ADMAIORA

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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 with REGENTIS representatives)
3	19/02/2020	Irene Bernardeschi, SSSA	Revision of the second draft and preparation of the third draft
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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

Table of Contents

1 Executive summary

In this Deliverable the prototypes of PEG-fibrinogen-based hydrogel formulations developed by REGENTIS and their main features are described.

In section 2, the key specifications identified for the hydrogels are reported, also with a description of the three material types developed, as well as of the methods and techniques used to optimize the hydrogel formulations based on the required specifications.

Then, in section 2.1, the results of preliminary biological tests performed to assess the viability of human adipose tissue-derived stem cells (ASCs) after cell encapsulation within PEG-fibrinogen printed hydrogels are reported. These tests also served to evaluate the right amount of photoinitiator, UV intensity and exposure time to be employed to have a material respecting the specifications.

After this preliminary analysis, an optimization process for PEG-based hydrogel formulations is described in the same section. This implied to vary: i) the total amount of Pluronic and ii) the ratio of non-cross-linkable and UVA-cross-linkable Pluronic. Material characterization in terms of mechanical and rheological properties, the diffusivity of proteins in the material and cell viability of human chondrocytes are also reported in section 2.1 for the different formulations. Results indicate that the formulations containing a lower amount of cross-linkable Pluronic show a higher swelling, a lower shear modulus (G') and a smaller Young modulus (E), corresponding to a few kPa.

Results on protein diffusivity, obtained by testing some selected formulations, are reported in section 2.2 and suggest that the decreased amount of Pluronic increases the "permeability" of the material to nutrients available in the medium surrounding the hydrogels. However, even the formulation showing the highest diffusivity between the ones tested, does not guarantee a sufficient level of human chondrocytes cell viability, highlighting a possible relevant role played by material chemistry, with the tested cell type.

In conclusion, the PEG-fibrinogen-based hydrogels do not result in good candidates usable as the matrix of the nanocomposite materials envisaged in ADMAIORA.

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 according to 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 highest priority has been given to the 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 a call conference between SSSA, 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 the Deliverable D2.1), and based on some preliminary biological results (see section 2.1) obtained by IOR on ASCs, 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 the nutrients available in the culture medium (see section 2.2). The idea was that the hydrogels not allowing a good diffusion of nutrients should be excluded because they would 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 PEG-fibrinogen based hydrogel formulations: material optimization

In the beginning, the Consortium analysed the biocompatibility of the original PEG-based formulations proposed by REGENTIS. All materials were prepared from sterile solutions in aseptic environment and considered sterile and suitable for cell cultures.

IOR performed preliminary biological tests for assessing the viability of human adipose tissue-derived stem cells (ASCs) after cell encapsulation within PEG-fibrinogen based hydrogels, namely Material 1 and Material 2.

Material 1 resulted not printable in its original form, due to the low viscosity of the solution[6]. For this reason, it was casted after mixing with human adipose tissue-derived human stem cells (ASCs) and UV cured within a 24-well plate (500 µl) by varying the time of exposure (2.5, 5, 7.5 and 10 min). Cell density was set to $1.0x10^6$ cells/ml. Figure 1 shows the cell behaviour post-printing up to 7 days.

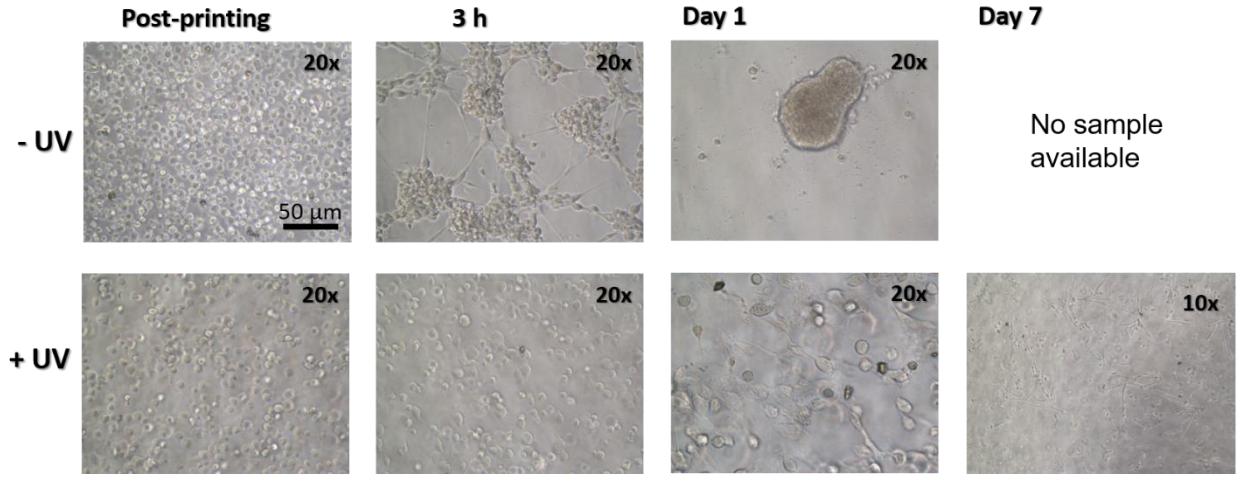

Figure 1: Bright images of cell behavior over time within Material 1. Curing time was set to 5 min.

Without UV curing, cells tended to move towards the top of the hydrogel, forming aggregates already after 3 hours. After such a time-point, we observed a modification of the cell morphology, from a round shape to aggregates with some spindle-shaped cells. Then the material dissolved due to the absence of physical crosslinking (so samples were thus available at day 7). On the other hand, upon UV crosslinking, cells resulted entrapped and well distributed in the hydrogel, showing a relatively nice morphology (round shape and bright aspect). Starting from day 1 we observed a modification of the cell morphology to a spindle-like shape. In the meantime, a Live/Dead analysis was performed to evaluate cell viability within the construct (Figure 2).

[6] 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.

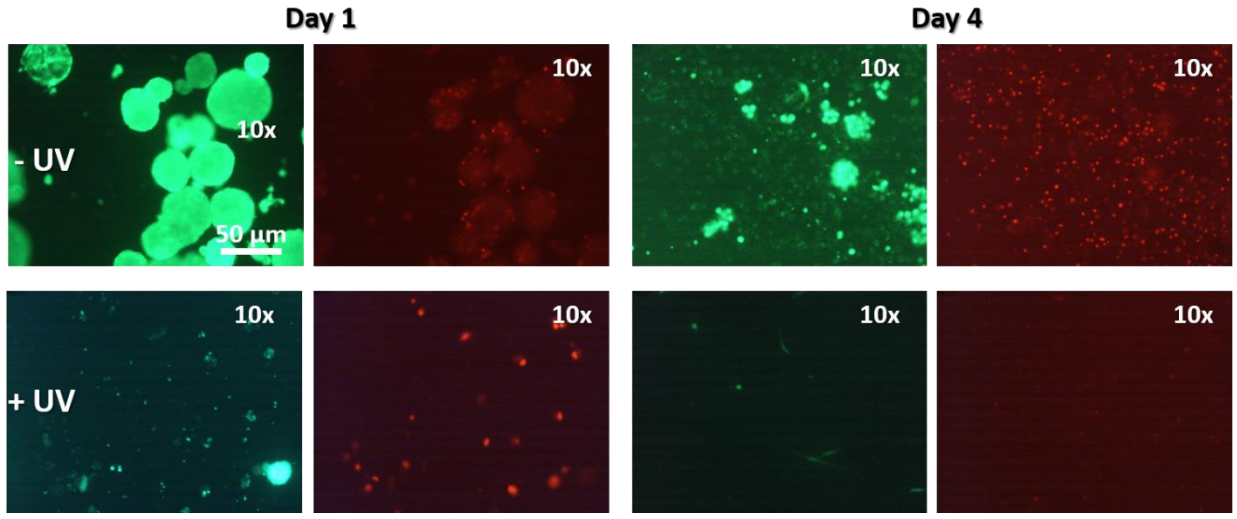

Figure 2: Live/Dead analysis of ASC within Material 1 after 1 and 4 days.

In the absence of the UV light, cells were alive (also cell aggregates) until day 4, when we observed an increment of dead cells. After UV curing, the visualization of cells within the hydrogel became more difficult due to the material properties; maybe UV curing could interfere with L/D test. In any case, a quite relevant number of dead cells were observed.

Such a hydrogel formulation was not considered suitable for the ADMAIORA project having a low viscosity, and not guaranteeing a high level of cell viability.

On the other hand, Material 2 was considered a printable material, as shown in the example reported in Figure 3.

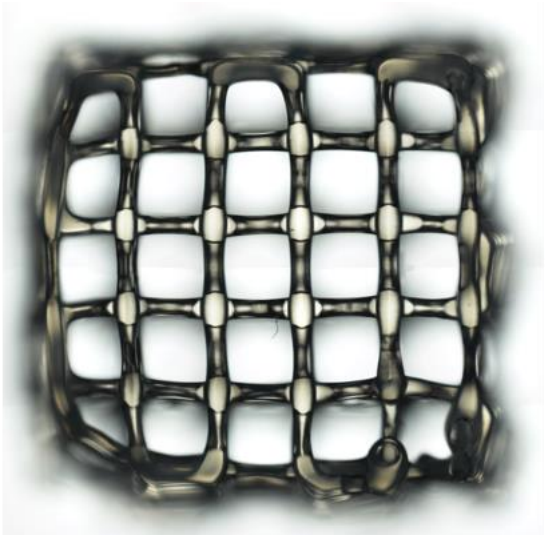

Figure 3: Representative images of a 3D printed scaffold made of Material 2. This material formulation was printed through a needle (diameter: 200 µm) with a pressure of 3 bar at a velocity of 5 mm/s, while maintaining a temperature of 26 °C.

Briefly, 1.5 x 10^6 ASCs/mL were mixed with Material 2 using three concentrations of Irgacure 2959 (0.1%, 0.05% and 0.01% wt.). ASCs were pre-mixed with the gel and then extruded by using a commercially available bioprinter (total volume of the structure: 5 x 5 x 1 mm^3).

Four-layered 3D constructs (each layer with a 200 µm thickness) were designed by using the BioCAD software (RegenHU, CH) with a length of 5 mm and a width of 5 mm, using an inter-

filament distance of 1000 μm. The 3D constructs were crosslinked after bioprinting, by exposing them at a 365 nm UV light for 5 min at 5mW/cm^2. ASCs viability was evaluated post-printing and after 2 and 6 days with a Live/Dead assay.

The results are reported in Figure 4.

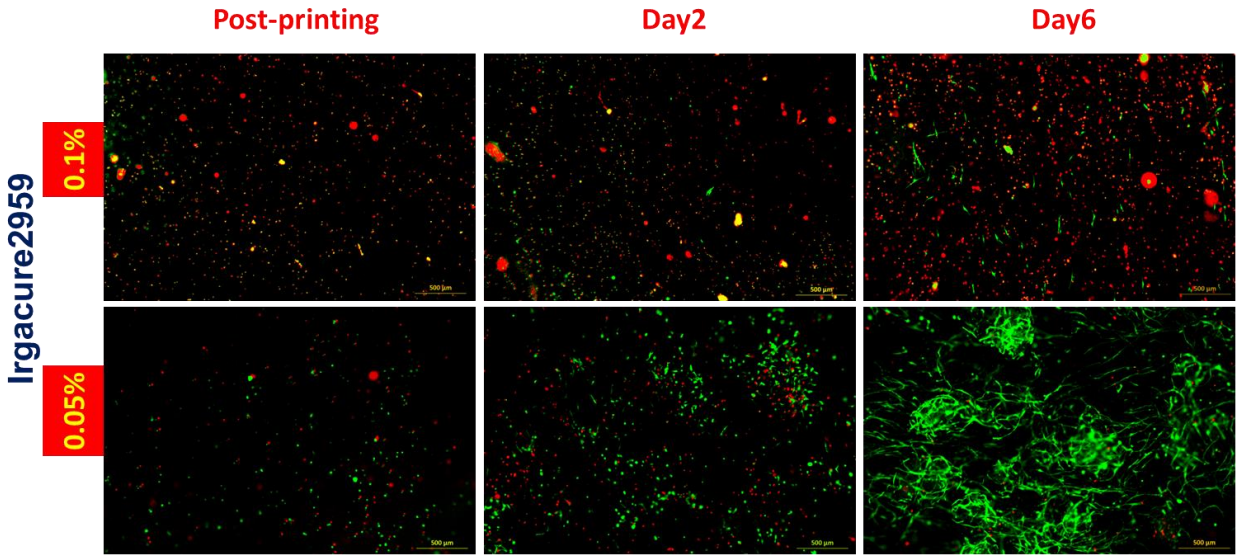

Figure 4: Live/Dead assay performed on Material 2 hydrogel, at different photoinitiator concentrations.

Cells were stably kept into the printed gel in the case of 0.1% and 0.05% concentration of the photoinitiator, while at a concentration of 0.01%, the cells flew out from the material (not shown). However, a high number of dead cells (in red) were observed when using 0.1% photoinitiator, starting from post-printing until day 6. Using 0.05% photoinitiator, cells initially suffered, but a higher number of live cells (in green) was observed at day 6. However, the cell morphology was clearly spindle-like, so not suitable for subsequent chondrocyte differentiation. The metabolic activity of encapsulated cells was also evaluated by MTT test. The results obtained confirmed a better cell behaviour in correspondence to a photoinitiator concentration of 0.05%, featured by a lower level of crosslinking (Figure 5).

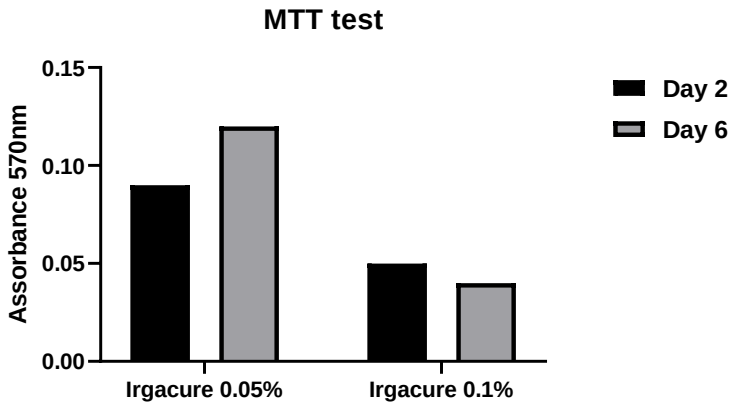

Figure 5: Results of MTT test carried out on 3D ASCs encapsulated in Material 2 with 0.05% and 0.01% photoinithiator Irgacure 2959.

In a second experiment, ASCs were extruded within Material 2 and then cultured for 14 days in differentiation (chondrogenic) medium (no FBS) with or without TGFβ3. TGFβ3 supplementation can initiate chondro-lineage differentiation. Only samples with 0.05% Irgacure were tested (post-printing, day1, day 7 and day 14) with two different cell concentrations (1.5 x 10^6 and 4 x 10^6 cell/mL). The hydrogel was cured post-printing with UV light at 5 mW/cm^2.

The Live/Dead assay (Figure 6) highlighted low viability after printing, and even lower at 7 days, especially without FBS (+TGFβ3 case).

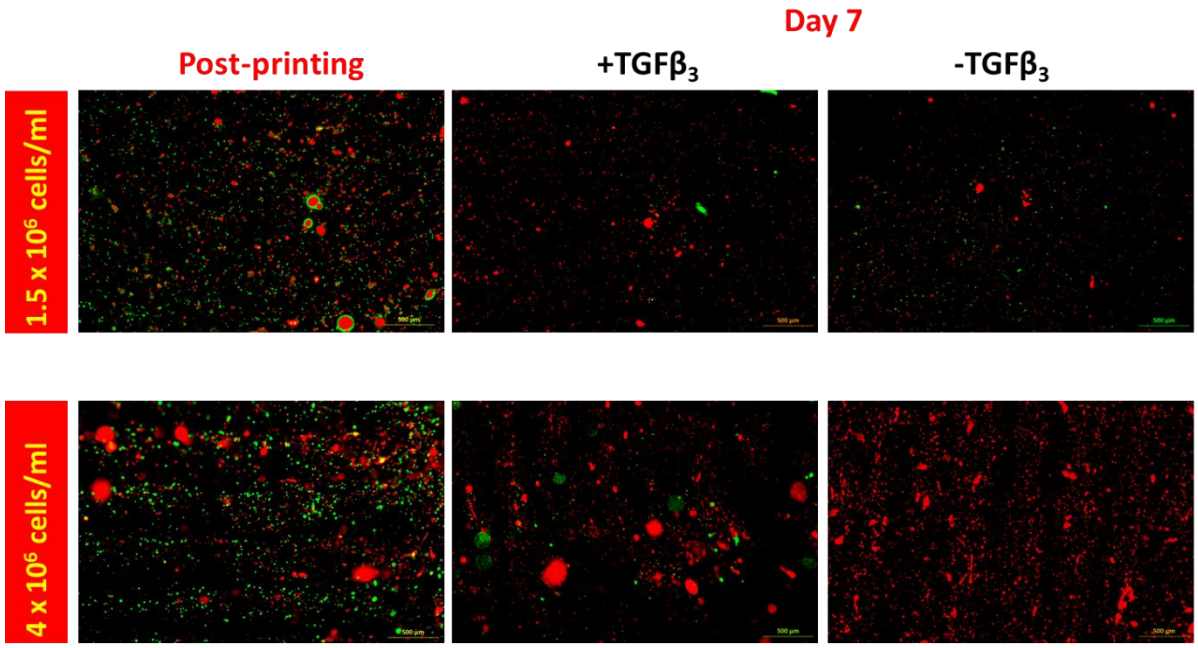

Figure 6: Live/Dead assay performed on REGENTIS Material 2 hydrogel, at different cell density, and in presence/absence of TGFβ3.

Figure 7 shows that cells were almost all dead after 14 days, in both cases.

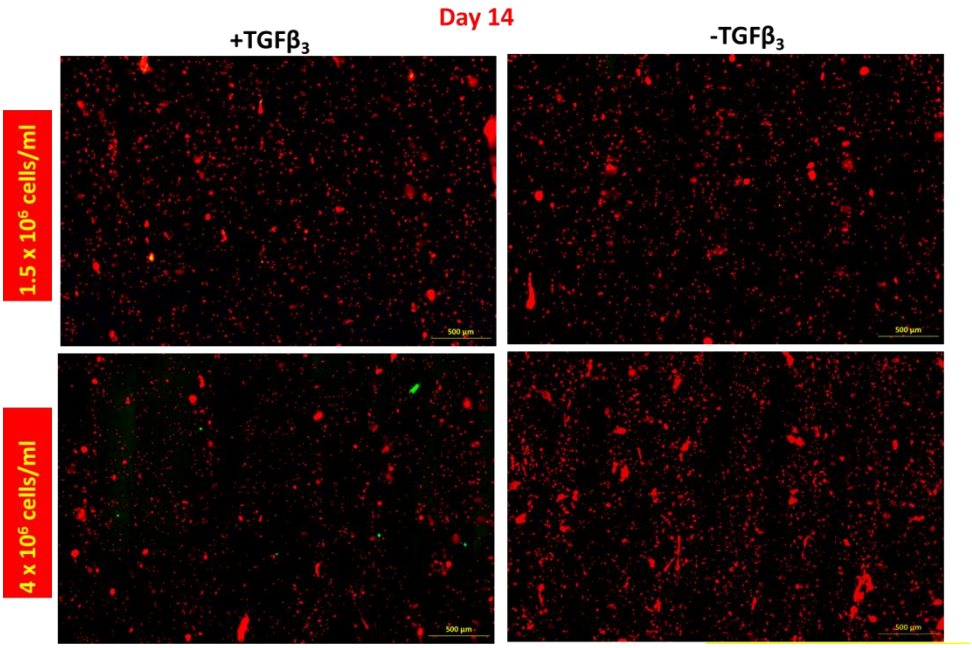

Figure 7: Live/Dead assay performed on REGENTIS Material 2 hydrogel, at different cell density, and in presence/absence of TGFβ3, 14 days after printing.

For these experiments, the control was based on micromass samples (diameter: ~ 250 μm), which demonstrated a good cell behaviour in both culture media.

This result can be probably due to the chemical cues of the material formulation that, combined with a relatively low diffusivity of nutrients within the construct after curing (due to an excessive matrix crosslinking), led to compacting the cells inside the hydrogel, making them suffer.

The exposure time was then lowered to 5 min in order to have a hydrogel formulation in which the cells remain viable for the whole hydrogel thickness of 2 mm, as suggested by clinicians. In addition, REGENTIS worked to modify the material formulation in order to respect the requirements imposed by the project.

Material 1 was excluded, since it resulted not printable and hard to modify. Concerning Material 2, instead, several formulations were prepared and characterized (Table 2), by varying: i) the total amount of Pluronic and ii) the ratio of non-crosslinkable and UVA-crosslinkable Pluronic (i.e. Pluronic-OH and Pluronic-diacrylate or Pluronic-DA, respectively).

Table 2: Different Material 2 formulations and their physical properties.

Formulation:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Total Pluronic Powder % in paste [w/w]	23	23	23	23	21	19.5	19.5	19.5	19.5	19.5	18	18
DA%/OH% out of total Pluronic	34/66	25/75	20/80	10/90	10/90	34/66	25/75	20/80	15/85	10/90	20/80	10/90
DA% in Paste [w/w]	8	5.7	4.6	2.3	2.1	6.6	4.8	3.9	2.9	1.95	3.6	1.8
Gel Transition Temperature [°C] [1] (not cured)	17	17	17	14	18	20	20	20	20	21	22	22

G' at RT (25 °C) [kPa] [1] (not cured)	20	20	20	21	17	13	13	14	13	11	10	10
G' after 5 min Cross linking at 5mW/cm² [1]	50	40	35	30	34	34	30	25	20	16	22	17
Swelling %*[2]	96	128	133	208	225	91	110	119	144	166	119	155

1- G' is measured using a rheometer, on a ~500 µm layer.

2- Swelling was measured on 200 µL plugs (600µm thickness) in PBS for 24h at 37°C, comparing their weight before and after.

Formulation 1 represents the original Material 2 tested with ASCs, as reported above. As expected, the decrease in the total Pluronic concentration yielded a higher gel transition temperature, from 14 (formulation 4) to 22 °C (formulations 11 and 12). All Material 2 formulations underwent swelling after being immersed in liquid (PBS or cell medium) at 37°C for 24 h. The formulations containing a lower amount of Pluronic-DA showed more significant swelling, caused by the higher amount of Pluronic-OH that diffused out of the net, allowing a higher swelling, and a lower G'. This aspect has also been considered during the subsequent protein diffusivity tests.
As can be seen in Figure 8, crosslinked plugs made from formulations 4, 5, 10 and 12 lost their shape after 48 hours in PBS, due to excessive swelling.

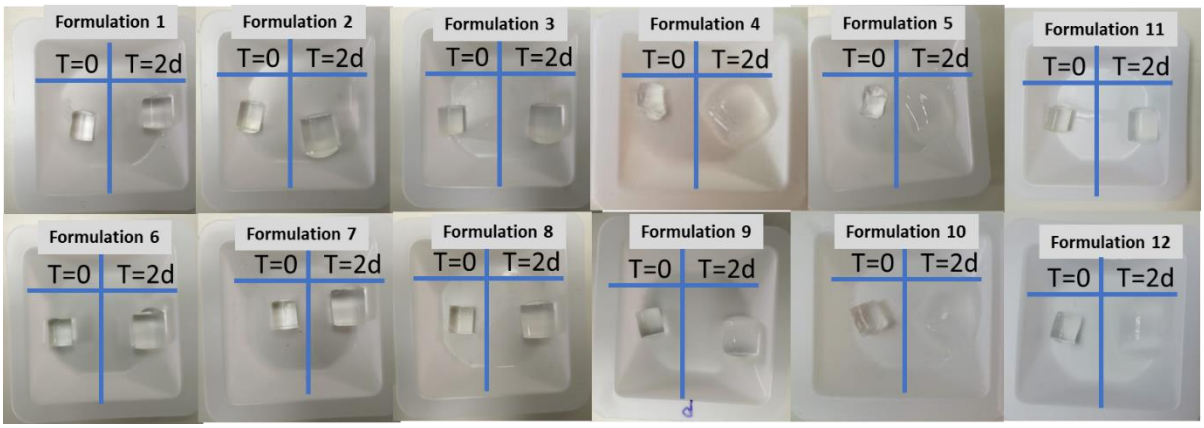

Figure 8: Images of cured hydrogels based on different Material 2 formulations at time zero (t=0) and 2 days post immersion in PBS.

Rheology tests were carried out by 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.
Figure 9 shows the Material 2 behaviour in terms of storage modulus G', according to different content of Pluronic F-127 and Pluronic-DA (the first number in the legend identifies the formulation, from 1 to 12).

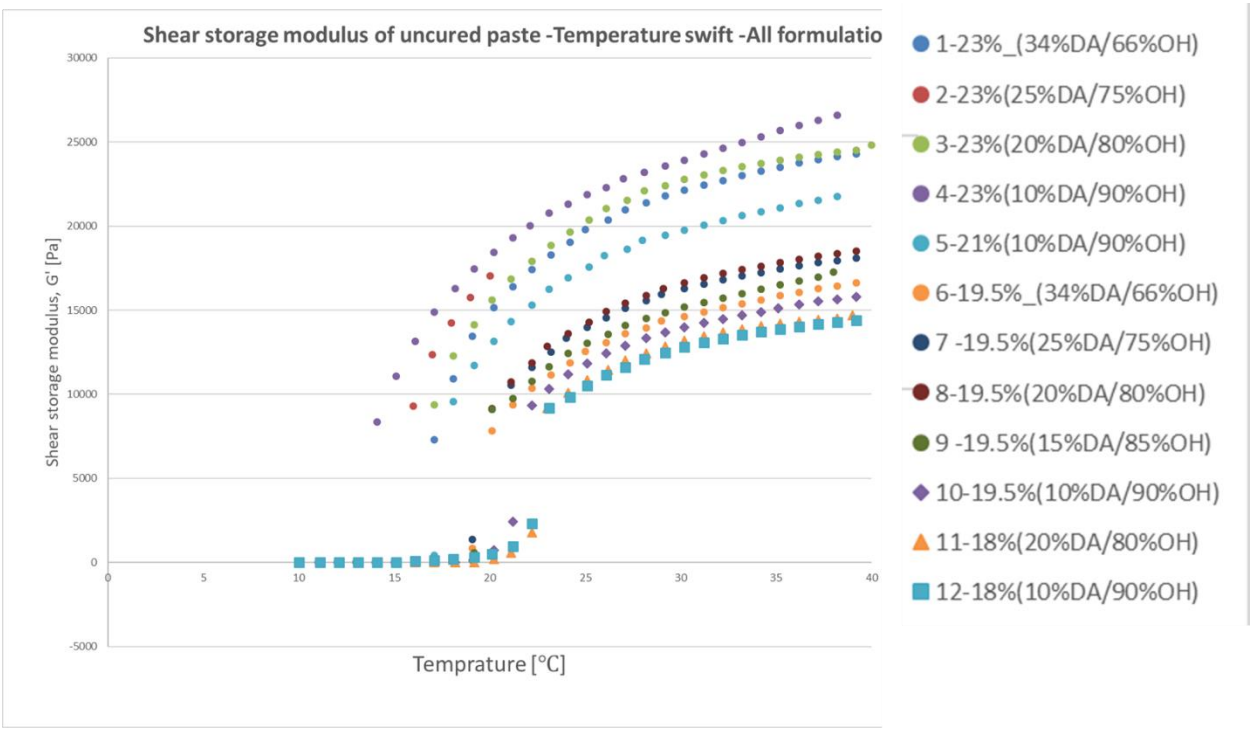

Figure 9: Shear modulus (G') as a function of temperature. The gel transition temperature varied depending on Pluronic concentration.

Figure 10 shows the Material 2 behaviour in terms of storage modulus upon curing with UV radiation, according to different content of Pluronic F-127 and Pluronic-DA (also here, the first number in the legend identifies the formulation, from 1 to 12).

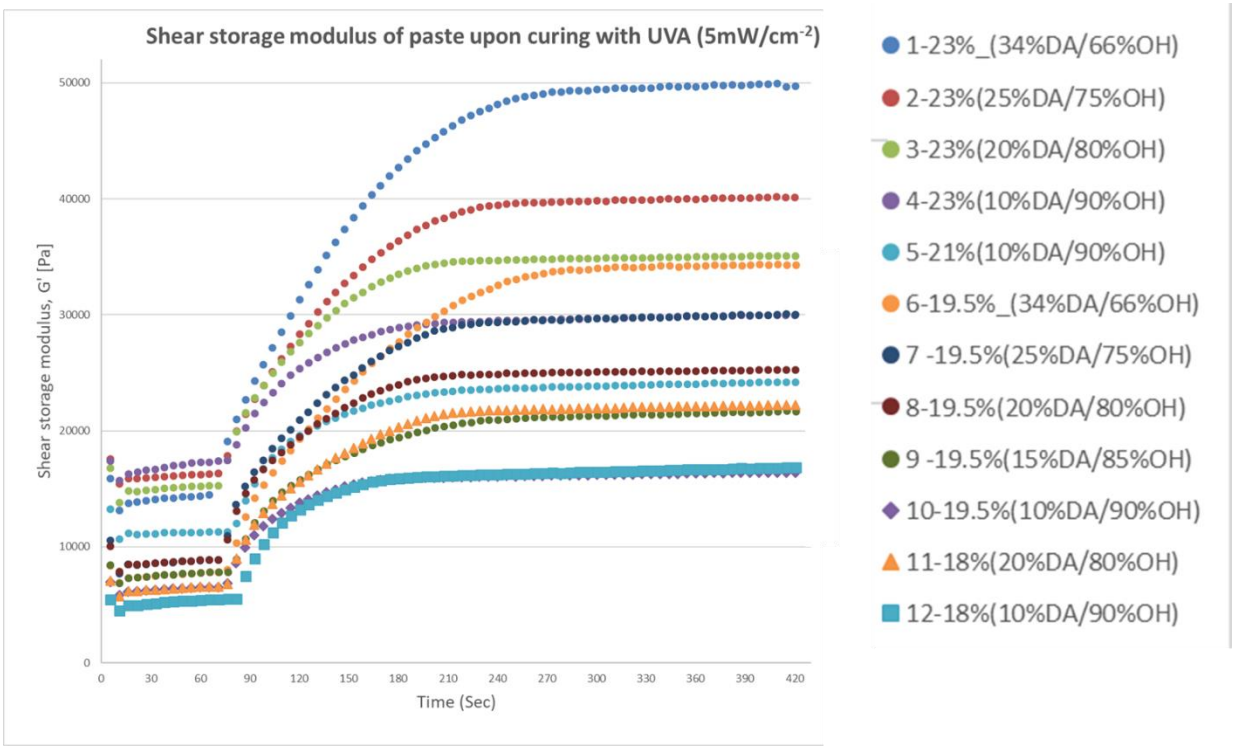

Figure 10: Shear modulus (G') as a function of curing time at room temperature. The modules varied depending on Pluronic concentration.

After these preliminary tests, formulations 1, 3, 7, 8, 9 and 11 were shipped to SSSA for further characterization.

Hydrogels were characterized in terms of compression modulus to assess their mechanical behaviour with respect to the initial specifications reported in Table 1. Uniaxial compression tests were performed using a traction test machine (Instron 2444), applying a compression rate of 1 mm/s. Before mechanical characterization, the samples were UV cured, then kept in PBS at 37 °C for 24 h. Figure 11 shows an example of a hydrogel crosslinked with UV light setting an intensity of 5 mW/cm^2 for 5 min.

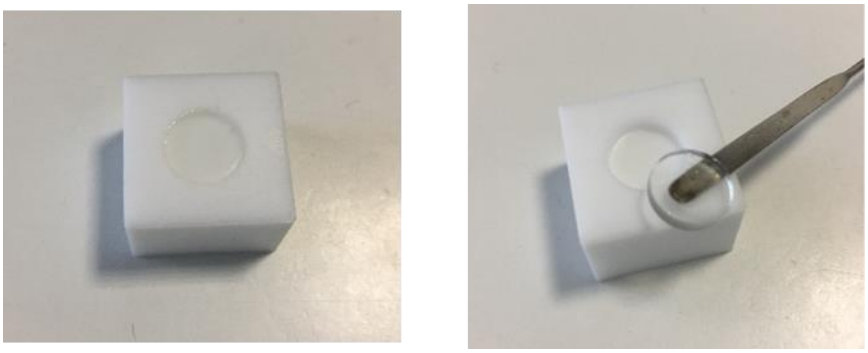

Figure 11: Sample of Material 2 (formulation 1), after UV curing.

Figure 12 shows the compressive modulus for the different hydrogel formulations.

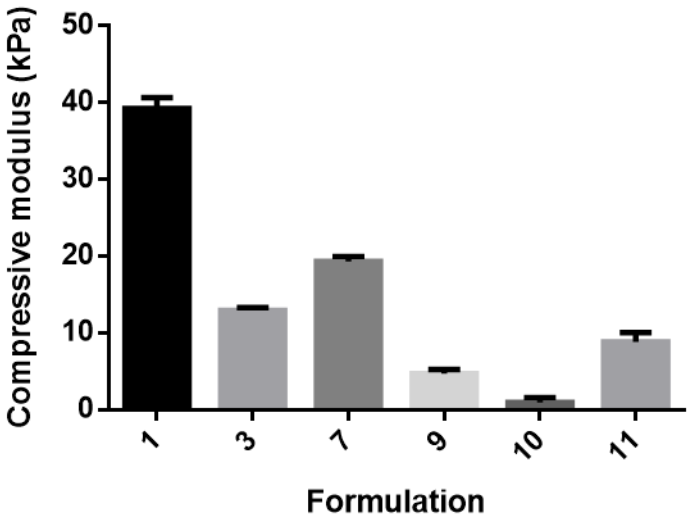

Figure 12: Compressive modulus of various formulations of Material 2.

This analysis was also helpful to assess the degree of crosslinking, by applying the following equation[7]:

$$N = \frac{EQ_v^{-1/3}}{RT}$$

where R is the gas constant (8.314 J/(mol*K)) and T is the temperature at which the mechanical modulus was measured (37 °C).

As noticeable, formulation 1 showed a higher Young's modulus respect to the other samples, due to the presence of a higher concentration of Pluronic. By decreasing its content, we were able to achieve values down to a few kPa, thus mechanically weaker formulations, which could result more suitable for cell culture and nutrient diffusion within the polymeric matrix.

Further analysis of swelling was carried out to evaluate the degree of swelling of hydrogels photo crosslinked with a thickness of 2 mm (Figure 13), summarized in Table 3.

[7] Vannozzi, Lorenzo, et al. "Self-Folded Hydrogel Tubes for Implantable Muscular Tissue Scaffolds." Macromolecular bioscience 18.4 (2018): 1700377.

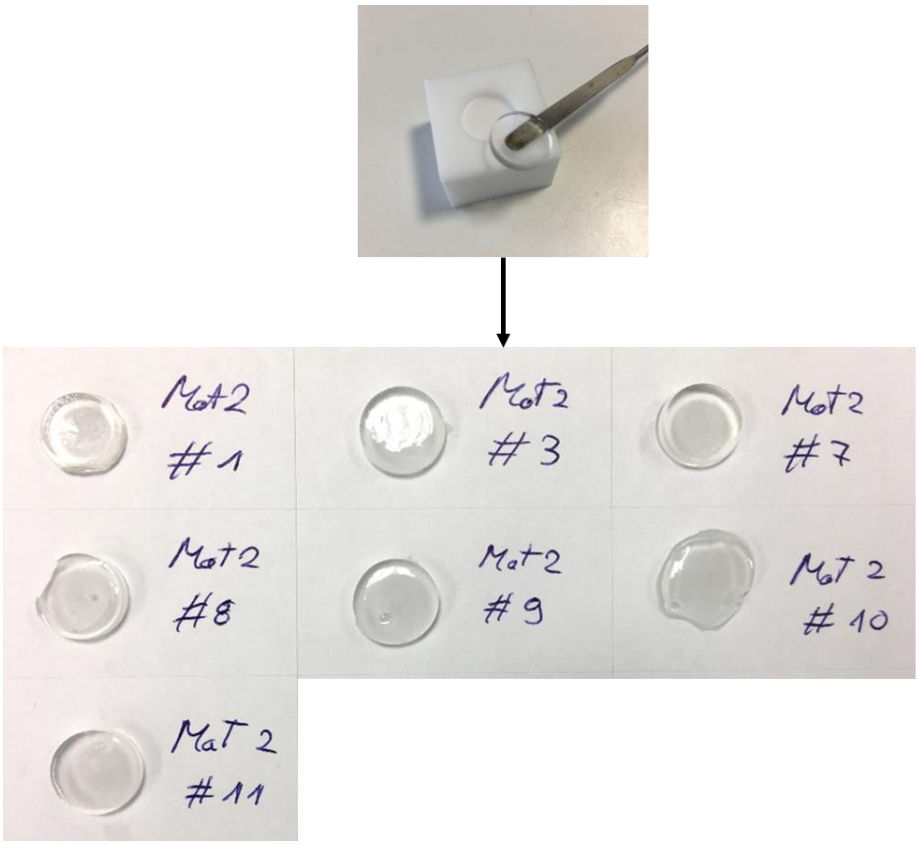

Figure 13: Swelling tests on hydrogels made of Material 2.

Table 3: Analysis of the swelling of each hydrogel formulation. The volume variation has been calculated respect to the initial volume of the hydrogel poured in the well (200 µL).

# Formulation	Diameter (mm)	Height (mm)	Final volume (mm^3)	Volume variation (%) from the initial one (well)
Material 2 #1	11.9 ± 0.1	2.2 ± 0.1	241 ± 6	+21 %
Material 2 #3	12.8 ± 0.2	2.7 ± 0.1	341 ± 16	+71 %
Material 2 #7	13.0 ± 0.1	2.6 ± 0.1	337 ± 11	+68 %
Material 2 #8	12.8 ± 0.3	2.5 ± 0.2	320 ± 30	+60 %
Material 2 #9	13.2 ± 0.1	2.5 ± 0.2	341 ± 17	+71 %
Material 2 #10	13.8 ± 0.3	2.8 ± 0.1	409 ± 6	+105 %
Material 2 #11	13.2 ± 0.1	2.6 ± 0.1	352 ± 5	+76 %

At this point, we decided to continue the analysis by selecting few candidates (formulation 1, 7 and 11) in order to analyse more in depth the features of Material 2 formulations that could be more suitable for cells. Such formulations were chosen by taking into account the lowest content of Pluronic as well as suitable swelling ratios for the clinical application. Formulations 9 and 10 resulted in very weak plugs upon crosslinking and the handling of such materials was more challenging. Formulation 1 was kept as negative control for comparison with the new formulations.

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.

In order to do this, 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.

Albumin from bovine serum, FITC conjugated (BSA-FITC, Mw: 66 kDa, hydrodynamic radius (Rh) ≈ 3.6 nm) solution was used as a model to assess protein/nutrient diffusion. The labelled solution (concentration: 100 µg/mL) was dropped onto the upper surface of each hydrogel (total volume of the 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 three times with PBS to remove the residual fluorescent dye. The cross-sectioned surface was then observed with a fluorescence microscope (Leica-TCS-SP5).
More in detail, protein diffusivity experiments were conducted by following this 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 min), and exposure for 15 min to visible light through a LED (this parameter was taken into account because during the material injection in the cartilage defect, the overall scenario will be kept illuminated by the surgeon);
- Swelling in PBS at 37 °C for 24 h;
- Incubation of the crosslinked hydrogels with 1.4 mL of BSA-FITC (concentration 0.1 mg/mL) solution for 30 min at 37 °C. Five independent samples were tested for each material 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 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 14.

[8] J. Yang et al. Influence of hydrogel network microstructures on mesenchymal stem cell chondrogenesis in vitro and in vivo. *Acta Biomterialia.* 91: 159-172 (2019)

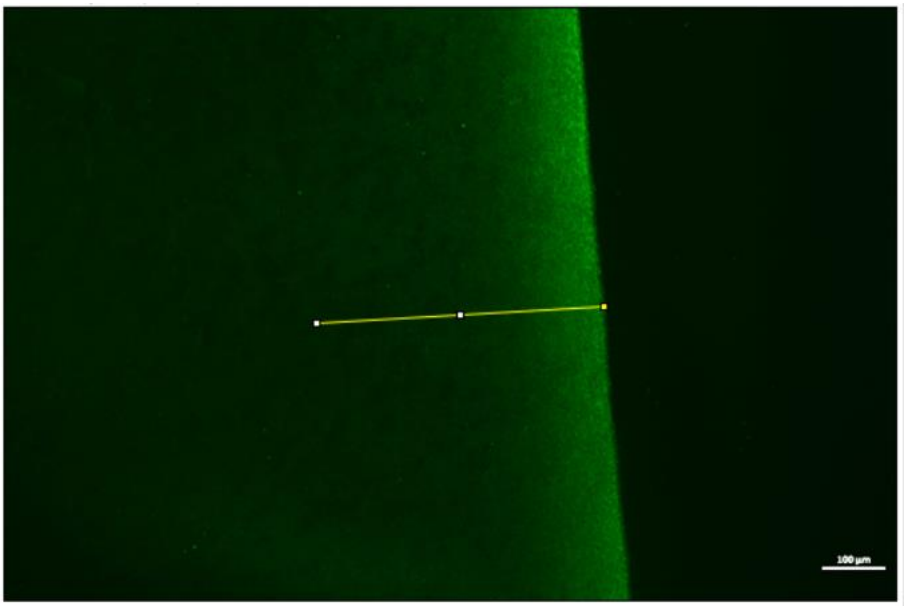

Figure 14: Depiction of the protein diffusion measurement, performed through ImageJ.

A threshold was defined in order to measure the maximum distance at which the protein diffused inside the hydrogel. This value was estimated by evaluating until where the fluorescence intensity resulted in double respect to the background value.

2.2.1 Protein diffusivity results

Figure 15 shows the diffusion of the BSA-FITC within the hydrogel for the three selected candidates (formulation 1, 7 and 11) of Material 2.

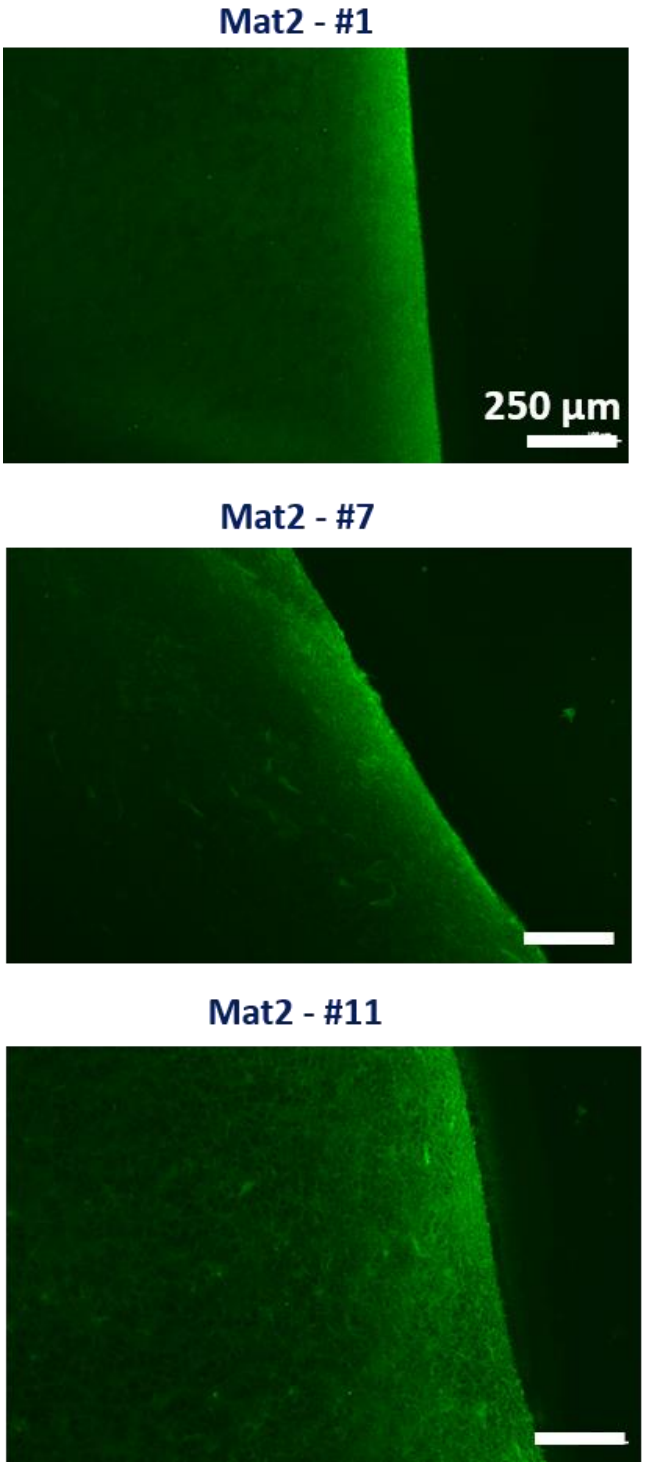

Figure 15: Representative images of the protein diffusivity test performed on the selected formulations of Material 2.

In parallel, we also analysed the blank hydrogels in order to get the correct background signals for each material, as reported in Figure 16.

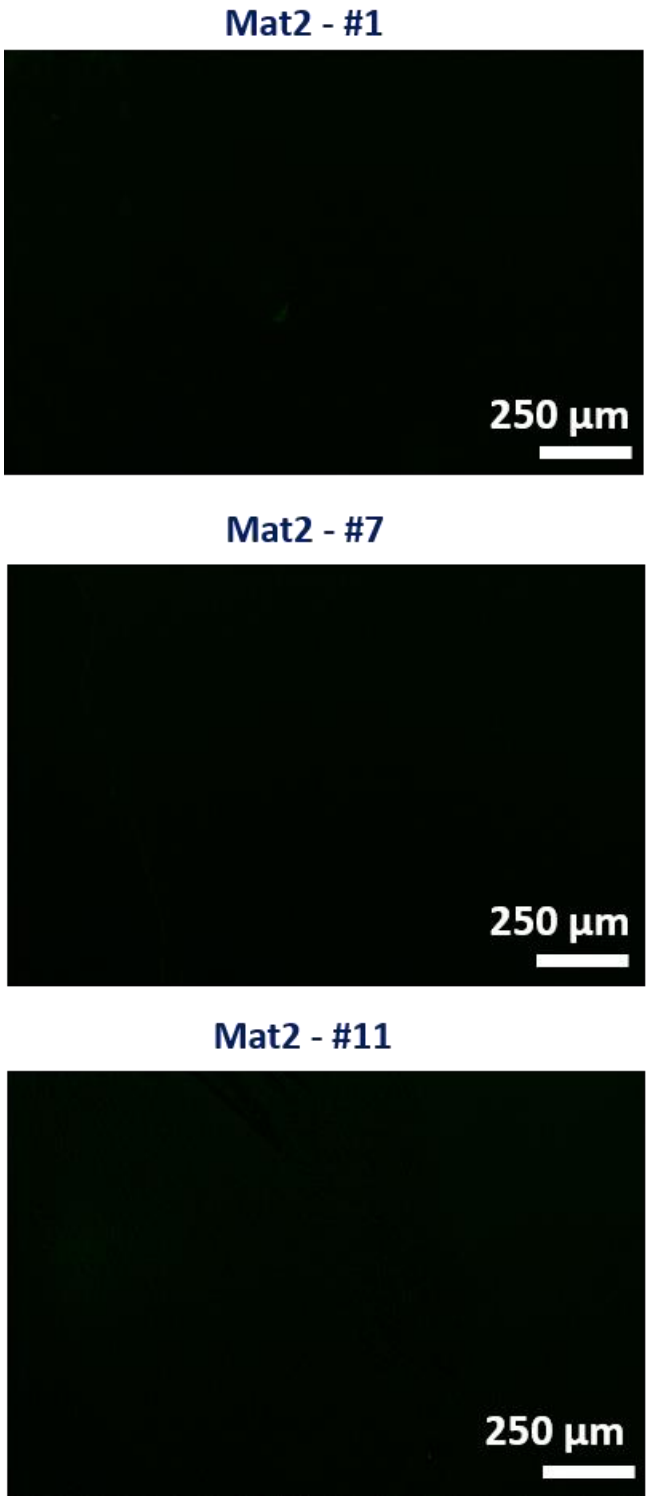

Figure 16: Flourescence measurement on hydrogels based on the selected Material 2 formulations, not incubated with BSA-FITC, to acquire background signals (corresponding to material auto-fluorescence).

Results on the penetration depth of BSA-FITC protein for the different hydrogels are reported in Table 4.

Table 4: Analysis of the penetration depth of BSA-FITC in the different Material 2 formulations.

Material 2 formulation	Penetration depth (µm)	Number of samples
1	424 ± 114	n = 5
7	500 ± 92	n = 4
11	825 ± 206	n = 5

The increase in "permeability" (intended as the capability of the material to allow diffusion of nutrients within its structure) of Material 2 correlated to a decrease of Pluronic content (*e.g.,* for formulation 11). Indeed, formulation 11 showed a double penetration depth with respect to formulation 1, used as control. This result was encouraging in view of cell viability tests.

2.2.2 Cell viability results

Human chondrocytes (Cell applications, Inc.) were used as a cell model to investigate the effects of the hydrogel formulation on chondrocyte viability, before adopting ASCs. For encapsulation experiment within the hydrogels, 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+cells solution was poured inside a polydimethylsiloxane (PDMS) mould, having an internal cavity with a 10 mm diameter and 2 mm height. The PDMS mould was previously sterilized with ethanol and UV light for 30 min. The photo-crosslinking was performed for 5 min at 5 mW/cm^2, afterward the culture medium was slowly added to submerge the hydrogel. Then, the sample was exposed for 15 min to visible light through a LED. The hydrogels were then placed in the incubator at 37°C. An example of the hydrogel obtained before and after being removed from the mould is shown in Figure 17.

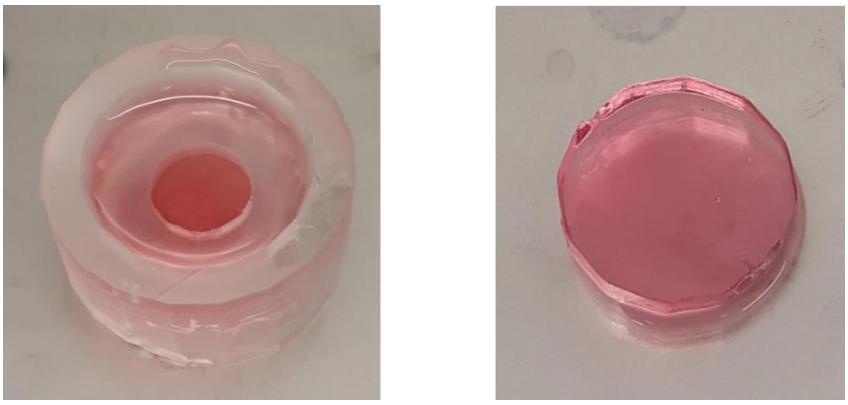

Figure 17: Images of a hydrogel (Material 2, formulation 1) crosslinked within a custom PDMS mould and incubated with cell culture medium.

A Live/Dead test was performed after 3 days to evaluate the chondrocyte viability within the hydrogel (viable cells were stained in green, while dead and necrotic cells were stained in red). The hydrogel was manually cross-sectioned, placed on a slide, and visualized with a fluorescence microscope equipped with FITC and TRITC filters.

Figure 18 shows the behaviour of chondrocytes embedded in a hydrogel made of Material 2 (formulation 1), from a top view.

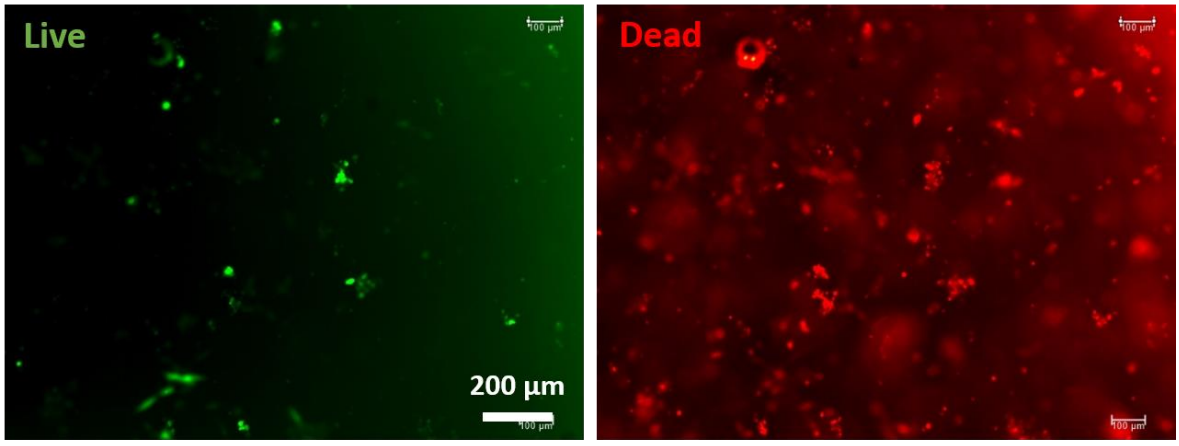

Figure 18: Live/Dead assay performed on Material 2, formulation 1 - top view. Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

It can be observed that a high number of dead cells were present in the sample, on the analysed plane. A more complete depiction of cell distribution and behaviour was obtained by analysing the hydrogel from a side view, after being sectioned (Figure 19).

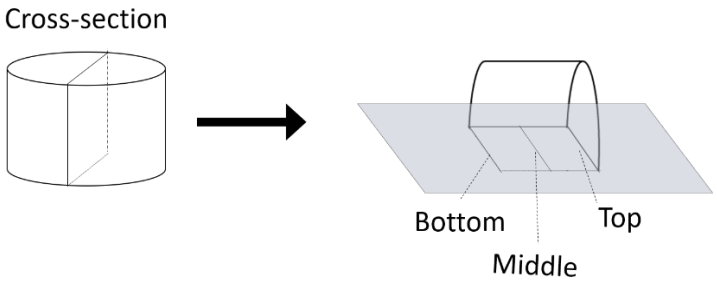

Figure 19: Depiction of the procedure adopted to analyze the cell viability across the hydrogel thickness.

Results are shown in Figure 20.

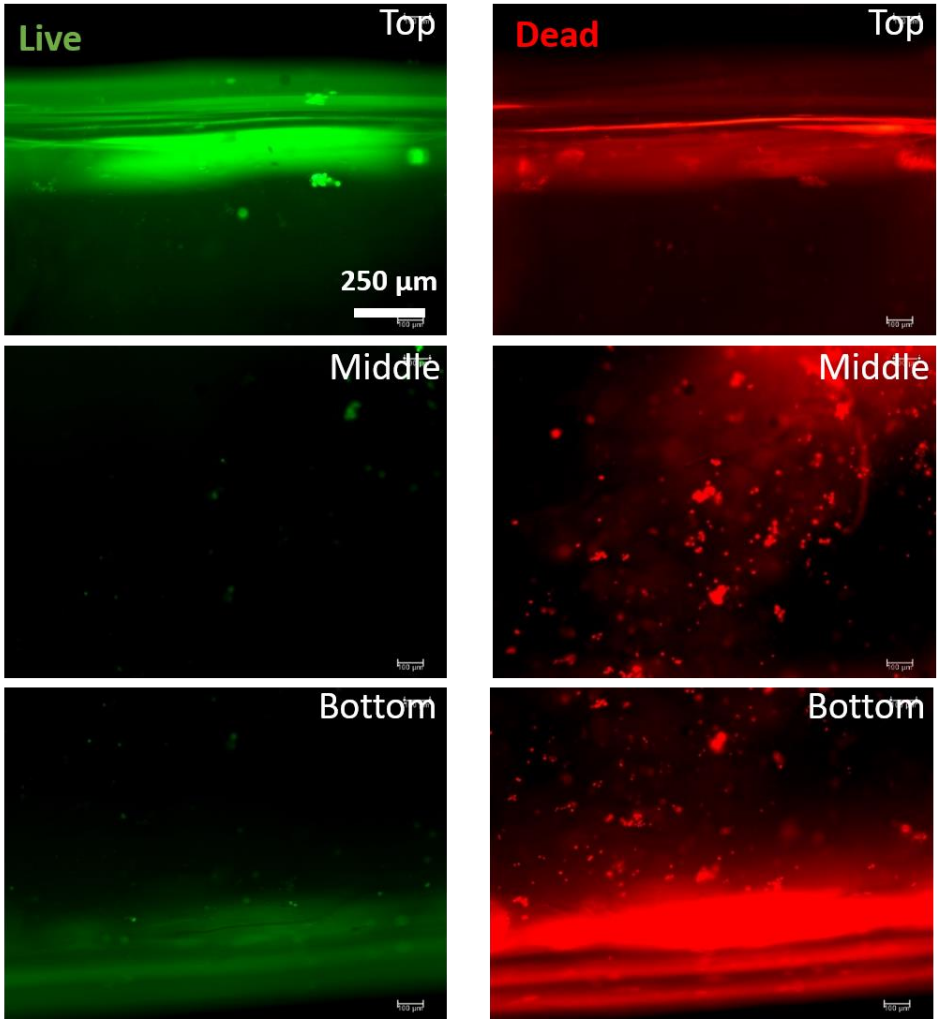

Figure 20: Live/Dead assay performed on Material 2, formulation 1 - side view of the hydrogel after being sectioned at different levels (top, middle and bottom). Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

Results highlighted that such a material formulation did not guarantee a high level of cell viability, also because of the relatively low penetration depth of the BSA-FITC, assessed in the previous section, which predicted a low "permeability" to nutrients.
A similar analysis was performed on formulations 7 and 11, to assess if the efforts made to optimize the material formulation might provide better results than the original one (formulation 1).

Figure 21 and Figure 22 show the results of the Live/Dead analysis, performed on the formulation 7, 3 days after cell seeding and photo-crosslinking.

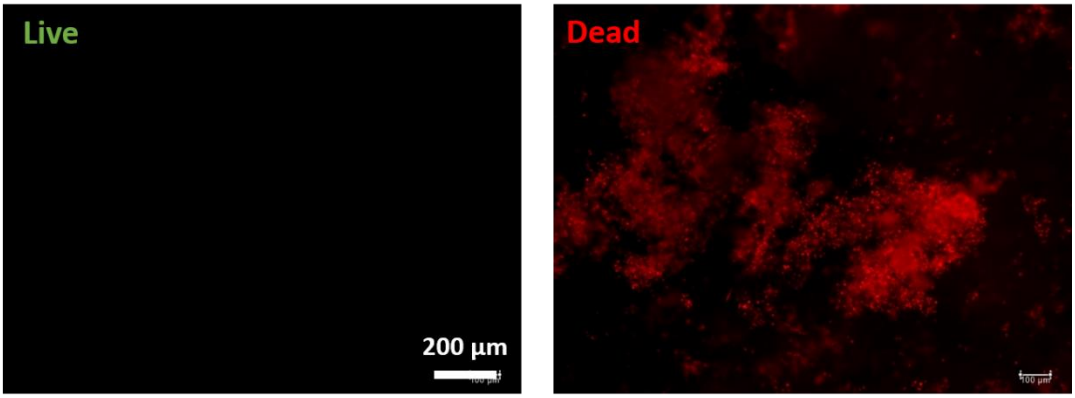

Figure 21: Live/Dead assay performed on the Material 2, formulation 7 - top view. Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

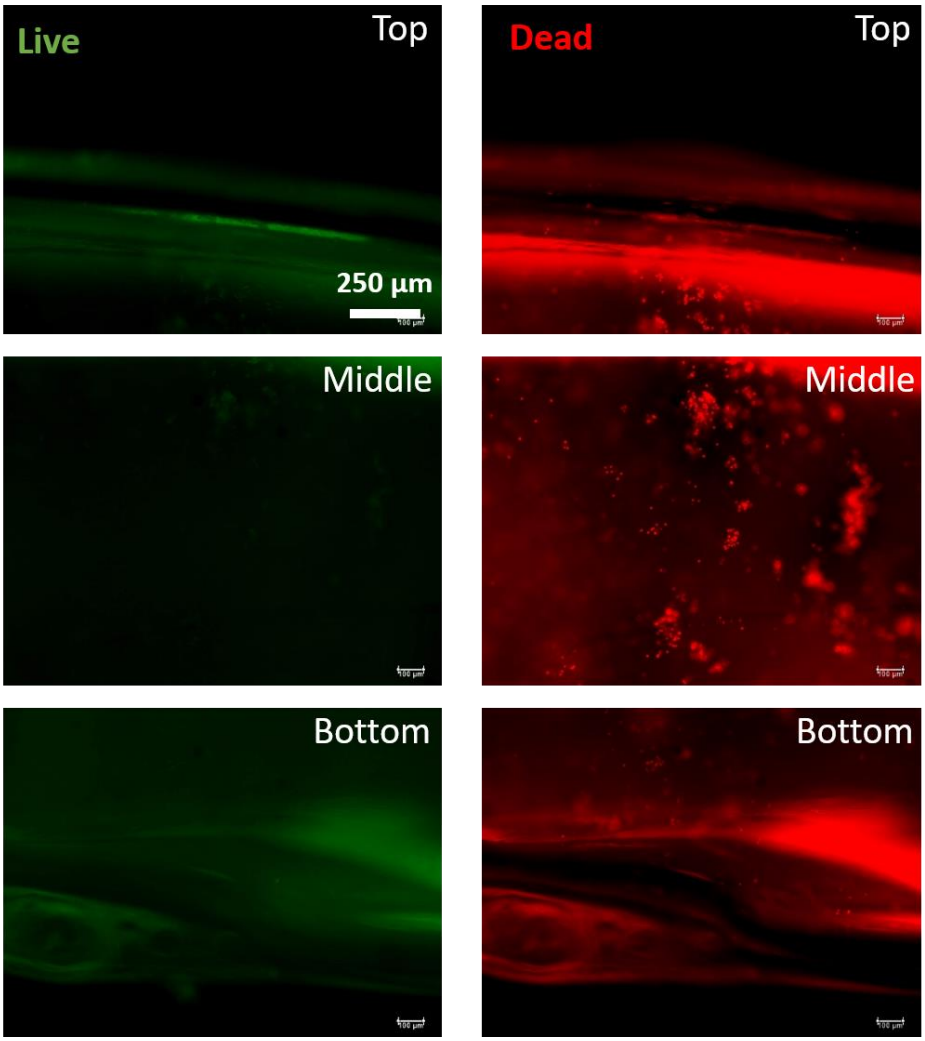

Figure 22: Live/Dead assay performed on the Material 2 - formulation 7 - side view of the hydrogel after being sectioned at different level (top, middle and bottom). Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

Also in this case, the material formulation did not guarantee adequate cell viability.

Figure 23 and Figure 24 show the results of the Live/Dead analysis performed on formulation 11, 3 days after cell seeding and photo-crosslinking.

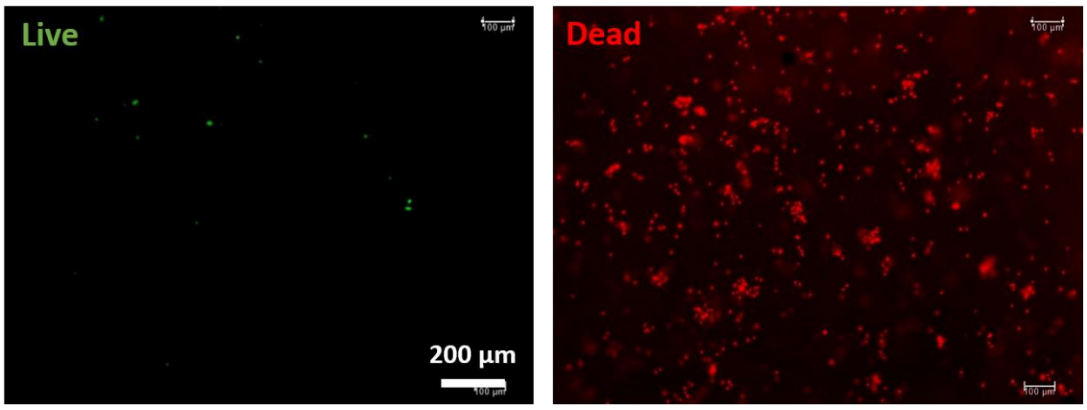

Figure 23: Live/Dead assay performed on the Material 2, formulation 11 - top view. Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

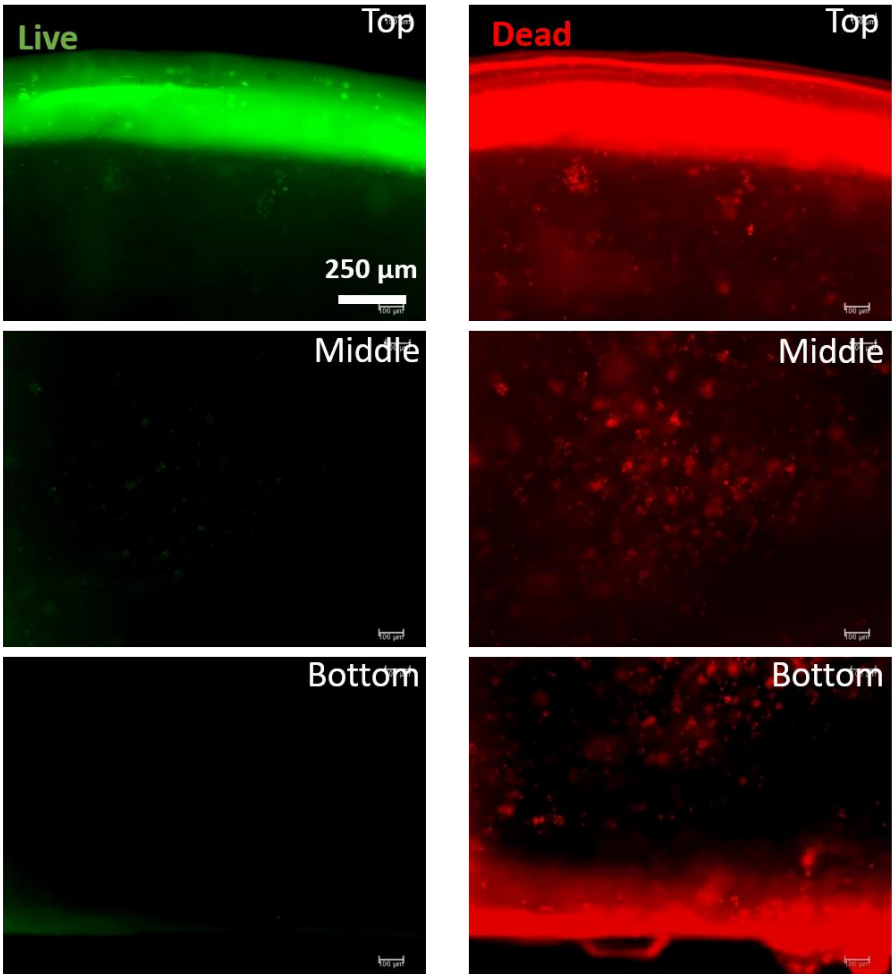

Figure 24: Live/Dead assay performed on the Material 2 - formulation 11 - side view of the hydrogel after being sectioned at different level (top, middle and bottom). Viable cells are shown in green (left), while dead and necrotic cells are shown in red (right).

Even in this last case, a high number of dead cells was observed, despite the encouraging protein diffusivity results shown in section 2.2.1. The increase of "permeability" of the hydrogel construct did not lead to a significant improvement of cell viability, highlighting a

possible relevant role due to the material chemistry. In this case, indeed, this aspect may result more important than other features related to the hydrogel network (such as nutrient diffusivity or mechanical properties), for the maintenance of a high level of cell viability over time.

3 Conclusions

In this Deliverable the results obtained from the characterization of the PEG-fibrinogen based hydrogels developed by REGENTIS are reported. In particular:

1. Material 1: A patented, bio-synthetic finely tuned combination of PEG-DA and denatured human fibrinogen, crosslinkable through UV light, which is a liquid formulation, also commercialized by REGENTIS with the name of Gelrin C.
2. Material 2: A patented, injectable and curable PEG-fibrinogen/Pluronic paste which is based on PEG-fibrinogen conjugate, Pluronic F127 polymer and a photo-initiator.

Preliminary biological tests performed for assessing the viability of human adipose tissue-derived stem cells (ASCs) highlighted a modification of cells morphology to spindle-like shape in Material 1 (both UV-cured and non UV-cured), which resulted not printable, due to too low viscosity. Thus, such material was excluded from further analyses.

Material 2 was printed with cells and different amounts of photoinitiator were tested. Results from Live/Dead assay and MTT test confirmed a good viability and methabolic activity for an Irgacure2959 concentration of 0.05%, compatible with a lower level of crosslinking, but that was enough for retaining cells in the hydrogel. Subsequent experiments with ASCs cultured in chondrogenic medium, however, highlighted a low viability after printing also in the presence of TGFβ3, with almost all cells dead after 14 days of culture. This behaviour can be attributed to chemical cues and also to a too high level of crosslinking of the material that led to a small diffusion of nutrients, as demonstrated from the results obtained testing the penetration of the BSA-FITC protein in some selected formulations of Material 2. REGENTIS worked to modify the material formulation in order to match the requirements imposed by the project, by varying: i) the total amount of Pluronic and ii) the ratio of non-cross-linkable and UVA-cross-linkable Pluronic (i.e. Pluronic-OH and Pluronic-diacrylate, respectively).

Rheological tests and mechanical characterization results indicated that the formulations containing a lower amount of cross-linkable Pluronic had a more significant swelling and a lower shear modulus (G') and a smaller Young modulus (E), corresponding to a few kPa. Protein diffusivity was evaluated on a few selected formulations of Material 2. Results highlighted an increased diffusivity of BSA-FITC protein correlated to a lower amount of Pluronic. However, even the formulation that showed the highest protein diffusivity did not guarantee a sufficient level of cell viability, as resulted from tests performed with human chondrocytes. This suggested that the chemico-physical properties of this material are not suitable for chondrocyte encapsulation.

From the results obtained, the hydrogels made from Material 1 and Material 2 did not result in suitable candidates for the project purposes.