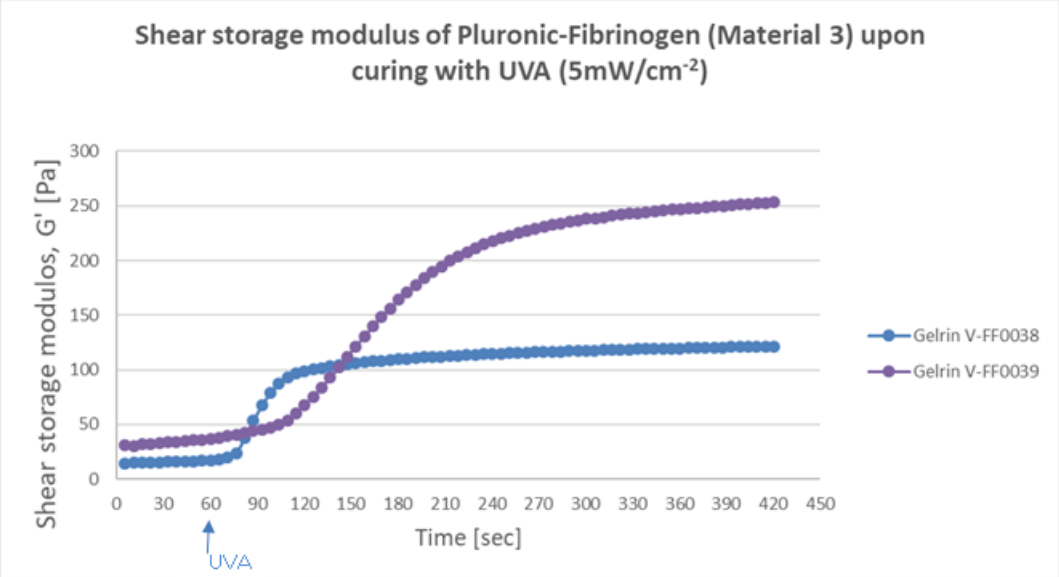

Figure 1: Mechanical properties as a function of curing time at room temperature.

There has been an inconsistence in the batch manufacturing, as visible by the different G' modulus achieved for the hydrogel formulation during the test.
A similar behaviour was found by analysing the mechanical properties as function of the temperature (Figure 2).

[6] Goldshmid, Revital, et al. "A method for preparation of hydrogel microcapsules for stem cell bioprocessing and stem cell therapy." Methods 84 (2015): 35-43.
[7] Vashi, Aditya V., et al. "Adipose differentiation of bone marrow-derived mesenchymal stem cells using Pluronic F-127 hydrogel in vitro." Biomaterials 29.5 (2008): 573-579.
[8] Im Lee, Jung, Hye Sung Kim, and Hyuk Sang Yoo. "DNA nanogels composed of chitosan and Pluronic with thermo-sensitive and photo-crosslinking properties." International journal of pharmaceutics 373.1-2 (2009): 93-99.

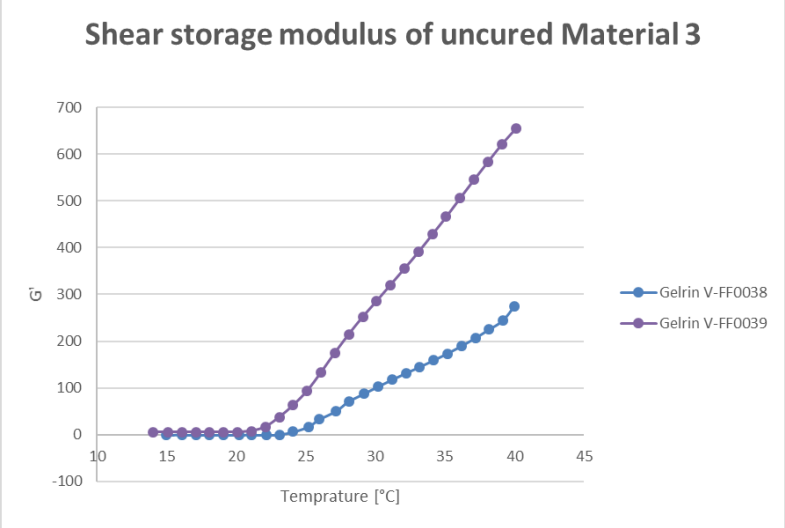

Figure 2: Mechanical properties as function of temperature.

After these preliminary tests, both formulations have been sent to SSSA for further characterization.
However, the batch FF0038 resulted more effective on being crosslinked with UV light radiation in a time compatible for the ADMAIORA project. For such a reason, the following tests were performed only on that batch.

The degree of swelling of such hydrogels after the photopolymerization was assessed on samples with a thickness of 2 mm and a diameter of 10 mm, crosslinked with a UV light intensity of 5 mW/cm^2 for 5 min (Figure 3).

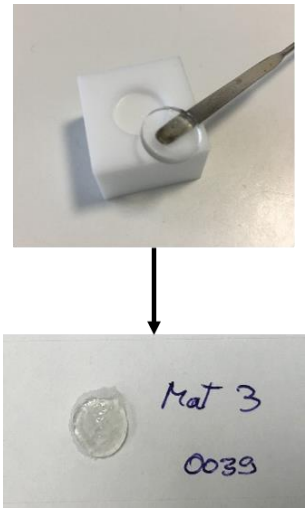

Figure 3: Swelling tests on hydrogels made of Material 3.

Such a hydrogel formulation tends to shrink with a volume variation of -38 % with respect to its initial volume when incubated in a saline solution (PBS) at 37 °C.

2.2 Protein diffusivity and cell viability tests

As previously discussed, it is of primary importance for the ADMAIORA project to find a hydrogel in which cells remain viable in the whole hydrogel thickness.

To this aim, a Teflon mould with appropriate dimensions was fabricated to mimic the theoretical volume of cartilage defects to be filled with the hydrogel. The mould had a diameter of 1 cm (defect size area: 0.785 cm^2) and a height of 7 mm.

A Teflon mould with appropriate dimensions was fabricated to mimic the theoretical volume of cartilage defects to be filled with the hydrogel. The mould had a diameter of 1 cm (represented defect size area: 0.785 cm^2) and a height of 7 mm.
Albumin from bovine serum, FITC conjugated (BSA-FITC, Mw: 66 kDa, hydrodynamic radius (Rh) ≈ 3.6 nm) solution was used to assess protein diffusion, as protein model. The labeled solution (concentration: 100 µg/ml) was dropped onto the upper surface of each hydrogel (total volume of solution: 1.4 ml). After 30 min (time calculated by adapting the one reported in [8], adapted to the volume of our gels), the hydrogel was washed with PBS three times to remove residual fluorescent dye. The cross-sectioned surface was then observed with a fluorescence microscope (Leica-TCS-SP5).
The protein diffusivity experiments were conducted by following the reported experimental procedure:

- Preparation of the hydrogel solution;
- Casting of the hydrogel solution in the Teflon mould;
- UV polymerization with fixed parameters (intensity: 5 mW/cm^2; time: 5 minutes), and exposure for 15 min to visible light through a LED light (This last parameter has been taken into account because during the material injection in the cartilage defect the overall scenario is kept illuminated by the surgeon);
- Swelling in PBS at 37 °C for 24 h;
- Incubation of the crosslinked hydrogels with the 1.4 ml of BSA-FITC (concentration 0.1 mg/ml) solution for 30 minutes at 37 °C. Total of 5 samples for each formulation.
- Removal of the BSA-FITC solution, and washing with PBS for 5 min
- Transversal cutting of the hydrogel, and immersion in OCT for freezing it in liquid nitrogen;
- Sectioning with the cryotome (slice thickness: 20-30 µm);
- Acquisition of the images with the fluorescence microscope. Exposure parameters: 1 s (4X magnification); 5 s (10X magnification). Gain: up to 37X

The main output of the experiment was the measurement of the protein diffusion distance from the top of the hydrogel. The average protein diffusion distance in the hydrogel was quantified using ImageJ software, as shown in Figure 4.

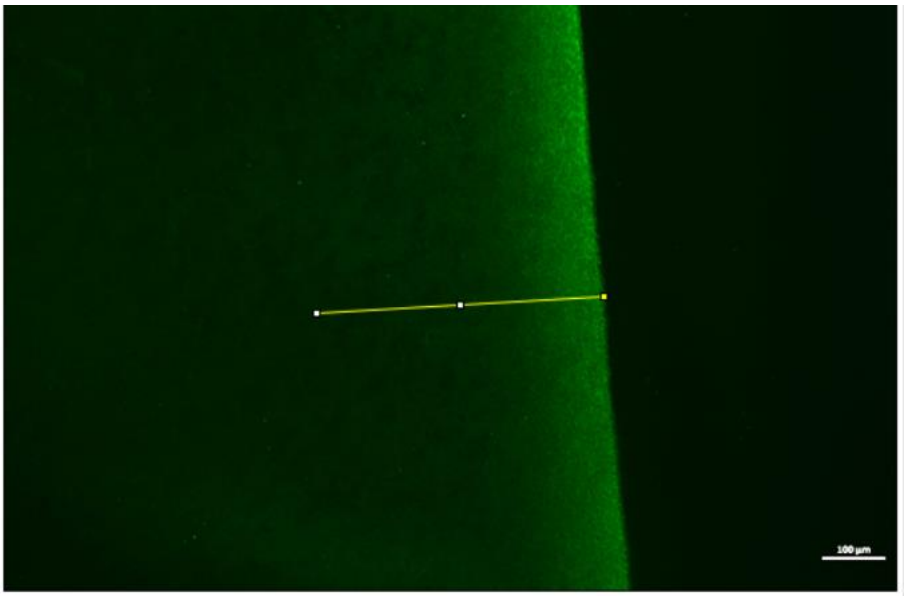

Figure 4: Depiction of the protein diffusion measurement, performed in ImageJ.

A threshold was defined in order to measure the maximum distance covered by the protein inside the hydrogel. Such a measure considered the fluorescence intensity when it doubled the value of the background.

2.2.1 Protein diffusivity test

Figure 5 shows the diffusion of the BSA-FITC within the hydrogel for the selected batch of Material 3.

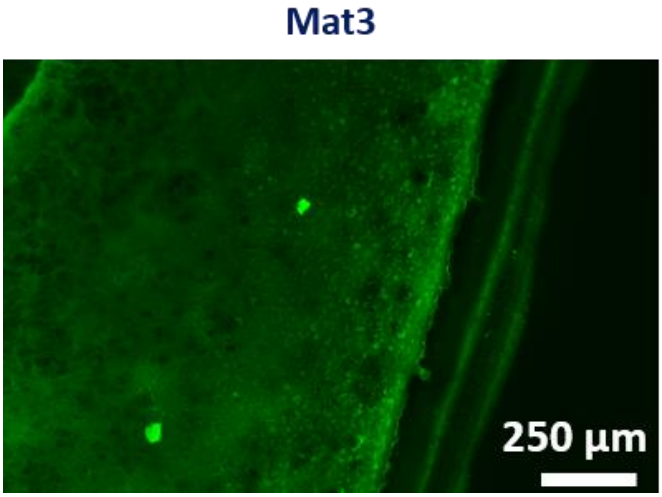

Figure 5: Representative images of the protein diffusivity test performed on the selected formulations of Material 3.

In parallel, blank hydrogels were also analysed in order to capture appropriate background signals, as reported in Figure 6.

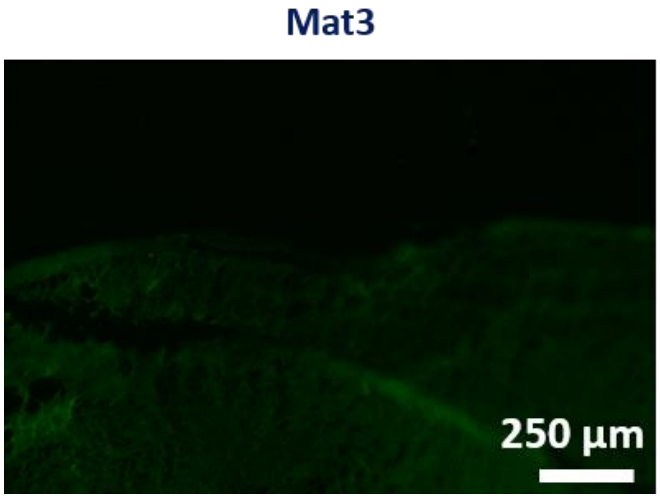

Figure 6: Flourescence measurement on hydrogels based on the selected Material 3 formulations, not incubated with BSA-FITC, to acquire background signals (corresponding to material auto-fluorescence).

It is noticeable the absence of marked gradients. Probably, the BSA-FITC permeates in the gel, without accumulating in the border as in the case of Material 2 (see Deliverable D3.1). This result looked promising in view of cell viability experiments.

2.2.2 Cell viability test

Human chondrocytes (Cell applications, Inc.) were used as a cell model to investigate the effects of the hydrogel formulation/thickness on chondrocyte viability before adopting ASCs. For the experiment with the hydrogel, a small cell pellet (density: $2*10^6$ cells/ml) was mixed with the hydrogel previously kept in a refrigerator at 4 °C to maintain it liquid. Subsequently, 200 µL of the hydrogel solution were poured inside a polydimethylsiloxane (PDMS) mould, with an internal cavity of 10 mm of diameter and 2 mm of height. The PDMS mould was previously sterilized with ethanol and UV light for 30 minutes. The photocrosslinking was performed for 5 minutes at 5 mW/cm^2, afterward the culture medium was slowly added to submerge the hydrogel. Then, the sample was exposed for 15 minutes to visible light through a LED. The hydrogels were then placed in the incubator at 37°C. An example of the obtained hydrogel before and after being removed from the mould is reported in Figure 7.

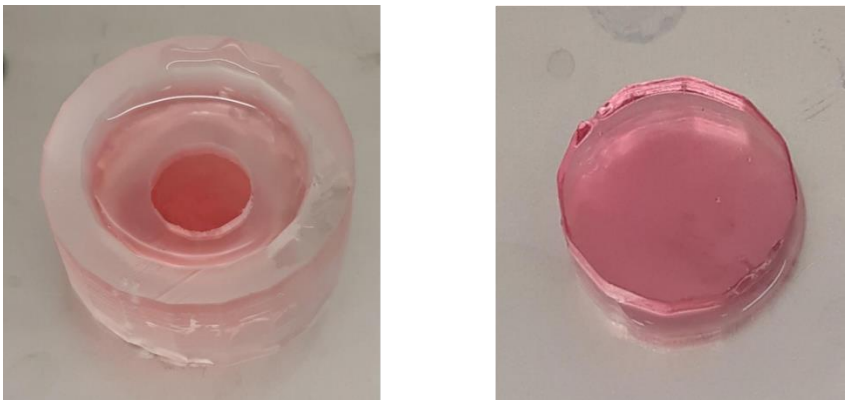

Figure 7: Images of a hydrogel (Material 2, formulation 1) crosslinked within a custom PDMS mould and incubated with cell culture medium.

A Live/Dead test was performed after 3 days to evaluate the chondrocyte viability within the hydrogel. The hydrogel was manually cross-sectioned, placed on a slide, and visualized with a fluorescence microscope equipped with FITC and TRITC filters.

Figure 8 shows the cell behaviour in a hydrogel in Material 3, from a top view.

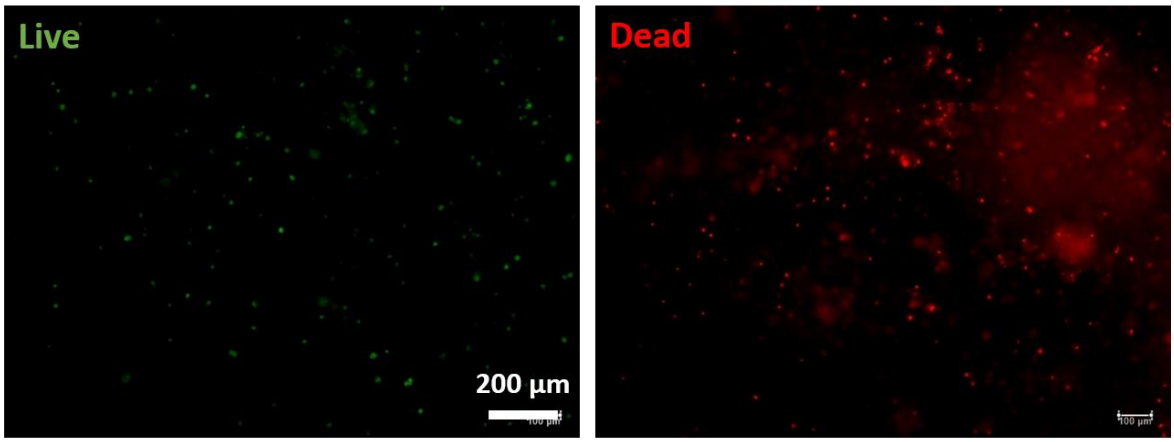

Figure 8: Live/Dead assay performed on the selected formulation of Material 3 - top view. Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

It can be observed the presence of few viable cells and a relatively high number of dead cells. A more complete depiction of cell distribution and behaviour was obtained by analysing the hydrogel from a side view, after being sectioned (Figure 9).

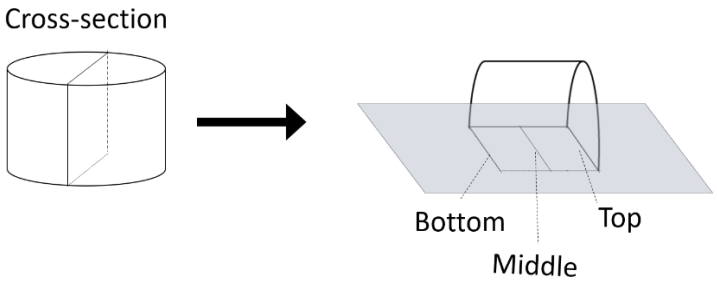

Figure 9: Depiction of the procedure adopted to analyze the cell viability across the hydrogel thickness.

Results are shown in Figure 10.

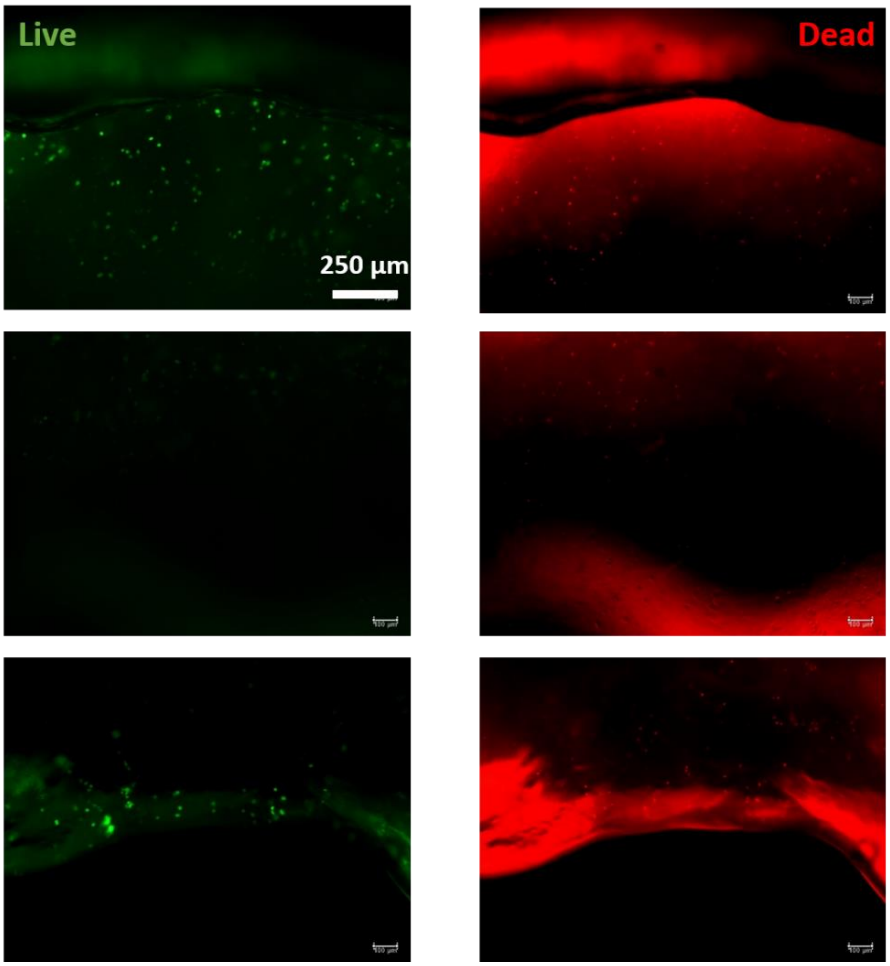

Figure 10: Live/Dead assay performed on the selected formulation of Material 3 - side view of the hydrogel after being sectioned at different levels (top, middle and bottom). Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

As evidenced by the images, such a material formulation does not guarantee a high level of cell viability. The increase in nutrient diffusion, guaranteed by the hydrogel construct, was not enough to guarantee a good cell viability, probably due to a sub-optimal material chemistry or photo-crosslinking residuals.

3 Conclusions

In this Deliverable the results obtained from the characterization of the Pluronic-fibrinogen based hydrogels developed by REGENTIS are reported. In particular, they concern Material 3, a patented thermosensitive Pluronic-F127-fibrinogen hydrogel, suitable for printing, also known with the commercial name of GelrinV.

As in the case of PEG-fibrinogen based hydrogels described in the Deliverable 3.1, the Pluronic-F127 based hydrogels formulations were tuned by changing the amount of Pluronic F127-OH and Pluronic F127-di-acrylate leading to a change in the thermo-responsive properties of each formulation.
From the two prepared batches, namely FF0038 and FF0039, rheological and mechanical properties have been characterized in function of the UV curing time and temperature, respectively. An inconsistence in the batch manufacturing emerged from the obtained results. Also, batch FF0038 resulted more effective in being crosslinked with UV light radiation in a time compatible with the ADMAIORA project requirements. For this reason, the subsequent characterization has been performed only on that batch of material.

From swelling analysis resulted that Material 3 tends to shrink after 24 h at 37°C in a saline solution, with a volume variation of -38%.

Protein diffusivity tests highlighted the absence of marked gradients. The BSA-FITC fully permeated in the gel, without accumulating at the borders, as obtained in the case of Material 2 (see Deliverable D3.1). However, even if this result was promising in view of cell viability, Live/Dead assay performed on human chondrocytes encapsulated in Pluronic-fibrinogen based hydrogels, showed a high number of dead cells. Thus, the Pluronic-fibrinogen based hydrogels do not represent good candidates for the project purposes.