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Deliverable Title	Photopolymerizable polymers biocompatibility						
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Overview/Abstract

Recent scientific evidences pointed out the key role of the cellular microenvironment on the proper development and functioning of tissues. Thus, one of the aims of tissue engineering is using novel biomaterials with tuneable properties for not only reproducing the extracellular matrix architecture and stiffness, but also its biochemical composition to create cell friendly niches; guiding cells self-organization into functional tissue units. The work gathered within this document is focused on that direction and summarizes the main actions established by task T2.2 (Polymer functionalization and biocompatibility evaluation).

Polymers developed in previous project stages based on click thiol-ene chemistry have been modified to covalently attach specific peptides (*e.g.* RGDs) to promote cell adhesion. Some polymerization tests varying the number of functionalized groups added to the polymer and upon different energy doses applied have been performed according to the protocols stablished in task T3.1.

Proof-of-concept tests evaluating the biocompatibility of the new polymer formulations showed no cytotoxic cellular response both in terms of adhesion and growth. However, due to the exceptional working conditions of the COVID19 pandemic scenario, not all the polymer samples synthesized were tested for biocompatibility and this task is still on-going.

Explanation for large delay in submitting deliverable

N/A

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1. Biofunctionalization of Norbornene-Pullulan polymers

The polymers developed in Task 2.1 can be used to form biocompatible hydrogels by photopolymerization. These hydrogels, however, do not promote the long-term adhesion and proliferation of cells and need to be functionalized with cell adhesion motifs. To this end, an adhesion peptide motif (the amino acid sequence Arginine-Glycine-Aspartic Acid (RGD)), present in extracellular matrix proteins such as fibronectin or vitronectin, was attached to the Norbornene-Pullulan (N-PLN) polymers, described in D2.1. This motif interacts with cellular surface proteins (integrins) and thus induces the formation of adhesion sites. Under such conditions many cell types display a more *in vivo-like* phenotype.

1.1 Direct Attachment of Thiol-Modified RGD Peptide to the Norbornene Groups of N-PLN

The most direct approach to create photopolymerized hydrogels functionalized with RGD is described in the following steps:

- Prepare a thiol-modified RGD Peptide
- Use a portion of the Norbornene groups of N-PLN to attach the thiol-modified peptide to the N-PLN with the thiol-ene photochemistry, as described in D2.1.
- Use the remaining Norbornene groups of N-PLN to crosslink RGD-modified N-PLN and form a hydrogel, as described in D 2.1.

1.1.1 Attachment of RGD Peptide to N-PLN in a Thiol-ene Photoreaction

Up to approximately 60% of RGD Peptides could be attached to a six-fold excess of pullulan-bound norbornene groups by a thiol-ene photoreaction (Figure 1).

At lower light intensities this percentage decreased. Higher light intensities caused a substantial increase in the viscosity of the sample solutions up to a gel-like structure preventing the size-exclusion chromathography (SEC) analysis. Attempts to improve the percentage of peptide attachment by, *e.g*. increasing the duration of the illumination or the concentration of the photoinitiator, always resulted in an increase of the sample viscosity.

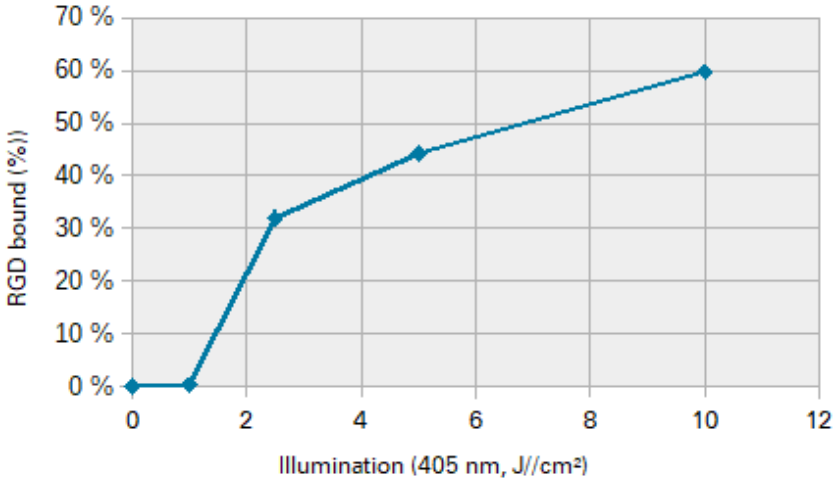

Figure 1: 300 nmol pullulan-attached Norbornene groups were illuminated together with 50 nmol thiol-modified RGD Peptide in the presence of 20 nmol LAP photoinitiator in a total volume of 50 µl at varying light intensities (405 nm). The reaction products were analyzed by SEC. The amount of free peptide was determined by integration of the corresponding peptide peak. The percentage of bound peptide was determined by subtracting the amount of free peptide in each sample from the amount of free peptide found in the sample without illumination (0 J/cm²).

1.2 Attachment of RGD Peptide in a Base-Catalyzed Michael Addition Reaction to N-PLN

Since the direct attachment of RGD Peptide to N-PLN in a thiol-ene reaction was not complete and appeared to polymerize the structure of the N-PLN, we decided to decouple the chemistry for peptide attachment from the chemistry of crosslinking and use a base-catalyzed Michael addition reaction for the attachment of the peptide. This was done using the following steps:

- Attach vinylsulfone groups to N-PLN
- Attach thiol-modified RGD Peptide to these N-PLN-attached vinylsulfone groups

1.2.1 Attachment of Vinylsulfone Groups to N-PLN

Vinylsulfone groups were introduced under alkaline conditions to the hydroxyl groups of N-PLN using a chemistry as shown in Figure 2. The extent of the reaction was controlled by a very short incubation time at the alkaline pH to make sure that a substantial portion of divinylsulfone would react with only one of their two vinylsulfone groups. The reaction yielded a polymer (VS-N-PLN) with a ratio of vinylsulfone groups to norbornene groups of 0.47:1.

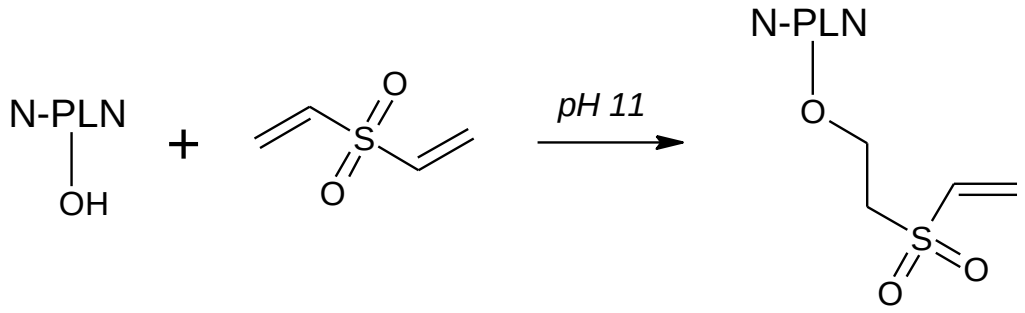

Figure 2: Base-catalysed Michael Addition reaction between hydroxyl groups of Pullulan and Divinylsulfone.

1.2.2 Attachment of RGD to VS-N-PLN

Vinylsulfone groups react with thiol groups under neutral pH conditions in a base-catalyzed Michael addition reaction. Therefore, the spontaneous attachment of thiol-modified RGD Peptides to VS-N-PLN was analysed.

A constant amount of pullulan-attached norbornene groups was incubated with different amounts of thiol-modified RGD peptides and the percentages of RGD attachment to the polymers were measured. Figure 3 shows that as long as there was an excess of vinylsulfone groups, a larger percentage (60-70%) of the RGD peptides could be attached to the polymer. When the RGD peptide was in excess with respect to the vinylsulfone groups, the percentage of the attached peptide decreased while the molar amount of bound peptide still increased.

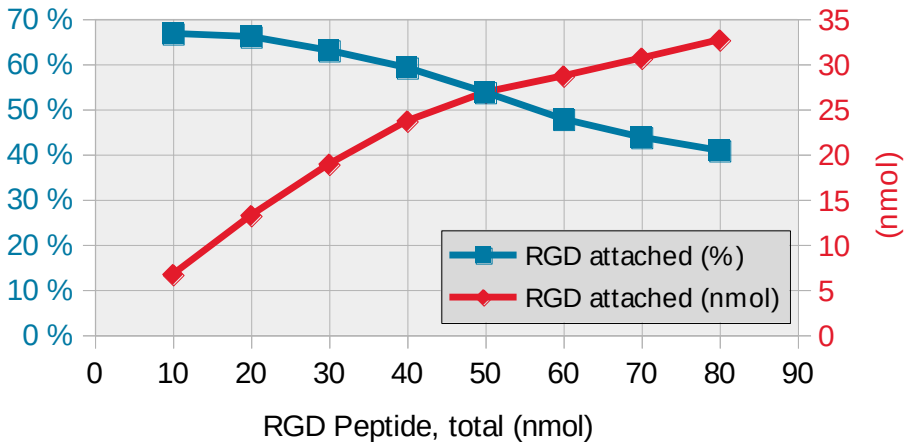

Figure 3: 40 nmol pullulan-attached Norbornene groups were incubated with different amounts of thiol-modified RGD peptide at pH 7.4 in a volume of 100 µl for 16 hr at room temperature. The samples were analysed for RGD binding to the polymeric vinylsulfone groups as described in ***¡Error! No se encuentra el origen de la referencia.****.*

This result suggested that the binding reaction could only be driven to completion by providing an excess of peptide.

Attempts to optimize the reaction by varying the reaction conditions (pH, time) did not significantly improve the yield of this reaction (data not shown). Other peptide lots, that were available only in small quantities, allowed slightly improved results (up to 75% attached RGD Peptide when 30 nmol peptide were reacted with 40 nmol vinylsulfone groups).

Based on these results, we decided to attach the RGD Peptides to VS-N-PLN in a bulk reaction and purify the functionalized polymer extracting the unreacted peptide by dialysis. This purified material is the one that will be provided to the consortium partners for further examination.

1.2.3 Bulk attachment of RGD Peptide to VS-N-PLN

For a pilot scale attachment of RGD Peptide to N-PLN, 887 mg of VS-N-PLN (142 µmol vinylsulfone groups) were incubated at pH 7.4 over night with 142 µmol thiol-modified RGD Peptide at room temperature (Fr. I).

¡Error! No se encuentra el origen de la referencia. shows that RGD peptide was found in Fraction II, RGD-N-PLN. During the preparation (dialysis, ultrafiltration, sterile filtration) 19-28% of the polymeric material had been lost (based on the yield of dry weight or norbornene groups). This loss explains the lower RGD yield compared to the previous results (see Figure 3). The vinylsulfone groups had been completely consumed by the reaction of the RGD peptide and their subsequent blocking with thioglycerol.

Table 1: Summary of bulk preparation of RGD-N-PLN

	Dry Weight	Norbornene	Vinylsulfone	RGD	Volume
Fr I: VS-N-PLN + RGD	*887 + 144 mg*	*308 µmol*	*142 µmol*	*142 µmol*	*20,8 + 12,8 mL*
FR. II: RGD-N-PLN	*832 mg (81%)*	*223 µmol (72%)*	*n.d.[1] (<3 µmol)*	*61 µmol [2] (3%)*	*22 mL*

In summary, a preparation of 22 mL had been obtained that contained 10 mmol/L pullulan-attached norbornene groups and 2.8 mmol/L of pullulan-attached RGD groups, *i.e.* a ratio of Norbornene to RGD groups of 3.66 to 1. At hydrogel standard concentrations of 4 mmol/L Norbornene and 0.5 mmol/L RGD this would be sufficient for a total of 123

[1] n.d.: not detectable.
[2] RGD amount was estimated using a color reaction (BCA Assay) with free RGD peptide as a standard.

mL hydrogel, if the RGD-N-PLN would be appropriately diluted with N-PLN prior to the crosslinking reaction.

1.3 Hydrogel formation with RGD-N-PLN polymers

The ability to form RGD-modified hydrogels was tested by crosslinking at a concentration of 4 mmol/L norbornene groups and 4 mmol/L crosslinker (PEG-Link or CD-Link) in the presence of 0.5 mmol/L RGD groups (Table 2). Two different light systems at 405 nm or 525 nm were employed. All tested combinations yielded hydrogels after illumination. In general, the RGD-modified hydrogels were slightly stiffer than unmodified hydrogels. Crosslinking with CD-Link or with the Eosin/green light system also resulted in hydrogels with a decreased stiffness compared to hydrogels fabricated with PEG-Link or the blue light system.

Table 2: Gel stiffnesses of RGD-modified hydrogels compared to unmodified hydrogels. See D2.1 for setup of stiffness measurements.

Sample	1	2	3	4	5	6	7	8
Norbornene (mmol/L)	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
PEG-Link (mmol/L)			*4*	*4*			*4*	*4*
CD-Link (mmol/L)	*4*	*4*			*4*	*4*		
RGD (mmol/L)	*0.5*		*0.5*		*0.5*		*0.5*	
Light System[3]	*LAP*	*LAP*	*LAP*	*LAP*	*Eosin*	*Eosin*	*Eosin*	*Eosin*
Complex Modulus (Pa)	*321*	*294*	*644*	*549*	*212*	*157*	*412*	*359*

2. Evaluation of biocompatibility of Norbornene-Pullulan

The biocompatibility of Norbornene-Pullulan gels was assessed by means of three different sets of experiments:

- Cell metabolic activity (photopolymerization process)
- Cell adhesion and growth on top of polymerized samples

[3] LAP: 0.5 mmol/L LAP; 7.92 J/cm² illumination at 405 nm. Eosin: 0.1 mmol/L Eosin Y/ 2mmol/L L-Tyrosine-methylester; 7.92 J/cm² illumination at 525 nm.

- Cell viability and spreading within the polymerized samples

First of all, the potential toxicity of the polymerization process combining LAP photoinitiator and irradiation of blue light (405 nm wavelength) was evaluated, using different energy dosages.

Then, cells behaviour both on top and within the polymer solutions after photopolymerization was assessed. For that purpose, Norbornene-Pullulan hydrogel samples (without cell-specific functionalization) were polymerized using a direct laser writing (DLW) system equipped with a 405 nm wavelength laser diode at IBEC, using similar illumination characteristics than the ones used in the light-sheet system assembled in GUF facilities. The molar composition of the gels was fixed at N-PLN : PEG-Link : LAP (5 mM : 4 mM : 0.2 mM), according to previous D2.1 findings. Table 3 summarizes the polymerization characteristics of the samples used.

Table 3: Polymerization characteristics of the printed samples.

Samples	Power (mW)	Scanning velocity (mm/s)
PLN-PEG_35i	*13*	*0.3*
PLN-PEG_45i	*22*	*0.3*
PLN-PEG_55i	*30*	*0.3*
PLN-PEG_0.15 speed	*22*	*0.15*

2.1 Toxicity of blue light at 405 nm

The toxicity of blue light as it is being used to photocrosslink with the LAP photoinitiator was studied in a toxicity assay based on the metabolic dye Alamar Blue. To this end, NIH-3T3 fibroblast cells were illuminated with increasing doses of light at 405 nm. The results (Figure 4) show that blue light up to a dose of 10 J/cm^2 does not affect the metabolic activity of NIH-3T3 cells. Beyond that level, the metabolic activity is clearly affected by the illumination, resulting in high cytotoxicity above 20 J/cm^2. The acceptable light dose is well within the light dose necessary to form hydrogels.

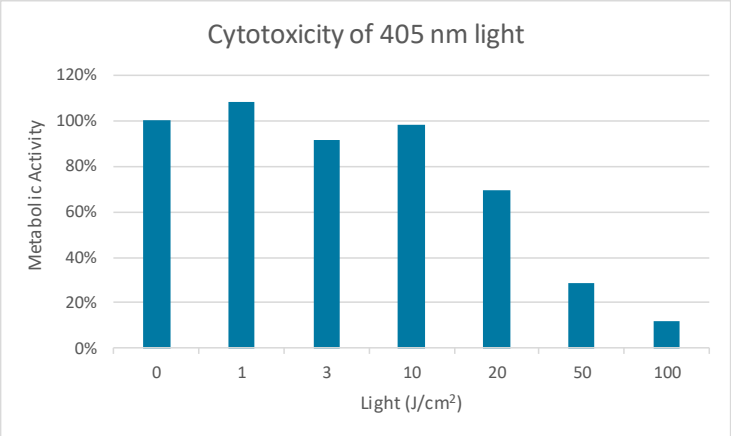

Figure 4: Relative metabolic activity of 3T3 cells, cultivated in a hydrogel (3 mmol/L PVA/CD-Link/0.5 mmol/L RGD), 20 hrs after illumination with increasing doses of light at 405 nm.

1.4 Cell adhesion and growth on top of N-PLN samples

Also, the adhesion and growth of HaCAT human keratinocyte on top of the photocrosslinked gels were tested. HaCAT cells were seeded on top of rectangular shaped N-PLN : PEG link : LAP (5 mM : 4 mM : 0.2 mM) samples (3.0 mm length, 0.6 mm width) at two different densities: $1.9 \cdot 10^6$ and $2.8 \cdot 10^6$ cells/cm^2 and kept in standard culture conditions (37ºC and 5% CO_2) for 7 days, exchanging the cell medium every two days. Samples were crosslinked using DLW system using both 22 mW and 30 mW and a laser speed of 0.15 mm/s. Pictures of the surface of the different samples were taken at different culture time points.

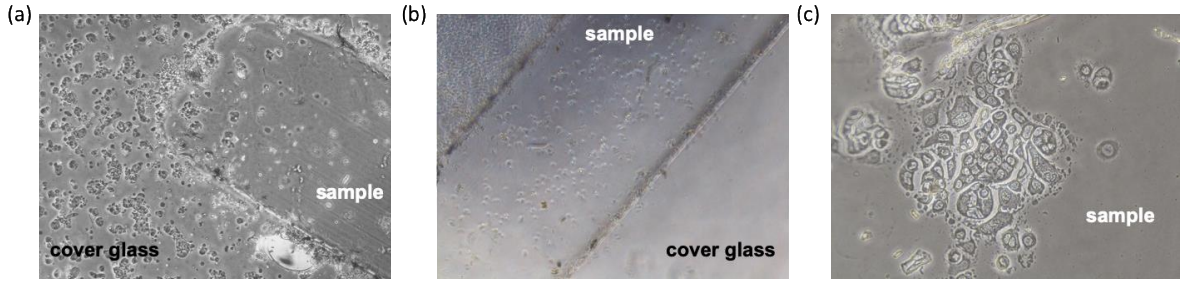

Figure 5: Different views of HaCAT cells performance on top of photocrosslinked N-PLN:PEG link:LAP (5 mM : 4 mM : 0.2 mM) gels using 30 mW of power at (a) the border of the sample and at (b) the center. Detail of an attached cell colony on the surface of the gel (c).

HaCAT cells did not colonize and grow well on top of these non-functionalized gels, probably due to their lack of cell-friendly functional groups, but also due to their low stiffness values (less than 1 kPa). Pictures in Figure 5 show, as an example, the results obtained for the samples crosslinked using 30 mW. Few cell colonies remained attached on the gels after 48h of seeding and started to spread (see Figure 5b-c), but they did not progress at further culture time points.

1.5 Cell viability and spreading within N-PLN samples

N-PLN : PEG-link : LAP prepolymer solutions were prepared at the molar concentrations of 5 mM : 4 mM : 0.2 mM in MilliQ, and mixed with a NIH-3T3 mouse embryonic fibroblasts cells, at a cell density of $2.6 \cdot 10^6$ cells/mL. Mixtures were irradiated using both 22 mW and 30 mW and a laser speed of 0.15 mm/s and kept in culture at standard conditions (37ºC and 5% CO_2). Cell viability on bioprinted cell-laden hydrogels was investigated using a Calcein-AM/Ethidium homodimer Live/Dead kit (Invitrogen) at days 1, 3, 7 and 10 after NIH-3T3 cells encapsulation. Confocal laser scanning microscope (LSM 800, Zeiss) with a 10x objective was used for imaging and a manual cell counter plugin in ImageJ software (http://imagej.nih.gov/ij, NIH) was used for image processing and cell viability quantification. Figure 6 shows, as example, the results obtained for the samples prepared using 22 mW.

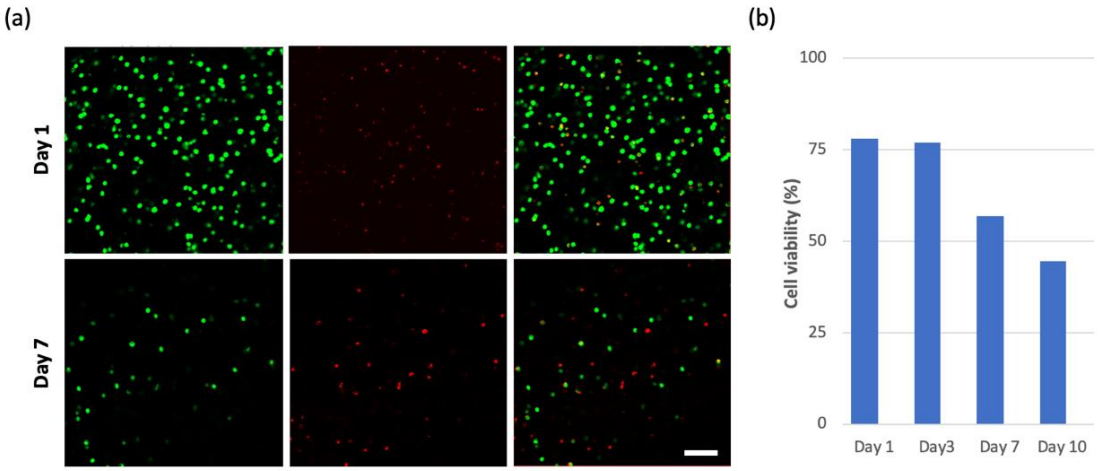

Figure 6: Cell viability assessment on N-PLN:PEG-link:LAP polymers. (a) Confocal Z projections showing live (green) and dead (red) NIH-3T3 cells at days 1 and 7 after encapsulation; (b) cell viability quantification. Scale bar = 100 µm.

Even if cell viability was not very high and decreased with the days of culture, a significant percentage of cells remained alive within the gels at day 10 of culture, thus indicating that the hydrogel per se is not cytotoxic. However, probably due to the lack of cell-adhesion cues and low stiffness values, cells did not spread and proliferate in these hydrogels formulations.

3. Ongoing and future work

Some experiments and actions planned for the completion of Task 2.2 (the one related with the present deliverable) and, in consequence, the successful achievement of the Milestone M4 “Custom-made hydrogels support cell adhesion and growth” have suffered a delay due to functionalization issues (WP2) and the SARS-Cov-2 global lockdown.

Specifically, this has affected the biocompatibility testing of the new functionalized polymer formulations. Thus, some extra effort will be devoted to mitigate these effects in the following months. In this sense, experiments involving the growth of skin representative cell types (HS27 human skin fibroblasts and HaCat epithelial cells) on the functionalized hydrogels are on-going.

Future work will involve the full characterization of the physicochemical properties of these gels when photopolymerized with the light-sheet instrument and the analysis of skin cell phenotype (tasks 3.1 3.2), together with the synthesis and characterization of other polymer formulations for creating photocrosslinkable interpenetrated networks (IPNs) (task 2.3).