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Overview/Abstract* *Delete as appropriate*

In the present document (Deliverable 3.1, D3.1) we report the main characterization results of the first batch of hydrogels produced by CED (WP2) during the first reporting period of BRIGHTER project.

A first, calibration of the crosslinking density of the photopolymerized hydrogels (~1 cm^2 area x 1 mm thick) was performed at IBEC facilities using light sources of equal wavelength (λ= 405 nm) and equivalent power characteristics as the Ligth-Sheet (LS) system developed by the BRIGHTER consortium (WP1). Main characterization parameters such as the swelling behaviour and the stiffness of the photocrosslinked gels were measured as a function of the exposure dose to determine the energy threshold to produce the formation of self-standing gels. Moreover, crosslinking tests of the same polymers were performed using the first-iteration LS Bioprinter located at the optical laboratory at GUF, providing a range of 3D biocompatible niches suitable for cell culture.

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Led by

Name	Núria Torras	**Partner**	IBEC	**Date**	21.09.2020

Reviewed by

Name	Francesco Pampaloni	**Partner**	IBEC	**Date**	29.09.2020
Name	Elena Martínez	**Partner**	IBEC	**Date**	28.09.2020
Name	Núria Torras	**Partner**	IBEC	**Date**	29.09.2020
Name	Javier Selva	**Partner**	IBEC	**Date**	30.09.2020

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1. Background, current situation

The present deliverable, D3.1, is the first document that encompass the work performed in WP3, which is devoted to the demonstration of the light-sheet bioprinting. A first step, which is very important, is to define the suitable range of photopolymerization parameters for ensuring proper hydrogel network for cells to grow. Thus, in there we report the characterization results in terms of hydrogel crosslinking and stiffness of the first batch of polymer formulations developed by WP2. By the combination of different exposure times and intensities, we studied the swelling behaviour and the mechanical performance of the gels (section 4), together with the diffusion of different FITC-Dextran molecules of different hydrodynamic radius (section 5). Part of this characterization work has been performed at IBEC facilities were a custom-made system parallel to the light-sheet one at GUF facilities was available for testing. This system (described in section 2 of the present document) has a laser diode of 405 nm wavelength and equivalent power characteristics as the ones the light-sheet system has, and will allow performing parallel experiments in the meanwhile the final version of the BRIGHTER's light-sheet printing system is ready. In addition, some experiments evaluating the effect of the exposure time and the energy dose on the hydrogel polymerization were performed at GUF facilities, using the first-iteration of the light-sheet bioprinting system developed by WP1. A final outlook of the further steps envisioned for the next characterization experiments is provided.

1. Bioprinting systems at IBEC facilities

To perform the first characterization tests and optimize the crosslinking parameters of the Norbornene-based polymer samples provided by WP2, we have available at IBEC facilities two different light-based photopolymerization systems:

(i) a Digital light processing stereolithography (DLP-SLA) 3D printer machine, and,
(ii) a direct laser writing (DLW) system.

The main characteristics of both systems and the main purpose of using each of them will be described in this section.

Digital light processing – Stereolithographic (DLP-SLA) 3D bioprinter

Based on a commercially available DLP-SLA 3D printer, this system was customized to allow the crosslinking of small volumes of soft and highly transparent polymers using visible light. The power density of the light source can be adjusted to work between 3.1 mW/cm^2 and 12.3 mW/cm^2 within the spectral range from 320 to 640 nm wavelengths. Additionally, a temperature controller was coupled to the system to keep the polymer solutions into a cuvette, the VAT, warmed at 37ºC, preserving them in liquid form, and providing a proper environment for using cell-laden polymers as bioinks. Figure 1 shows a schematic representation of the DLP-SLA 3D printing system together with an example of a print.

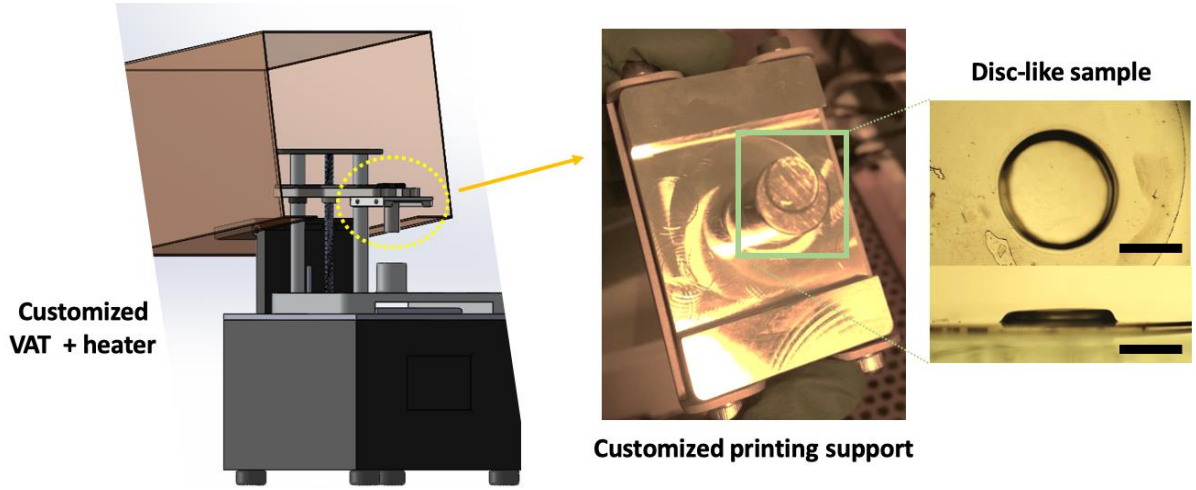

Figure 1 – *Schematics of the DLP-SLA 3D bioprinting system available at IBEC facilities. The printing support and the polymer cuvette (VAT) were customized for printing structures using lower sample volumes. Example of disc-like printed sample of 3 mm of diameter and 300 μm of thickness (right).*

Direct laser writing (DLW) system

This custom made direct laser writing (DLW) system was assembled at IBEC facilities with the main objective of characterizing the crosslinking capabilities of different soft and transparent polymers. It consists on a 405 nm wavelength laser diode coupled to automated translational stages. By means of a collimator, a lens tube and a 10x objective, and varying the power and the translational stage speed, the laser beam can be controlled

and be directed to the surface of a movable printing support, where the hydrogel will be crosslinked, within a working power range between 1 mW to 40 mW. The apparatus is connected to a PC from which the laser trajectory, and its writing velocity and acceleration can be controlled by means of homemade LabView interface. A picture of the current system is shown in Figure 2a together with its corresponding schematics (Figure 2b).

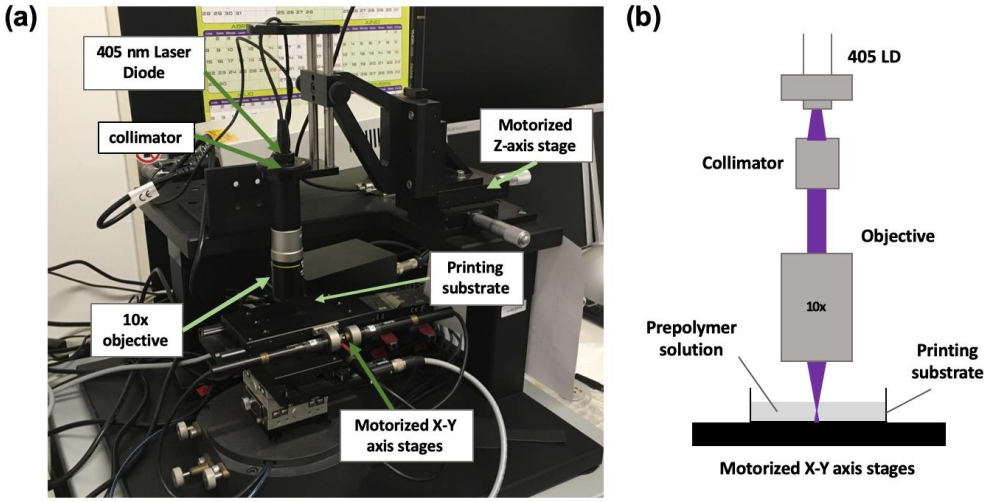

Figure 2 – *Custom made 405 nm wavelength Direct laser writing (DLW) system available at IBEC facilities. (a) Picture of the real system with its main parts, and (b) schematic representation of the system.*

Some examples of the geometries that can be obtained using this crosslinking system are shown in Figure 3; mainly an extrusion of simple 2D structures.

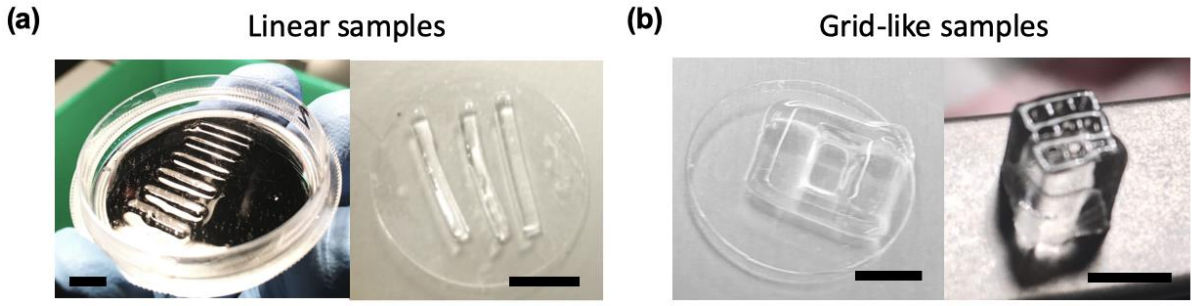

Figure 3 – *Examples of samples geometries that can be printed using DLW system. (a) linear samples of up to 1mm width, thicknesses from 100 µm to few millimetres, with variable length, and (b) grid-like samples (extruded simple 2D geometries). Scale bars = 5 mm.*

2. Identification of the best polymer formulation for BRIGHTER purpose

First crosslinking tests were performed using the DLP-SLA printing system to compare the polymerization results with the previous ones obtained by IBEC group using acrylate-based hydrogels. The polymer formulations used for that purpose were the following ones, according to the materials provided by WP2. For all of them, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator was used to allow photopolymerization:

- Pullulan-Norbornene + PEG-Link (N-PLN_PEG),
- Pullulan-Norbornene + Hy-Link (N-PLN_Hy),
- PVA-Norbornene + PEG-Link (N-PVA_PEG).

First of all, the printing parameters such as the layer thickness and the exposure time per printed layer were optimized for each formulation to find the best printing conditions. Once adjusted, prints of disc-like hydrogels were performed for each formulation. Figure 4 shows some examples of the prints obtained for each different polymer formulation.

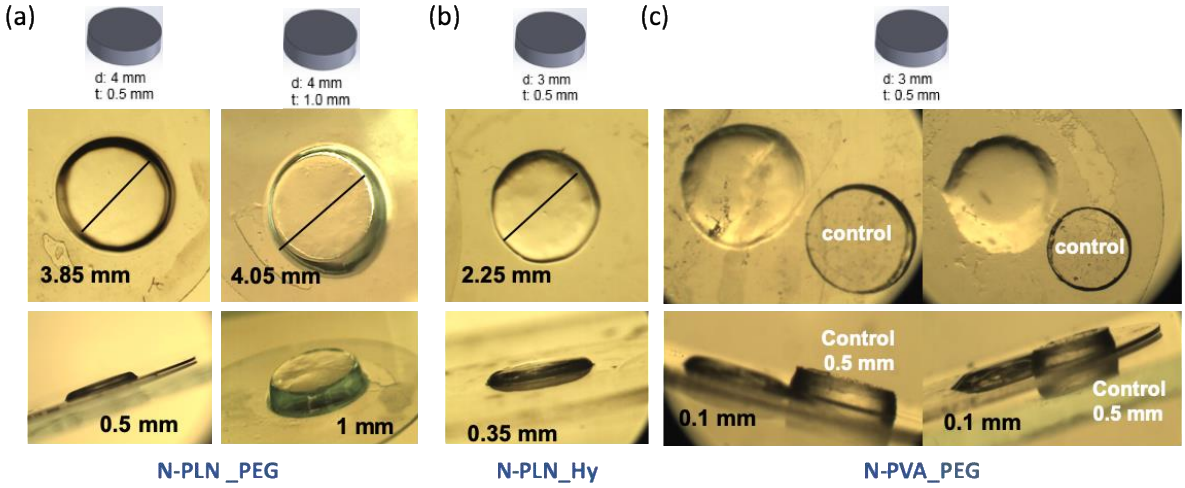

Figure 4 – *Polymerization tests using DLP-SLA system of the different samples provided by WP2: (a) N-PLN_PEG, (b) N-PLN_Hy, and (c) N-PVA_PEG. Top sketch shows the theoretical samples design and dimensions, whereas pictures below, show the real prints with their corresponding dimensions.*

Top and lateral views of the prints proved differences on the crosslinking behaviour of the three different polymer formulations tested (see table 1), evidenced by the resulting gel consistencies and shape definitions.

Table 1- *Composition and molecular concentrations of the polymers used for testing, according to WP2 findings.*

Polymer ID	Polymer composition	Molecular content
N-PVA_PEG	N-PVA : PEG-link : LAP	5 mM : 5 mM : 5 mM
N-PLN_Hy	N-PLN : Hy-link : LAP	4 mM : 4 mM : 0.2 mM
N-PLN_PEG	N-PLN : PEG-link : LAP	5 mM : 4 mM : 0.2 mM

As observed in Figure 4c, samples containing N-PVA_PEG polymer were not able to properly crosslink, resulting in soft gels unable to keep their geometry that cannot be manipulated. N-PLN_Hy samples crosslinked better, but still were too soft, resulting in thinner and smaller prints (Figure 4b). N-PLN_PEG gels, however, polymerized better and perfectly resembled the designs after printing (Figure 4a). The crosslinking was consistent, and samples could be manipulated for further testing.

Same samples were also tested using the DLW system obtaining similar results and thus confirming the suitability of N-PLN_PEG gels for further experiments.

3. First characterization results of photopolymerized hydrogels

Volumetric swelling measurements

Once identified as best formulation, volumetric swelling behavior of N-PLN_PEG-link polymers were investigated by monitoring the variations in sample dimensions in both, longitudinal and tangential directions. By means of this assay, the impact of the polymerization conditions on the water uptake capacity of the resulting networks can be identified for better designing the polymerization patterns in later stages of the BRIGHTER project.

For that, the 405 nm wavelength DLW system was used. Rectangular shaped samples of 3.0 mm length, 0.6 mm width and 0.5 mm thickness were printed using the polymerization conditions in Table 2. Right after photopolymerization, hydrogels were washed out using warmed PBS and carefully wiped with a KimWipe tissue (Kimtech Science) to remove any excess of liquid. Pictures from top and lateral sides of the samples were taken using a Stereo Microscope (Olympus, SZX2-ILLB) to have the initial length, width and height values, L_0 , W_0 and H_0, respectively. Samples were then kept submerged in PBS at 37°C (mimicking the standard cell culture conditions) to induce swelling until reaching the equilibrium, exchanging the PBS buffer solution every day. To monitor the process, pictures from both longitudinal and tangential directions were taken at appointed times (0, 30, 60 minutes and after 2, 3, 4, 6, 24, 48 and 72 hours). Three replicates (n=3) were printed per time point.

The volumetric swelling ratio, S_V (%), of the printed hydrogels was determined using the following expression,

$$S_V(\%) = \frac{(V_t - V_0)}{V_0} \times 100$$

were V_0 is the initial sample volume, and V_t is the sample volumes at time 't'. $S_V > 100\%$ mean volume increase, whereas $S_V < 100\%$ volume decrease.

Table 2- *Polymerization conditions for the swelling measurements.*

Gel formulation N-PLN:PEG_link:LAP	Sample ID	Power (mW)	Scanning velocity (mm/s)
5 mM : 4 mM : 0.2 mM	i35	13	0.3
	i45	22	0.3
	i55	30	0.3
	i45s	22	0.15

Figure 5 shows the volumetric swelling evolution of the different polymerization conditions tested to visually compare similarities and differences on their swelling profile. As observed in the graphs, after 72 h, the equilibrium swelling was reached for all the polymerization conditions tested, resulting in very similar swelling profiles. Figure 6

shows a comparison of the volumetric increase for each condition at 24 h, 48 h and 72 h, from where one can confirm that the equilibrium swelling was reached. Despite the condition i45 seems to undergo a higher water uptake, the deviations on the measurements for this particular condition were also high, resulting non-significative the variation among the four different conditions.

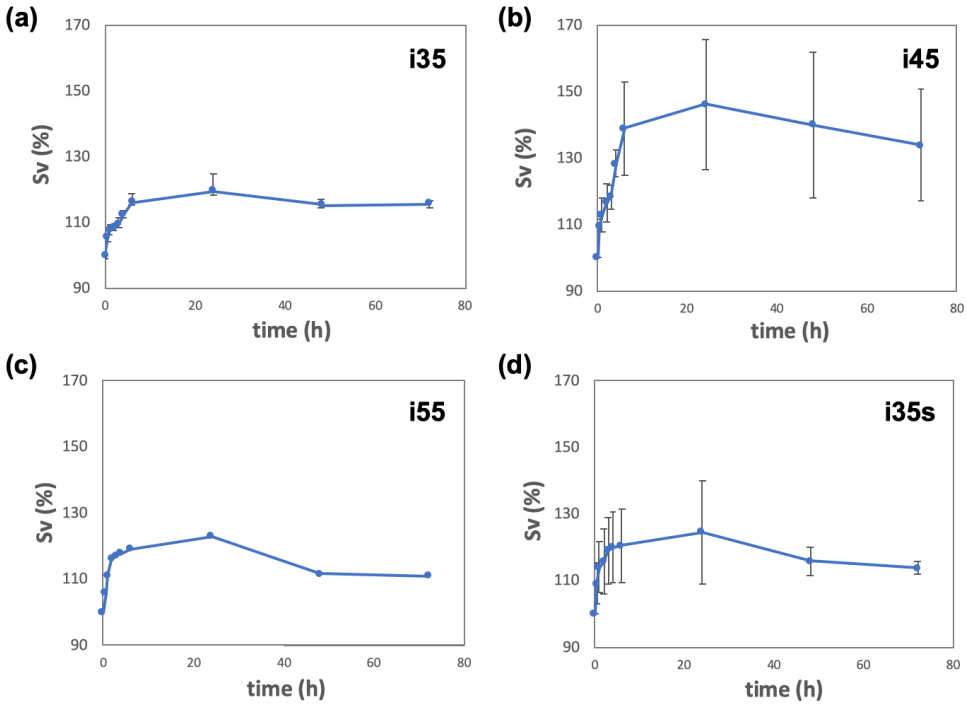

Figure 5 – *Volume increase monitoring (in percentage) of the different N-PLN : PEG link : LAP (5 mM : 4 mM : 0.2 mM) polymerization conditions tested. (a) sample i35, (b) sample i45, (c) sample i55 and (d) sample i35s.*

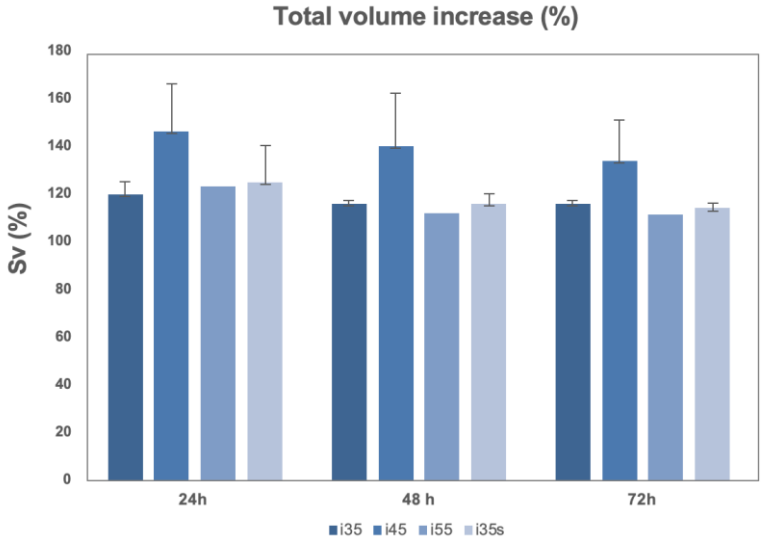

Figure 6 – *Comparison of the final volumetric ratio for each tested condition.*
These results obtained are within the same range of the reported swelling values for other types of hydrogels with lower macromer content, such as Gelatine methacryloil (GeLMA) and poly(ethylene glycol) diacrylate (PEGDA), commonly used as tissue engineering materials and 3D bioprinting applications [1].

Stiffness calibration

ATOMIC FORCE MICROSCOPY

In parallel to the swelling behavior, the mechanical performance of the samples was investigated using atomic force microscopy (AFM). AFM is a well-known technique for the local measurement of the mechanical properties of materials at the surface level. Several AFM indentations were performed on the surface of rectangular-shaped N-PLN : PEG link : LAP (5 mM : 4 mM : 0.2 mM) samples (3.0 mm length, 0.6 mm width and 0.5 mm thickness), obtained with the DLW system. Samples were crosslinked on functionalized glass coverslips to avoid detachment, using the fabrication conditions listed below (Table 3).

Table 3- *Polymerization conditions for the swelling measurements.*

Gel formulation N-PLN:PEG_link:LAP	Sample ID	Power (mW)	Scanning velocity (mm/s)
5 mM : 4 mM : 0.2 mM	i35	13	0.3
	i45	22	0.3
	i55	30	0.3
	i45s	22	0.15
7.5 mM : 6 mM : 0.2 mM	i45h	22	0.3

A JPK Nanowizard 4 machine (JPK Instruments) was used to perform the measurements with a V-shaped cantilever with a quadratic pyramidal tip of 35° (θ) face angle, and a nominal spring constant (k) of 0.08 N m^{-1} (PNP-TR-20, Nanoworld). After calibration of the sensitivity, series of local force–displacement (F–z) curves were measured at several points on the surface of the samples, indenting up to 5 µm. The values of the local surface stiffness were obtained by applying Hook's law and the Hertz model for a quadratic pyramidal tip to the force–displacement curves, assuming a Poisson's coefficient of ~ 0.5.

Graph in Figure 7 shows the results obtained for the different samples measured (two replicates each except the last one), revealing the soft nature of the crosslinked gels, with resulting Young's Modulus below 1 kPa. Grey dots referred to different indentation points per sample (n ≧ 10). Due to its softer nature, some of the samples could not be measured for tip indentation difficulties, and in some others, the deviation in the values obtained was too large to be considered as good data.

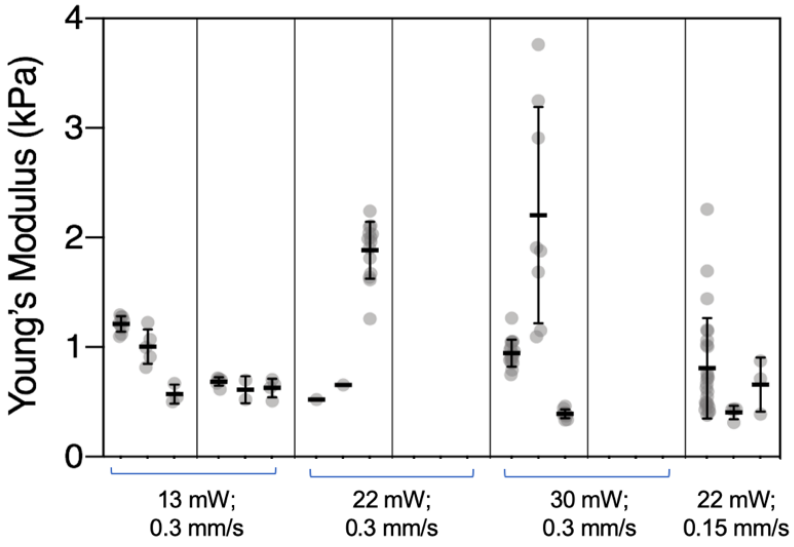

Figure 7 – *AFM measurements of N-PLN : PEG link : LAP (5 mM : 4 mM : 0.2 mM) samples. Soft gels were obtained with an averaged Young's Modulus below 1 kPa.*

Taking into account the results obtained and the soft nature of the samples, an alternative technique was investigated to obtain the bulk (global) properties of the gels. For that purpose, a rheological technique was chosen.

RHEOLOGY

To determine the bulk mechanical behavior and flow characteristics of the printed hydrogels, rheology measurements were performed using samples with the same polymerization characteristics of the ones already evaluated with AFM technique. However, this time the shape of the printed samples was modified due to testing requirements (increase the surface contact between the measurement plates).

Squared-like samples of 8 mm side and 0.6 mm linewidth were printed using our standard N-PLN : PEG link : LAP formulation (5 mM : 4 mM : 0.2 mM) and the

polymerization parameters in Table 3 and allowed to reach equilibrium swelling for 5 days in PBS at 4ºC. After swelling, rheological tests were performed on a MCR302-PP08 rheometer (Anton Paar) equipped with parallel sandblasted plates of 8 mm diameter (see Figure 8). Measurements were taken using sinusoidal signals and applying a sweep to the amplitude of deformation (or shear strain) between 0.01% and 500% strain while keeping constant the angular frequency to 10^{-1} s. The running temperature was fixed at 23ºC (RT) throughout the measurements. Values of both Storage G' and Loss G'' modulus were obtained as a function of the deformation (strain), from which the complex modulus, G*, and the corresponding elastic modulus, E, were derived assuming a Poisson's coefficient value of 0.5.

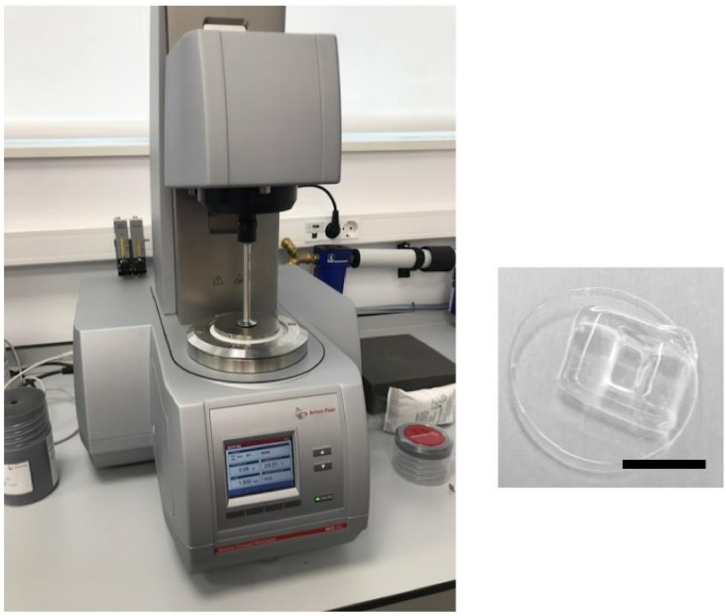

Figure 8 – *Picture of the MCR302-PP08 rheometer (Anton Paar) -left- and example of the samples used for these measurements.*

As observed in Figure 9a, the shape described by the different samples during the testing was consistent and followed the same pattern. The Loss modulus, G'', curves, which represent the viscous behaviour of the samples, were pretty similar in all cases, without showing significant variations between samples; whereas the Storage modulus, G', differed in values (not in shape), increasing values with the increasing energy dosage used for crosslinking the samples. This effect is more evident when the Elastic modulus of each tested condition is compared (see Figure 9b).

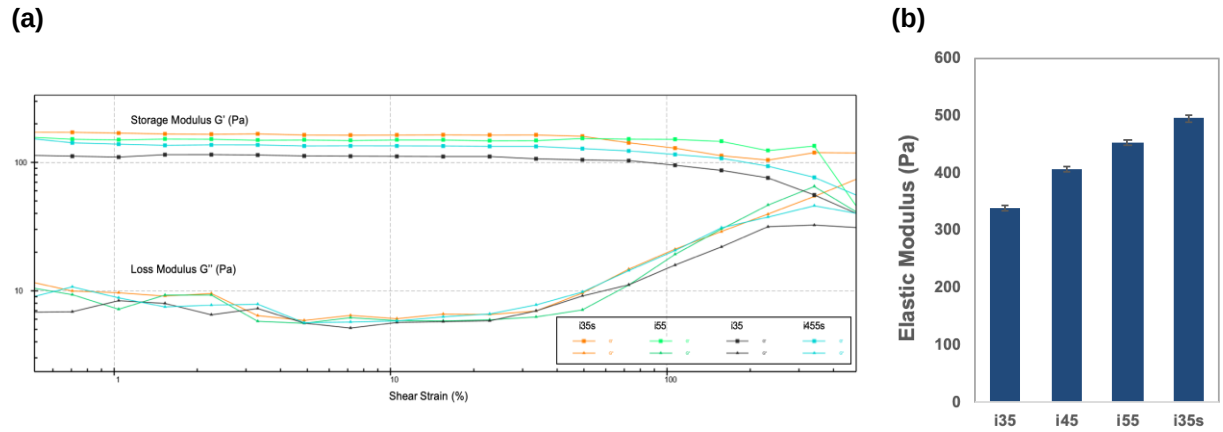

Figure 9 – *Rheology measurements. (a) Plots of the Storage and Loss Modulus, G' and G'' respectively, obtained per each sample type. (b) Values of the Elastic Modulus determined from the rheological analysis.*

These results obtained are in agreement with the reported values for other materials that are typically employed in tissue culture, such as Matrigel® (20 Pa to 300 Pa) and Collagen I (0.5 Pa to 100 Pa), also measured by rheology [2-3]. Figure 10 shows a visual comparison of the stiffness values between various epithelial tissues and the most common materials used in tissue culture.

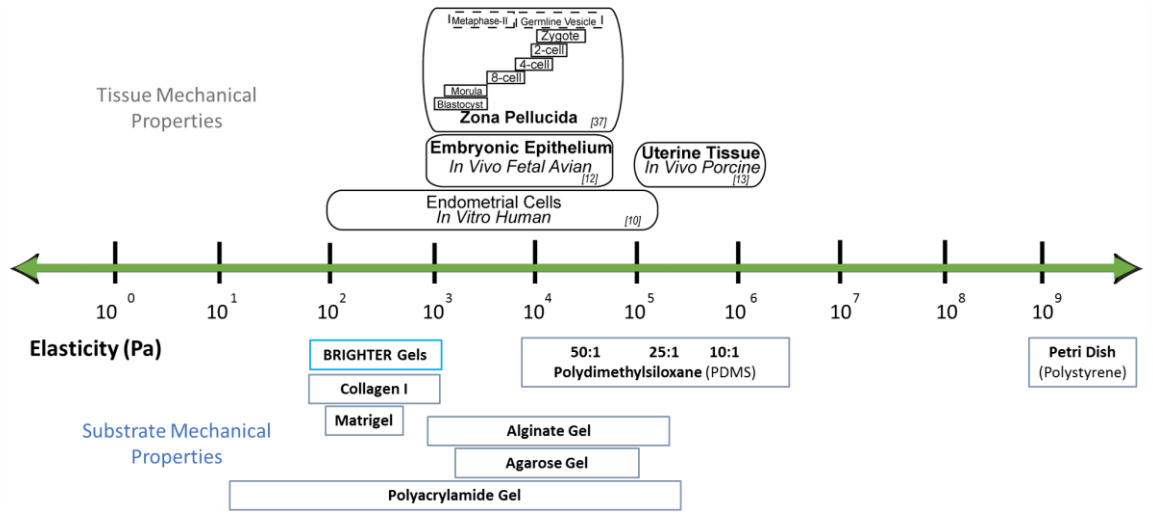

Figure 10 – *Stiffness comparison of various epithelial tissues with the elasticity of materials typically employed in tissue culture. Figure adapted from [4].*

4. Crosslinking of the hydrogels using Light-sheet exposure

Once identified the best polymer formulation, some experiments were performed to evaluate its crosslinking using the first-iteration of the Light-sheet printing system at GUF facilities. The main parameters studied were the intensity of the incident light and its exposure time, which, combined, determine the suitable range of energy dosages for BRIGHTER project purposes.

$$Energy\ dose = intensity * time$$

Thus, to investigate the dose, the light intensity and the exposure time were varied independently.

Polymerization process

To study the effect of light intensity and exposure time required to reach full polymerization, a diffusion test was used to visually identify the polymerized shape and therefore perform tests in a faster manner. First, the prepolymer solutions were inserted into custom-made FEP cuvettes (see Deliverable D1.8) and the focus of the detection objective was made on the internal side of the cuvette's wall. Then, 100 LS planes were polymerized keeping 5 µm spacing between each of them. The sample holder moved along the Z axis towards the detection objective, ultimately polymerizing the defined Z stack. After polymerization, a fluorescent dye (FITC-dextran of 20 kDa) was injected on top of the hydrogels, resulting the part that did not allow the immediate free diffusion of the FITC-dextran dye being identified as the polymerized construct (see Figure 11).

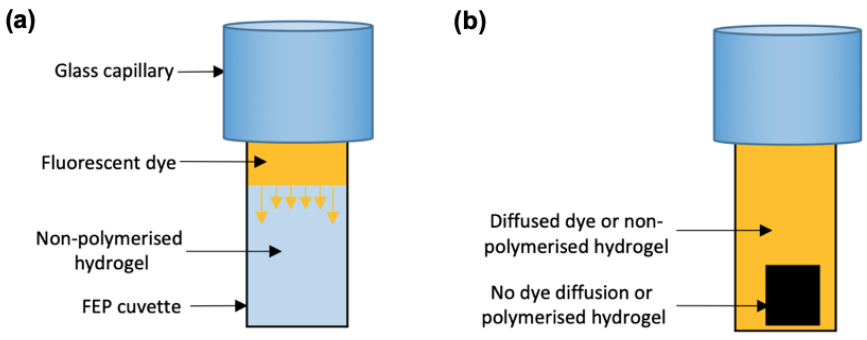

Figure 11 – *Sketch of the fluorescent diffusion showing the polymerized shape.*

The two parameters tested, the intensity and the exposure time, were assessed stepwise and for each step, the area was measured from the side. According to the printed pattern, the expected area should measure 2.5x105 μm^2 which equalled to 500 µm (height of the light sheet) x 500 µm (length of the Z stack). In this case, two different hydrogel formulations provided by WP2 were investigated, PVA-N:PEG_ink (5 mM: 5 mM : 1 mM) and PLN-N:PEG_link (5 mM : 4 mM : 0.4 mM) for a better comparison.

When varying only one parameter (either the intensity or only the exposure time), a threshold under which no full polymerization was reached could be observed (see Figure 12). When varying the intensity with a fixed exposure time of 5 seconds, a threshold was identified at 1.7 mW, above which there was no significant variation between the different intensities tested. However, when the intensity was fixed, in this case, to 0.7 mW (See Figure 12d), the average surface area of the polymerized object decreases with the exposure time, evidencing the relevance of this parameter on the polymerization process. It is worth noting that using this test, the polymerization indicates a crosslinking dense enough for a 20 kDa FITC-dextran dye not to immediately diffuse. The hydrodynamic radius of a 20 kDa FITC-dextran molecule was given by the supplier as 3.3 nm; therefore, the pore size of the polymerized hydrogel was comprised at or above this value.

The diffusion test gave a good indication of the minimum intensity and exposure time required to polymerized through the cuvette, which permitted to print bigger constructs without needing to use the double-sided lasers. However, further experiments are needed to comprehend the extend of the X-axial and Z-axial resolutions.

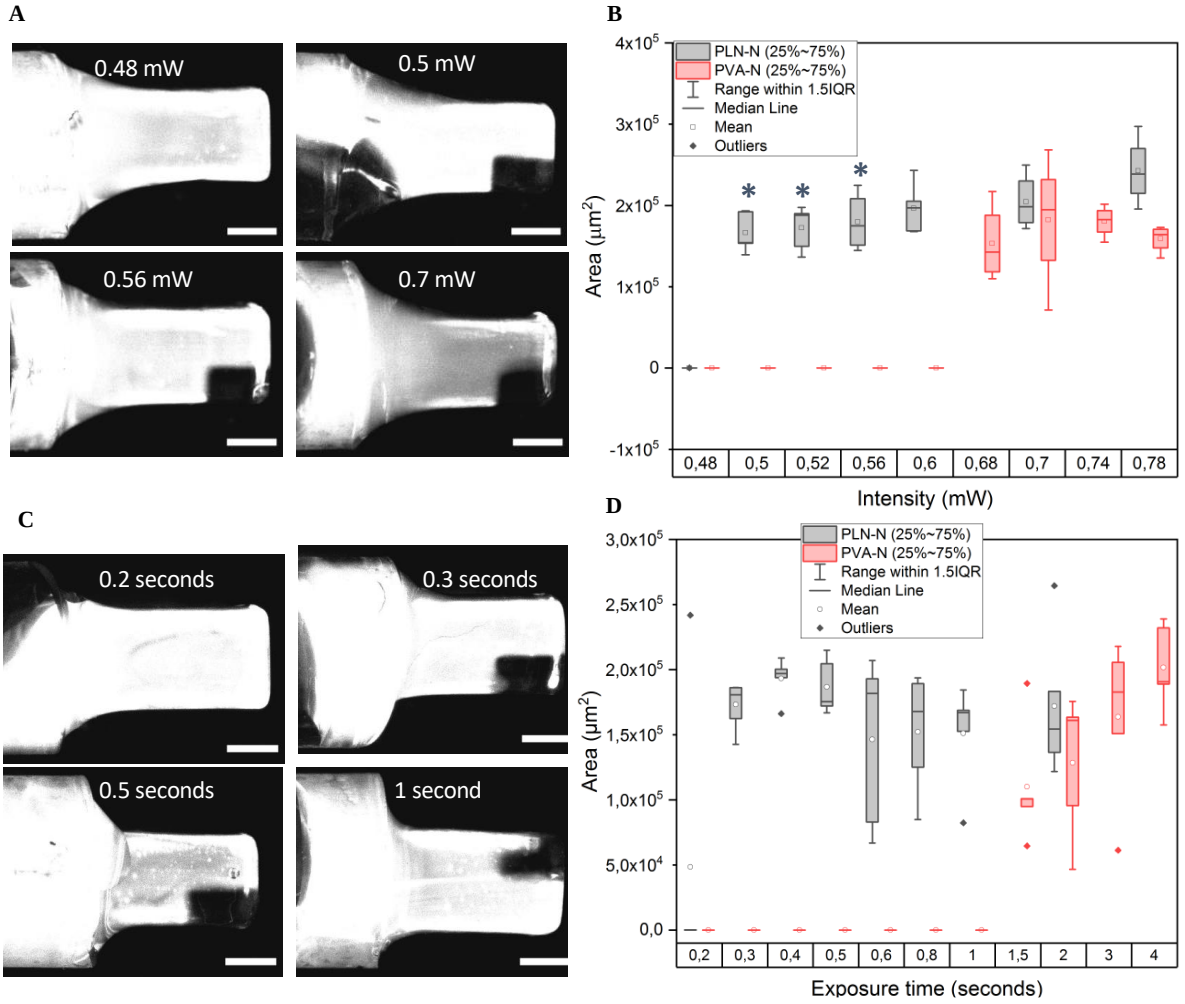

Figure 12 –*The crosslinking of the hydrogel upon above threshold laser intensity and exposure time led to full polymerization. Polymerization of a cuboid of 500 µm x 500 µm x 500 µm, with fixed exposure time of 5 seconds (A-B) and fixed intensity of 0.7 mW (C-D). (A) Representative stereomicroscope images of the FEP foil cuvettes after photopolymerization of the PLN-N hydrogel at different intensities for 5 seconds exposure time and the addition of FITC-Dextran of 20 kDa. Scale bars: 500µm. (B) Area of the polymerized hydrogel depending on laser intensity. (p<0.05, n=5). (C) Representative stereomicroscope images of the PLN-N hydrogel with different exposure times for a fixed intensity of 0.7 mW, after adding FITC-Dextran of 20 kDa. Scale bars: 500µm. (D) Area of the photopolymerized object as function of the exposure time. (p<0.05, n=5).*

Effects of the intensity and the exposure time

To further understand the independent effect of intensity and exposure time on the photopolymerization process, fluorescence recovery after photobleaching (FRAP) was used to determine the diffusion profile of FITC-dextran by focusing on the mobile fraction and half recovery time. Indeed, the mobile fraction of the diffusing molecule indicates the

extent to which said molecule regained full initial intensity, and therefore suggests the capacity of the molecule to interact with elements of the hydrogel. The half recovery time represents the time needed for the molecules to reach 50% of the final recovery intensity (which is not necessarily the initial intensity, see mobile fraction). The half recovery time provides information on the way the fluorescent molecules observed diffuse in the hydrogel.

First, the hydrogel was polymerized at a fixed dose of 6 J/cm^2) while the intensity and exposure time varied (430 ms and 0.7 mW, 500 ms and 0.6 mW or 600 ms and 0.5 mW), using the diffusion of FITC dextran 3-6 kDa of molecular weight (see Figure 13a). The main factor determining the photopolymerization of the hydrogel seemed to be the intensity, as the mobile fraction and the half recovery time decreased with decreasing intensity, indicating a looser hydrogel network. Next, the behaviour of the hydrogel under increasing doses was investigated, using a bigger dye (FITC dextran of 20 kDa of molecular weight). In this case, the selected energy doses were: 1 J/cm^2, 6 J/cm^2, 10 J/cm^2 and 15 J/cm^2. The availability of free fluorescent molecules to diffuse decreased with increasing dose (increasing half recovery time), from a dose of 10 J/cm^2. As a control of the experiment, the diffusion of the different FITC-dextran molecules in water was also measured. The variation in the mobile fraction of the two controls showed that this parameter is quite unreliable. The half recovery time measurements gave an indication of the baseline for which the samples were not polymerized. A longer half recovery time would indicate partial or complete polymerization (which was the case for all the experiments).

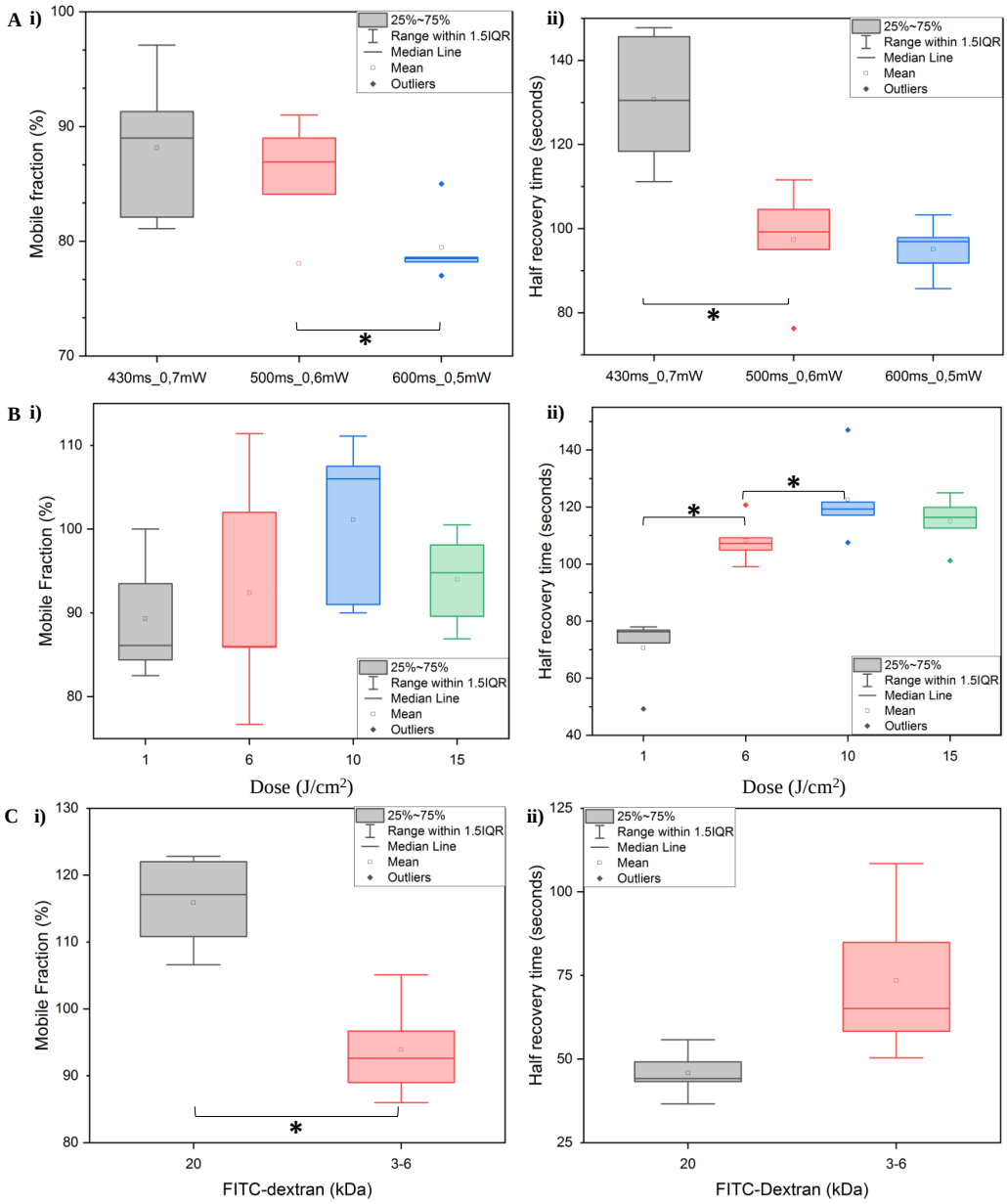

Figure 13 – *FRAP analysis of the diffusion of different FITC dextran molecules revealed the behaviour of the hydrogel under different conditions. (A) FRAP analysis of the diffusion of FITC dextran of 3-6 kDa through a hydrogel polymerized at the same dose but different combinations of laser intensities and exposure times indicated a drop in diffusion with increasing intensity. (p<0.05, n=5). (i) Mobile fraction of the hydrogels polymerized at a dose of 6 J/cm² with different intensity and exposure time combinations and its corresponding half recovery time (ii). (B) FRAP analysis of the diffusion of FITC dextran of 20 kDa at different doses (the exposure time remains constant) (p<0.05, n=5). (i) Mobile fraction of the hydrogels polymerized at different doses and its corresponding half recovery time (ii). (C) FRAP analysis of the diffusion of FITC dextran of 3-6 kDa and 20 kDa in ultrapure water (p<0.05, n=5). (i) Mobile fraction in ultrapure water and its corresponding half recovery time (ii).*

The FRAP experiments were performed using the first-iteration of the light-sheet microscope, by turning off the scanning of the gaussian beam so the photobleaching resulted in a single beam. The imaging was taken using the scanned beam; therefore demonstrating the versatility of the LS device – photopolymerization and imaging in one go. The data analysis was conducted using a Python script from which the diffusion rate of the fluorescent molecules through the gels were deduced, leading to the pore size within the hydrogel network. Figure 13 summarizes the main data obtained from this experiment.

Refractive index of the hydrogel formulations

The refractive indices of the two hydrogel formulations (before and after photopolymerization) and of the FEP foil were measured using a refractometer, to check if some variation was produce during the crosslinking process. The refractive indices of PLN-N and PVA-N polymers were very close, on average 1.339 and 1.338, respectively. The FEP-foil used to produce the cuvettes was also measured, keeping a constant refractive index of about 1.34, which matched the manufacturer's specifications (Figure 14).

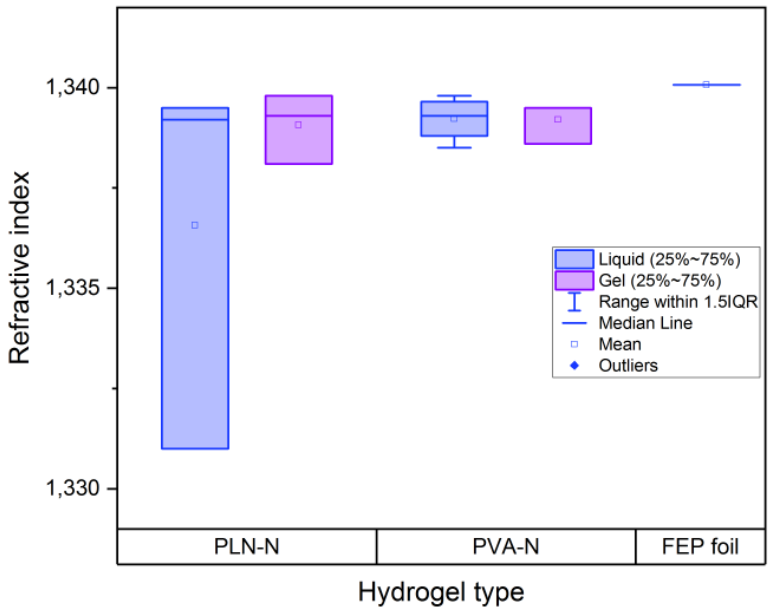

Figure 14 – *Refractive index measurements of the different hydrogels and the FEP-foil.*

5. Conclusions

In the present document, we reported the first characterization results of the different polymer formulations developed for BRIGHTER project by WP2. With the different experiments performed we have demonstrated that:

- the polymer formulations can be photopolymerized using light with the same wavelength (405 nm), intensity and energy doses as the ones proposed for BRIGHTER's light-sheet bioprinting system, resulting in self standing and handling hydrogels. Moreover, some polymerization tests were performed using the first-iteration of the light-system at GUF facilities, proving the suitability of both, the materials and the novel photopolymerization technique.

- the resulting hydrogels have a stiffness within the range of most commonly used materials for cell culture and tissue engineering applications, such as Matrigel®, Collagen I and Gelatine, with values ranging from 300 Pa to 550 Pa.

- the water uptake capacity of the different polymer formulations was also assessed by measuring the volumetric swelling. Results revealed that BRIGHTER's hydrogels experience a volume increase which is also in the range of other hydrogels with lower macromer content reported in the literature, such as GelMA and PEGDA co-networks, that are typically used in 3D bioprinting applications.

- by FRAP experiments we have demonstrated the suitability of this technique for characterizing the pore size of the hydrogels networks.

6. Ongoing experiments

New generation of hydrogels have been recently developed for WP2 (batch 2), which include functional RGD groups to provide cells with some adhesion motifs, and thus promoting their growth and differentiation when seeded on top and/or embedded within. Within the following months, this second batch of functional polymers will be characterized in similar way to the first batch (swelling behaviour, stiffness range, crosslinking, etc.) and the results compared with those reported in the present document, to see how the addition of the RGD functional groups is affecting the polymerization of the gels.

7. References

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