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Platelet Lysate-Loaded Photocrosslinkable Hyaluronic Acid Hydrogels for Periodontal Endogenous Regenerative Technology

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ABSTRACT

The integrity and function of the periodontium can be compromised by traumatic injuries or periodontitis. Currently available clinical therapies are able to stop the progression of periodontitis and allow the healing of periodontal tissue. However an optimal strategy capable of restoring the anatomy and functionality of the lost periodontal tissue is still to be achieved.

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3 Herein is proposed the development of an injectable hydrogel system able to release a growth
4 factors and cells to the periodontal defect.
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10 This injectable system is based on a photocrosslinkable hydrogel, prepared from methacrylated
11 Hyaluronic Acid (me-HA) and incorporating Platelet Lysate (PL). The delivery of growth factors
12 and cells in situ is expected to enhance regeneration of the periodontium. Various formulations
13 of me-HA containing increasing PL concentrations were studied for achieving the formation of
14 stable photocrosslinkable hydrogels. The produced hydrogels were subsequently characterized to
15 assess mechanical properties, degradation, protein/growth factor release profile, antimicrobial
16 activity and response towards human Periodontal Ligament fibroblasts (hPDLFs). The results
17 demonstrated that it was possible to obtain stable photocrosslinkable hydrogels incorporating
18 different amounts of PL that can be released in a sustained manner. Furthermore, the
19 incorporation of PL improved ($p < 0.02$) the viscoelastic properties of the hydrogels and enhanced
20 their resilience to the degradation by hyaluronidase (HAase). Additionally, the PL showed to
21 provide antimicrobial properties.
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39 Finally, hPDLFs, either seeded or encapsulated into the developed hydrogels, showed enhanced
40 proliferation over time ($p < 0.05$), proportionally to the increasing amounts of PL present in the
41 hydrogel formulations.
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50 KEYWORDS

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53 Photocrosslinkable hydrogels, Platelet lysate, Hyaluronic acid, Periodontal ligament,
54 Endogenous regenerative technology
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INTRODUCTION

The periodontium is a complex and dynamic oral structure comprising soft and hard tissues, the cementum, a functionally oriented periodontal ligament, alveolar bone and gingiva. The main function of this structure is anchoring the teeth to the jaw bones, while withstanding the forces originated by the masticatory process¹. The integrity and function of the periodontium can be compromised by trauma or disease, such as periodontitis, an inflammatory disease predominantly caused by gram-negative bacteria that causes the destruction of these tooth supportive tissues potentially leading to tooth loss¹⁻².

Current therapeutic options, which include the implantation of autografts, synthetic bone fillers and guided tissue regeneration (GTR), are not able to fully regenerate periodontium morphology and function. In recent years endogenous regenerative technology (ERT) has arisen as a new paradigm in periodontal regeneration. This new concept has its foundations in tissue engineering and aims to induce or encourage periodontal regeneration by superimposing specific chemical (e.g. growth factors) and biophysical cues³. These signals are expected to encourage homing of stem and progenitor cells leading to the formation of new periodontal ligament and cementum³.

Platelet rich hemoderivatives (PRHds), namely platelet rich plasma and platelet rich fibrin, have been widely investigated for periodontal ERT as important sources of autologous growth factors and provisional fibrin matrices^{1, 3}. Nevertheless, the traditional PRHds clots retract, impairing the needed stability for periodontal tissue ingrowth⁴. In this research work we propose the development of photocrosslinkable hyaluronic acid hydrogels enriched with platelet lysate as a stable system for the delivery of endogenous GFs, directed for periodontal ERT.

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It is advocated that current ERT scaffolding materials needs sophistication and that should be employed in a patient-tailored fashion using preferably own patients' biological material³. In this sense, platelet lysate (PL) offer great potential in regenerative medicine as an alternative source of growth factors (GFs)⁵⁻⁶. These PL-origin GFs, include fibroblast growth factor (FGF), vascular endothelial growth factor, platelet-derived growth factor, transforming growth factors- β 1 and - β 2, insulin-like growth factor, epidermal growth factor, epithelial cell growth factor, hepatocyte growth factor and bone morphogenetic proteins⁷⁻⁹ are known to be involved in essential stages of wound healing and regenerative processes such as chemotaxis, cell proliferation and differentiation¹⁰⁻¹¹. Moreover, platelets release numerous cell adhesion molecules (fibrin, fibronectin and vitronectin) which can provide a provisional matrix for the adhesion and migration of cells¹⁰. In addition, platelet concentrates (PCs) have also been reported to exhibit antimicrobial properties¹² and the PL, as a product of PCs activation, is expected to have the same antimicrobial properties, contributing for the prophylaxis of the wound site. In fact, the use of PL holds several advantages over other (PRHds) which include the ease to standardize of the production process and the higher consistency in GF content between batches⁵, that is expected to yield more predictable clinical outcomes.

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Since PL is obtained as a liquid solution, it was incorporated in a photocrosslinkable HA matrix. HA is a glycosaminoglycan copolymer of D-glucuronic acid and N-acetyl-d-glucosamine that is present in connective tissues and plays an important role in several cellular processes including, cell proliferation, morphogenesis, inflammation, and wound repair¹³. HA-based biomaterials have demonstrated positive results for several potential applications in the regeneration of hard or soft tissues¹⁴. Moreover, given HA anti-inflammatory, anti-edematous,

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3 and anti-bacterial effects, it has been also proposed for the treatment of lesions caused by
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5 periodontal diseases¹⁵⁻¹⁷.
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8 The aim is to characterise these PL-rich scaffolds with regard to mechanical properties, release
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10 of proteins, periodontal cell response and antimicrobial action against dental plaque bacteria.
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12 This new ERT scaffold offers a new and promising periodontal treatment modality that should
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14 encourage tissue regeneration through the release of PL-derived GFs while providing
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16 concomitant anti-microbial action. Furthermore, functionalization of HA with methacrylic
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18 groups allows the production *in situ* of stable photopolymerizable hydrogels, enabling the
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20 application in periodontal defects in a clinical scenario.
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27 MATERIALS AND METHODS

28 **Materials**

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31 HA obtained from *Streptococcus equi* (Mw = 1.5 to 1.8 MDa), methacrylic anhydride Irgacure
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33 2959 (2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone), hyaluronidase type IV from
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35 bovine origin (HAase), Phosphate Buffered Saline (PBS), phalloidin-tetramethylrhodamine B
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37 isothiocyanate 4,6-diamidino-2-phenylindole, dilactate (DAPI) and the dialysis tubing cellulose
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39 membrane were all purchased from Sigma (Sigma-Aldrich, USA). Sodium hydroxide (NaOH)
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41 and hydrochloride acid (HCl) were purchased from VWR Chemicals (BDH, Prolabo -
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43 international, USA). Alpha MEM (α -MEM) culture medium and fetal bovine serum (FBS) were
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45 purchased from Gibco (Life Technologies, UK). Deuterium oxide ($^2\text{H}_2\text{O}$) was purchased from
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47 LaborSpirit lda (PT) and the polydimethylsiloxane (PDMS) from Down Corning (USA). The
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49 Muller-Hinton agar plate was obtained from Oxoid (UK).
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Preparation of Platelet Lysate (PL)

PL was obtained from different lots of platelet concentrates provided by Serviço de Imunohematologia do Centro Hospitalar de São João (CHSJ, Porto, Portugal), based on a previously established protocol. To produce PL, batches of platelet concentrates obtained by plasma apheresis with a density of 10^6 cells/ μL and biologically qualified according to Portuguese legislation (Decreto-Lei No. 100/2011) were processed as previously described¹⁸⁻¹⁹. Very briefly, platelet concentrates from three different donors were pooled and exposed to three repeated freezing and thaw cycles (frozen with liquid nitrogen and thawed in a 37°C water bath) to promote the lysis of the platelets and release of GFs. Afterwards, the lysis product was centrifuged at 1400 rcf for 10 min and the supernatant stored at -20°C until further use¹⁸⁻¹⁹.

Methacrylation of Hyaluronic Acid (HA)

The method followed for the methacrylation of HA was based on a previously described protocol²⁰, (depicted in Figure 1A), consisting in the addition between 5- to 10-fold molar excess (5x and 10x) of methacrylic anhydride (MA) to a solution of 1 wt% HA in distilled water (dH_2O). The pH was adjusted between 8 and 8.5 with 5N NaOH added dropwise. The reaction occurred during 24 hours at 4°C provided by an ice bath. Subsequently, the reaction products were precipitated using cold ethanol (at -20°C). Then the precipitate was dissolved in dH_2O and dialysed using a membrane with a cut-off of 14,000 kDa for a week against mili-Q water, replaced 3 times a day, to remove the unreacted reagents and by-products. Finally, the solution was filtered, frozen at -80°C and the methacrylated HA (me-HA) recovered upon lyophilisation.

Characterization of the me-HA

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Fourier transform infrared spectroscopy (IR-Prestige-21, FTIR Shimadzu) was used to record the infrared spectra of HA and me-HA. Briefly, a small portion of each batch was mixed with potassium bromide, and processed into pellets. The spectra were obtained in the range of 400 to 4000 cm^{-1} at a 4 cm^{-1} resolution with 32 scans. ^1H NMR spectra were recorded with a Varian Inova 500 at 70°C. me-HA solutions were prepared for analysis by dissolving 5 mg of me-HA in 1 mL of $^2\text{H}_2\text{O}$. The degree of methacrylation (*Dmet*) was defined as the percentage of methacryloyl groups per HA disaccharide repeat unit and was calculated from the ratio of the relative peak integration of the methacrylate protons (peaks at ~ 6.20 , ~ 5.77 , and ~ 2.05 ppm) and HA's methyl protons (~ 1.98 ppm).

Development of the photocrosslinkable me-HA hydrogels incorporating PL

The development of the photocrosslinkable me-HA hydrogels incorporating PL was optimized by changing the HA (5x and 10x MA molar excess) solution concentration (1 and 2 wt%), the concentration of photoinitiator Irgacure 2959 (0.1 and 0.2 wt/v%), the power of the UV light, the distance to the UV light source, and the concentration of PL incorporated in the solvent solution (Table 1). PL was incorporated in the solvent solution in increasing volumetric concentrations ranging from pure water (0% PL) to pure PL (100% PL). To obtain hydrogels, dry me-HA was dissolved in the solvent solution containing the photoinitiator. Then, 25 μL of me-HA solution were injected into a circular (5mm diameter) PDMS mold and exposed to a UV light (Omniscure series 2000 EXFO S2000-XLA, Omnicure, Canada) to trigger the photocrosslinking, producing disk-shaped hydrogels. The produced formulations, incorporating 0, 50 and 100% PL, were designated PL_0 , PL_{50} , and PL_{100} , respectively.

Table 1. Summary of the formulations studied for the optimization of the hyaluronic acid hydrogels incorporating PL (HAPL). The concentrations of me-HA and Irgacure 2959 are presented as weight/volume percentage. The PL concentrations are volumetric concentrations of pure PL (100%PL) in water (0%PL). All the formulations described below were prepared using both the batches of me-HA (5x and 10x molar excess).

me-HA (wt/v%)	Irgacure (wt/v%)	PL (v/v%)
1%	0,10%	0%
		50%
		100%
2%	0,20%	0%
		50%
		100%
1%	0,10%	0%
		50%
		100%
2%	0,20%	0%
		50%
		100%

Characterization of the HAPL hydrogels

Only the 10x me-HA batch allowed obtaining the hydrogels by photopolymerization, using either 0.1 or 0.2% of photoinitiator, so this batch was selected for all further studies. Considering that Irgacure 2959 presents some cytotoxicity²¹, it was also decided to use the lower photoinitiator concentration for the following characterization steps.

Evaluation of the mechanical properties by DMA

The viscoelastic properties of the developed hydrogels (PL₀, PL₅₀ and PL₁₀₀ with 1% or 2% of me-HA and with 0.1% of Irgacure) were evaluated by dynamic mechanical analysis (DMA)

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3 (TRITEC8000B, Triton Technology, UK), equipped with the compressive mode. DMA spectra
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5 were obtained during a frequency scan ranging between 0.1 and 15 Hz for all time points. The
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7 experiments were performed under constant strain amplitude, corresponding to approximately
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9 1% of the original height of the sample. Samples were tested while immersed in PBS and at
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11 37°C, to simulate the physiological conditions.
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15 16 17 **Swelling and weight loss**

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19 The results obtained from the DMA analysis revealed better mechanical properties for the 2%
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21 me-HA formulation and thus this was selected for the subsequent studies, namely degradation,
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23 protein release and cell response. Thus, formulations of hydrogels with increasing concentrations
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25 of PL (PL₀, PL₅₀ and PL₁₀₀), were prepared into disc-shaped samples of 5mm in diameter and 1
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27 mm thickness, as above described, and placed in 24 wells plate.
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32 Periodontal ligament fibroblasts express hyaluronidase (HAase) and generate HAase activity
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34 that regulates extracellular hyaluronan metabolism²². Given the presence of this enzyme in the
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36 periodontium, the degradation promoted by a HAase was investigated. Similar assay was
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38 conducted in PBS. Each sample was incubated in 1.6 mL of PBS at 37°C, pH 7.4. For the
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40 enzymatic degradation assays, the same formulations were incubated at 37°C in 1.6 mL of a
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42 HAase solution of 100 U/mL in PBS.
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47 The assays were carried out using 4 samples of each formulation immersed in each of the
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49 solutions. The samples were retrieved after 1, 3, 7, 14 and 21 days of incubation.
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52 The wet weight of the samples was registered (PI-214 analytical balance, Denver Instrument
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54 Company, USA) at each pre-determined time point. The dry weight of the samples was also
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3 registered after allowing samples to dry overnight at 37°C. The percentage of weight loss was
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5 calculated according to equation (1):
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$$\text{Weight loss} = \frac{(m_i - m_f)}{m_i} \times 100 \quad (\text{Equation 1})$$

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15 where m_i is the initial weight and m_f the final weight.
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21 The water uptake ratio was also calculated following equation (2) by dividing each sample wet
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23 mass (m_{wet}) by the final dry hydrogel mass (m_{dry}).
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$$\text{Water uptake ratio} = \frac{m_{wet}}{m_{dry}} \times 100 \quad (\text{Equation 2})$$

28 29 30 31 32 **Quantification of protein release**

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34 Protein release from PL₀, PL₅₀ and PL₁₀₀ was quantified after 30min, 1h, 4h, 8h, 1, 7, 14 and
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36 21 days of incubation in PBS at 37°C. For this purpose, at each time point, a volume of
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38 supernatant was collected and stored at -20°C. The total protein content was quantified using a
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40 micro BCA protein assay (Thermo Fischer Scientific, USA), following the manufacturer's
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42 instructions. Additionally, the release of fibroblast growth factor-2 (FGF-2), present in the PL,
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44 was also quantified using an enzyme-linked immunosorbent assay kit (Human FGF-basic,
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46 ELISA Development Kit, by PeproTech, USA), according to manufacturer's instructions.
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54 **Evaluation of the response of human periodontal ligament fibroblasts (hPDLFs)**

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3 The response of hPDLFs to the photocrosslinked me-HA/PL hydrogels was assessed upon
4 either encapsulation or seeding of the cells onto the hydrogels surface and further cultured for up
5 to 14days.
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10 The hPDLFs (ScienCell Research Laboratories) at passage 3 were seeded on disc-shaped (5
11 mm diameter) samples of the formulations PL₀, PL₅₀ and PL₁₀₀ produced as previously
12 described, at a cell density of $5 \times 10^4 \text{ cm}^{-2}$. A 50 μL drop of a cellular suspension containing 1×10^4
13 cells was seeded on the surface of each sample, previously placed in a 24 wells plate, and
14 allowed to adhere for 1h. After this period, 450 μL of α -MEM basal medium (supplemented with
15 10% of FBS and 1% antibiotic-antimycotic) were added to each well. The 24 wells plates
16 containing the cell-seeded hydrogels were further incubated at 37°C, 5% CO₂ for 1, 4, 7 and 14
17 days, renewing the culture medium every 3 days. Cells cultured on polystyrene cover-slips
18 (Sarstedt) were employed as positive control.
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31 For the encapsulation, hPDLFs cells were resuspended in 2% me-HA solutions containing 0,
32 50 and 100% PL to obtain a final cell density of $4 \times 10^6 \text{ cells.ml}^{-1}$. Then, 25 μL (1×10^5 cells) of
33 the cellular suspension in each hydrogel solution formulation was injected into circular moulds
34 (5mm diameter) and exposed to UV light, as previously described to obtain the hydrogel
35 samples. The cell-laden hydrogels were subsequently transferred to individual wells of 24-well
36 plates, each one containing 500 μL of basal medium. The 24-wells plates were incubated at
37 37°C, 5% CO₂ for 1, 4, 7 and 14 days renewing the culture medium every 3 days.
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48 The metabolic activity of the cells seeded/encapsulated in the hydrogels and further cultured
49 was evaluated using the Alamar blue assay (AbDseroTec, USA), following the manufacturer's
50 instructions. Briefly, at each time point, the culture medium was discarded, the samples were
51 washed twice with PBS and then incubated in a 10% Alamar blue solution in basal medium (450
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3 μL of basal medium, and 50 μL of Alamar blue) at 37°C, 5% CO₂ for 150 min. The fluorescence
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5 of the supernatant solution was read in triplicates in a microplate reader (Synergy HT, Biotek,
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7 USA-) at 560 nm of excitation and 590 nm of emission.
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10 The cellular proliferation was also evaluated as a function of the dsDNA quantification using
11 the PicoGreen dsDNA quantification kit, according to manufacturer's specifications (Life
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13 Technologies, USA).
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17 Finally, the morphology and the migration of the cells either encapsulated or seeded on the
18 surface of the hydrogels were investigated by confocal microscopy, upon staining with DAPI and
19 phalloidin. For this purpose, samples retrieved after each of the pre-set culturing times were
20 fixed with 10% formalin (in PBS) for 30 min at room temperature. Afterwards, the samples were
21 washed 2 times with PBS to remove the formalin and 300 μL of phalloidin solution (1:100 in
22 PBS) were added per well and incubated 1 hour at room temperature. Then phalloidin solution
23 was discarded and the samples were washed 3 times with PBS. A DAPI solution (1:1000 in PBS)
24 was prepared and 300 μL were added per well and incubated 5 min. The samples were washed 3
25 times and the prepared for visualization under a confocal microscopy (TCS SP8 from Leica
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27 Mycosystems CMS GmbH) with vectashield mounting medium.
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43 **Antimicrobial Assay**

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45 The antimicrobial activity of PL soluble factors released form from the HA hydrogels was
46 evaluated using the radial diffusion assay, according to Kirby-Bauer method²³. Five different
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48 bacteria species were used: the gram-positive bacteria *Bacillus megaterium* (Internal collection),
49 Methicillin Resistant *Staphylococcus aureus* (MRSA) (Internal collection), and Vancomycin
50 Resistant *Staphylococcus aureus* (VRSA) (internal collection) and the gram-negative species
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3 *Pseudomonas aeruginosa* T6BT12, *Escherichia coli* DH5 α) and the fungus *Candida albicans*
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5 (Internal collection). With the exception of *P. aeruginosa*, which was isolated from
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7 environmental samples, all the other microorganisms were isolated from clinical samples. Prior
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9 to the antimicrobial activity testing, these microorganisms were cultured aerobically in Luria-
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11 Bertani broth at 37 °C overnight with agitation (150 rpm). Afterwards, they were centrifuged at
12
13 8000 rpm for 5 min, and washed three times with PBS. Microbial cultures were adjusted to a
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15 concentration corresponding to ca. 10⁷ CFU.mL⁻¹, and pipetted with 0.4 % agar into a petri dish
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17 containing 5 mL of Muller-Hinton (MH) Agar plate.
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22 The PL₀, PL₅₀ and PL₁₀₀ hydrogels and the negative control (PBS) were placed on MH-agar
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24 plates and cultured with each of microbial strain at 37 °C for 16 h, upon which the inhibition
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26 halo measure and the general macroscopic response was recorded. Experiments were performed
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28 in triplicate.
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34 **Statistical analysis**

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36 All the experiments were performed with at least three replicates. All the cell culture
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38 experiments were performed simultaneously in order to reduce the variability intra-assay and 3
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40 independent studies were performed, exactly as described. Results are expressed as mean \pm
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42 standard error of the mean (SEM). Statistical analysis was performed by repeated measures Two-
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44 way ANOVA comparison test (* p < 0.05, ** p < 0.01 and *** p < 0.001 for statistically
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46 significant differences) using the software Graph Pad Prism 6.
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53 RESULTS

55 **Development of the photocrosslinkable me-HA hydrogels**

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HA methacrylation

In this study, unmodified hyaluronan was methacrylated reacting a 1% HA aqueous solution at pH 8, with 5x and 10x of molar excess of MA for 24h at 4°C.

The methacrylation of HA was confirmed by the FTIR spectra, where the deep peak at 1715 cm^{-1} represents the carbonyl ester group resultant from the methacrylation (Figure 1C). Moreover, the ^1H NMR spectra of the me-HA batches (Figure 1D) exhibited the presence the characteristic peaks corresponding to the two protons of the double bond region (δ 5.77 and 6.20 ppm) of the MA group absent in the non-modified HA spectrum.

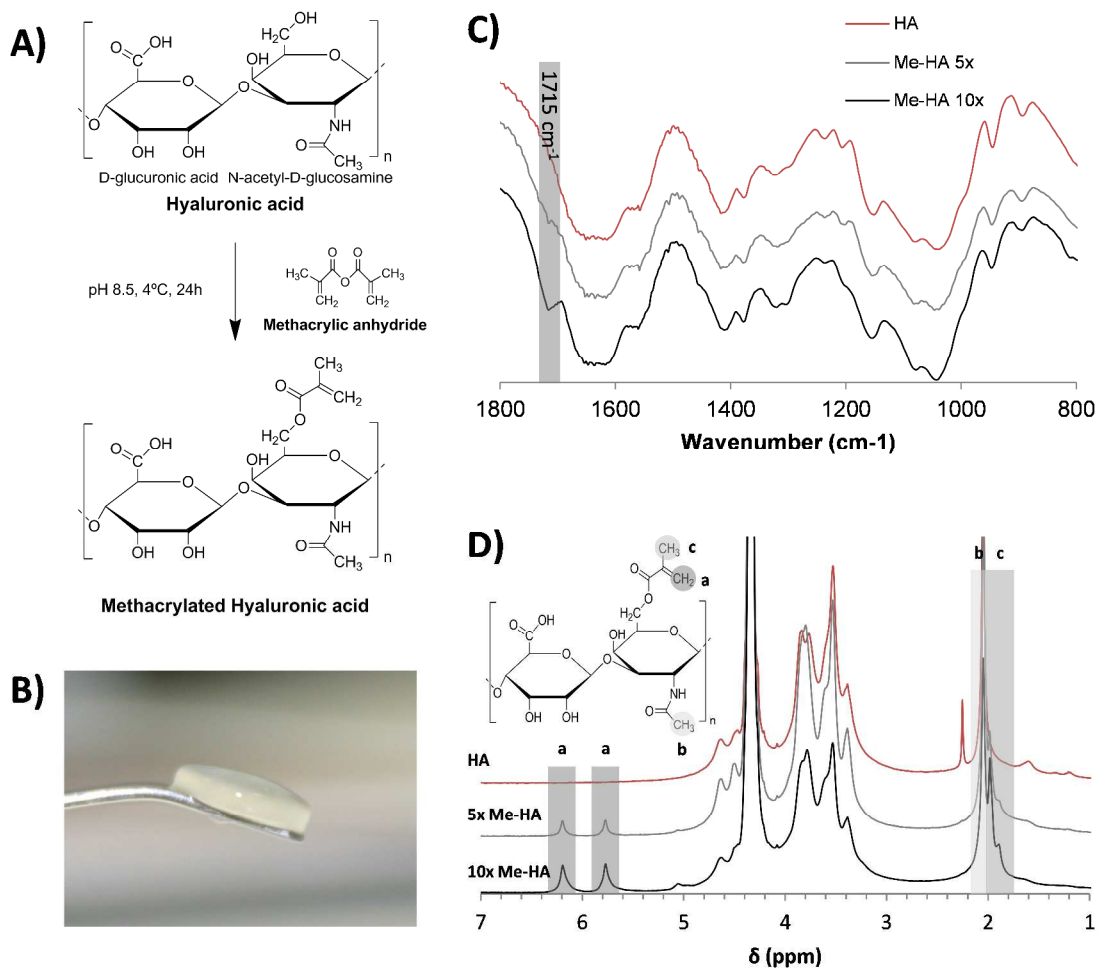


Figure 1. A) Scheme of the methacrylation process of Hyaluronic acid using methacrylic anhydride. B) Representative image depicting a typical me-HA/PL hydrogel obtained by photopolymerization. C) FTIR Spectra of HA and me-HA produced with 5 and 10x molar excess of (5X and 10X me-HA). D) ^1H NMR spectra of HA, 5x me-HA and 10x me-HA: a) Vinyl groups of MA (δ 5.77 – 6.20 ppm); b) Methyl group of the N-Acetyl-d-Glucosamine (δ 2.05 ppm); and c) methyl group of MA (δ 1.94 ppm).

The degree of methacrylation was calculated from the ratio of the relative peak integration of the methacrylate protons (peaks at \sim 6.20, \sim 5.77, and \sim 2.05 ppm) and the methyl protons of N-

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3 Acetyl-D-glucosamine (~1.98 ppm). A *Dmet* of 14% was obtained for the me-HA batch
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5 produced with 5x excess of MA (5x me-HA), while the batch produced with 10x excess MA
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7 (10x me-HA) presented a *Dmet* of 24%.
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10 11 12 **Mechanical properties of the developed hydrogels**

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15 Dynamic mechanical analysis (DMA) experiments were performed in a hydrated environment
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17 at 37°C, in an array of biologically relevant frequencies, in order to assess the viscoelastic
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19 properties of the samples in a physiological-like environment. Both storage (elastic) modulus, E' ,
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21 and the loss factor, $\tan \delta$, were obtained at different frequencies. E' is a measure of the materials
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23 stiffness. The loss factor is the ratio of the amount of energy dissipated (viscous component)
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25 relative to energy stored (elastic component); $\tan \delta = E''/E'$.
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30 The obtained results (Figure 2) showed the effect of different concentrations of me-HA and/or
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32 PL on the stiffness of the developed hydrogels. When the concentration of me-HA was increased
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34 from 1% to 2% the elastic storage modulus of the hydrogels also increased above three to four
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36 times, from approximately 100 kPa to 428– 600 kPa, in formulations incorporating PL (PL₅₀ and
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38 PL₁₀₀). The concentration of PL in the hydrogels also showed to influence the elastic modulus
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40 that was found to increase proportionally with the amount of PL. The formulation that exhibited
41
42 the highest elastic modulus corresponds to the formulation containing 2% of me-HA dissolved in
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44 100% PL.
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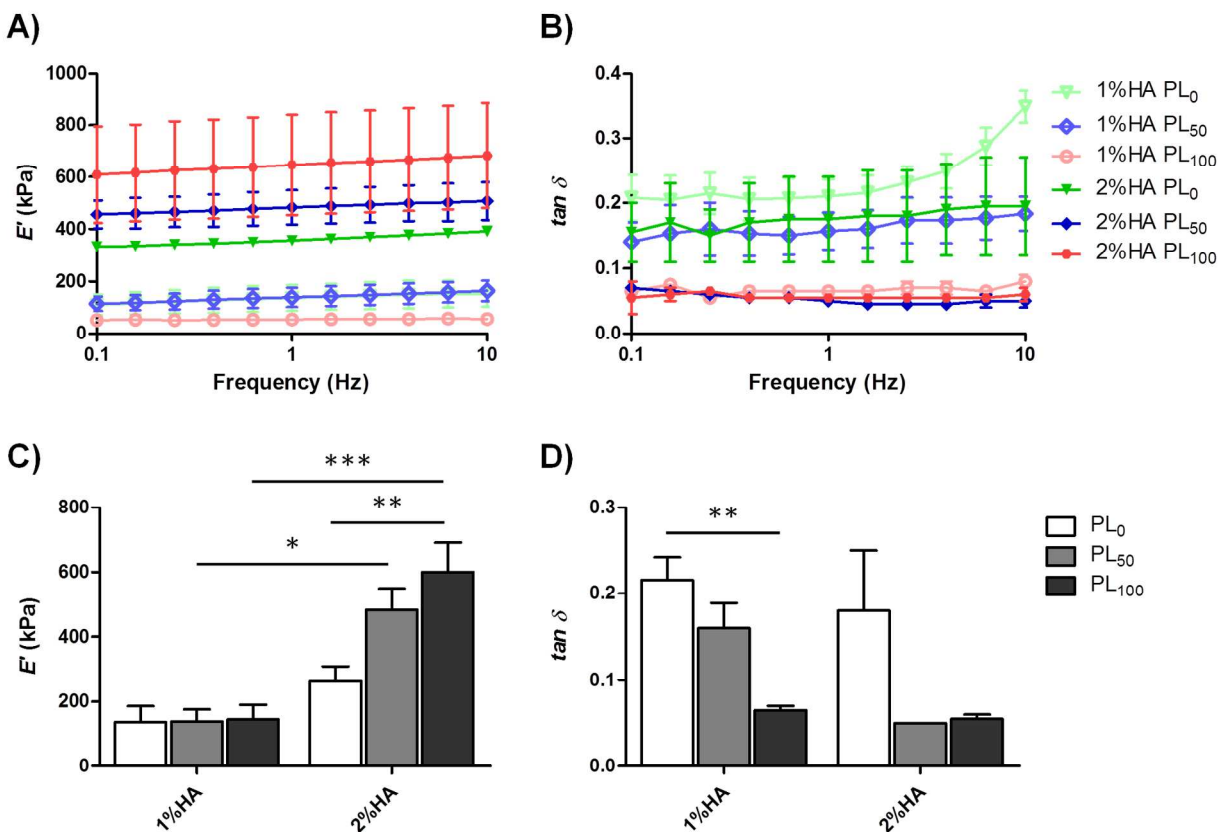


Figure 2. Variation of elastic (E') modulus A) and loss factor ($\tan \delta$) B) with frequency of 1% and 2% HA hydrogels incorporating 0%,50% and 100% v/v PL (PL₀, PL₅₀ and PL₁₀₀) measured by dynamic mechanical analysis. Differences observed on elastic (E') modulus C) and loss factor ($\tan \delta$) D) at 1 Hz. * $p < 0.05$, ** $p < 0.02$; *** $p < 0.001$

Degradation behavior

The weight loss and swelling ratio profiles of the PL₀, PL₅₀ and PL₁₀₀ hydrogels after incubation in PBS or HAase (100U/mL) solution at 37°C for 1, 3, 7 and 14 days are presented in Figure 3.

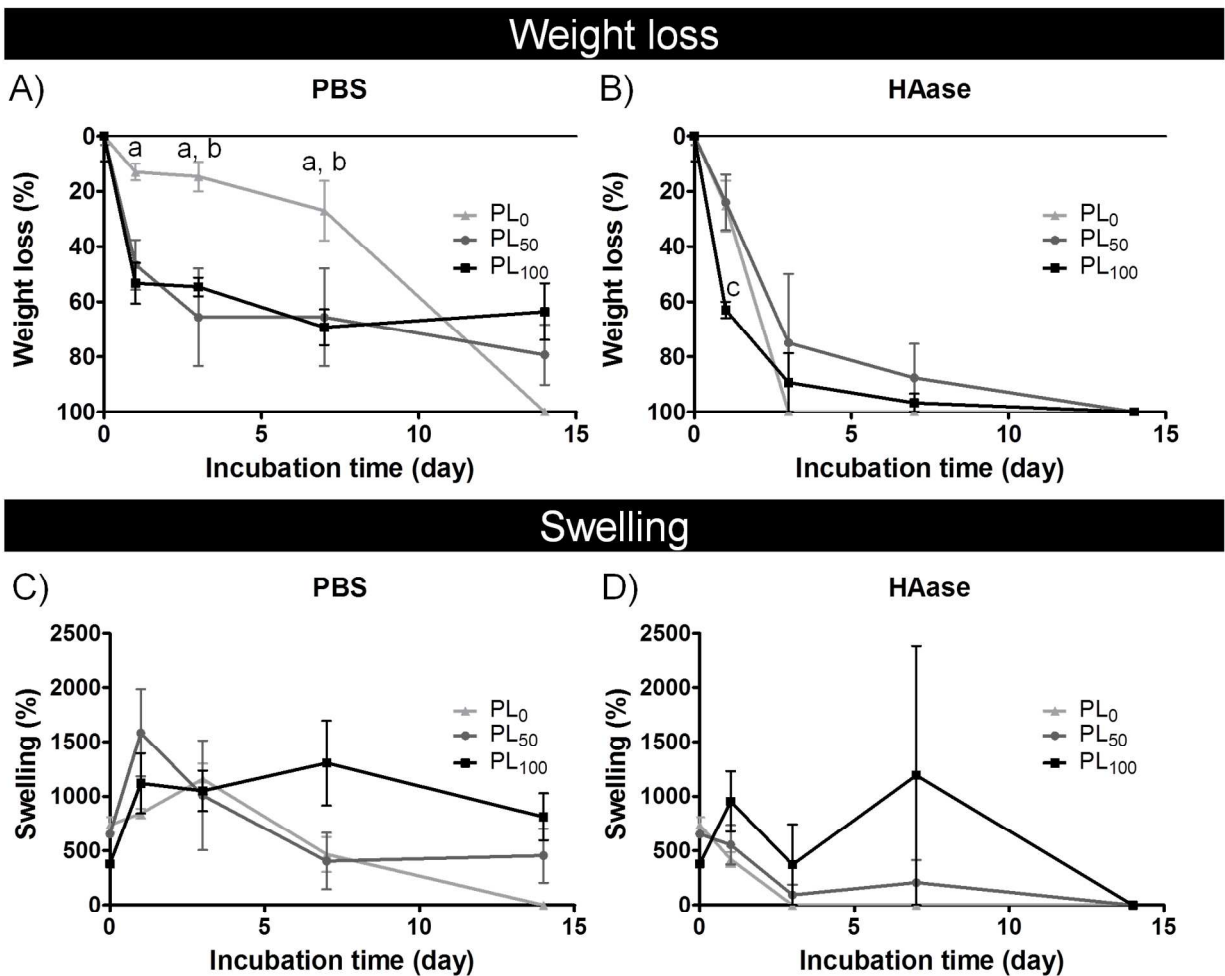


Figure 3. Weight loss (A and B) and Swelling ratio (C and D) profile of PL₀, PL₅₀ and PL₁₀₀ hydrogels in: PBS (A and C) and in HAase solution (100U/ ml) (B and D). a) statistically different ($p < 0.05$) from PL₁₀₀; b) statistically different ($p < 0.05$) from PL₅₀; c) statistically different ($p < 0.05$) from PL₀ and PL₅₀

Weight loss

Overall, the results obtained showed that the incorporation of PL in me-HA hydrogels influences its stability. Although the PL₀ hydrogels showed lower weight loss until the 7th day of immersion in PBS, they were completely degraded after 14 days (Figure 3A). On the other hand,

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3 despite the weight loss profile of the formulations incorporating PL is characterized by an initial
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5 loss of around 70% of the dry weight in the first 3 days, the PL₅₀ and PL₁₀₀ hydrogels tend to be
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7 more stable along immersion time in PBS.
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10 The weight loss results obtained upon immersion in HAase, revealed that PL₁₀₀ formulation
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12 displays higher degradability, upon the first day. Nevertheless, it was found that samples
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14 containing PL were only completely degraded after 14 days, while all the hydrogels of the PL₀
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16 formulation were completely degraded after only 3 day of immersion in the enzymatic solution.
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22 **Swelling ratio**

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24 In the beginning of the assay, the swelling of freshly produced PL₁₀₀ hydrogels was
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26 significantly lower than the formulations with lower PL concentration. When immersed in the
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28 PBS solution the PL₀ and PL₅₀ hydrogels, didn't show significant statistical differences among
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30 them for all the time points studied. Accordingly, both hydrogels formulations presented a
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32 similar profile characterized by a peak around day 1 (1500% for PL₅₀) and day 3 (1000% for
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34 PL₀), followed by a decrease of swelling until the end of the assay, due to the total degradation of
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36 the material. On the other hand, PL₁₀₀ hydrogels had a later peak at day 7, reaching near 1400%
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38 of swelling.
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43 Regarding the swelling in HAase solution, the values were statistically similar for PL₀, PL₅₀
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45 and PL₁₀₀ hydrogels. Nevertheless, while the formulations PL₀ and PL₅₀ depicted a similar
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47 behavior, presenting a constant decrease in the swelling values from the beginning of the assay,
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49 the PL₁₀₀ formulation reached an average swelling of 1400% at day 7, before starting to
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Protein release

The total amount of protein released from me-HA/PL hydrogels over time is represented in Figure 4.

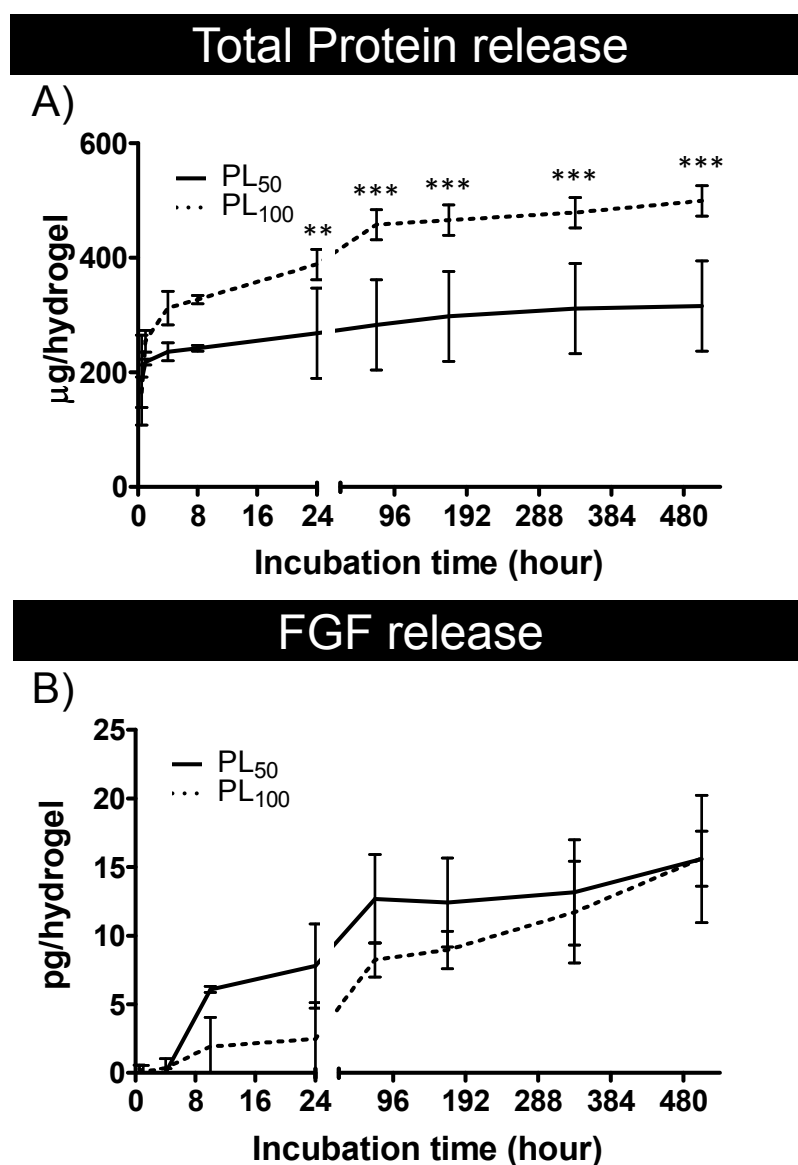


Figure 4. Total protein released from the hydrogels containing PL, assessed using the Pierce® BCA protein assay kit (A) incubated in PBS. Fibroblasts Growth Factor (FGF) release, assessed

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3 using the PeproTech ELISA Development kit (B) incubated in HAase solution (100U/ml). **
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5 p<0.02; *** p<0.001
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11 Both PL₅₀ and PL₁₀₀ hydrogels displayed a similar release profile that is characterized by an
12 initial “burst” of protein released during the first hour, that represents around 15% for PL₁₀₀
13 hydrogels and 25% for PL₅₀ hydrogels of the total protein contained, followed by a sustained
14 release up to 14 days. While no statistically significant differences were observed between the
15 formulations during the first day of release, there was a substantial difference in the amount of
16 protein released by the PL₁₀₀ formulation, which is proportional with the amount of protein
17 incorporated in the formulations.
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28 In order to evaluate the release of PL-specific GFs from the developed HA hydrogels, and the
29 interaction of the GFs with the HA mesh, hydrogels were incubated either in PBS or in 100
30 U/mL HAase solution and the release products were quantified by ELISA.
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35 The results for the release of FGF-2, depicted in Figure 4B, showed that the PL₅₀ and PL₁₀₀
36 had a different profile for FGF-2 release. The FGF-2 released by PL₅₀ was characterized by an
37 initial burst of release up to day 3, as observed. After day 3, the release kinetics reached an
38 apparent plateau, and a slow sustained delivery remained up to day 21. On the other hand, PL₁₀₀
39 hydrogels showed a sustained release, progressing in a linear way, during all the duration of the
40 assay, without signs of deceleration. Nevertheless, despite the PL₁₀₀ hydrogels have higher
41 amount of total protein incorporated, they depicted a FGF-2 release similar to the PL₅₀ hydrogel.
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54 **Cell response to the developed hydrogels**

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The response of hPDLFs, either surface seeded or encapsulated onto the PL₀, PL₅₀ and PL₁₀₀ hydrogels was assessed. In both the cases, the increasing amounts of PL in the hydrogels had a positive effect in the cells metabolic activity and proliferation rate as shown in Figure 5.

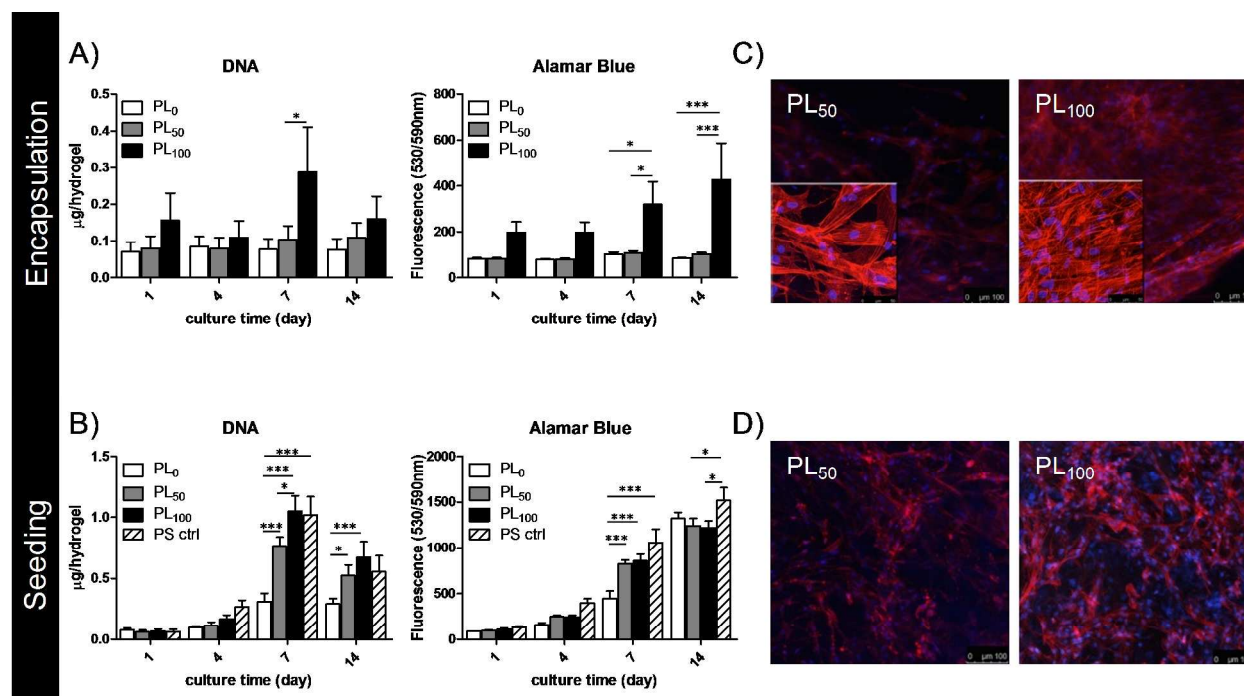
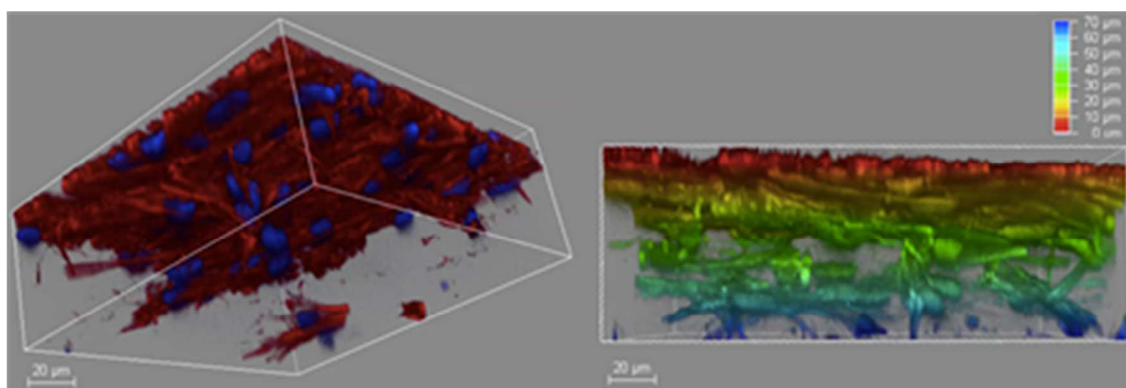


Figure 5. Response of hPDLFs seeded/encapsulated on the hydrogels with the formulations PL₀, PL₅₀, PL₁₀₀. A) DNA quantification and metabolic activity of encapsulated cells. B) DNA quantification and metabolic activity of seeded cell. C) Representative pictures of hPDLFs encapsulated in PL₅₀ and PL₁₀₀ hydrogels, stained with DAPI (Blue) and Phalloidin (Red). for 21 days. The small micrographs on the bottom left depict the spindle-like shape morphology of the hPDLFs encapsulated into the hydrogels. D) hPDLFs seeded on PL₅₀ and PL₁₀₀ hydrogels and cultured for 21 days, stained with DAPI (Blue) and Phalloidin (Red).

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3 The results presented in Figure 5A show that there were no significant differences between the
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5 PL₀ and PL₅₀ hydrogels with respect to proliferation and metabolic activity of encapsulated cells.
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7 Remarkably, PL₁₀₀ hydrogels exhibited higher cell growth and metabolic activity than PL₀ and
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9 PL₅₀ hydrogels. Regarding the morphology of the encapsulated cells, Figure 5B shows that
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11 hPDLFs dispersed and stretched inside of the hydrogels, following the alignments of the fibrous
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13 structures observed macroscopically in the hydrogels.
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17 The Figure 5C shows the behaviour of the hPDLFs cells when seeded at the surface of the PL₀,
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19 PL₅₀ and PL₁₀₀ hydrogels. No significant differences were seen in terms of seeding efficiency on
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21 the hydrogels and on the PS positive control.
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25 The analysis of hPDLFs distribution throughout the PL-enriched hydrogels, obtained by
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27 confocal microscopy from PL₁₀₀ hydrogels 21 days after being seeded on the surface, is
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29 represented in Figure 6. This picture shows that hPDLFs seeded in the surface of the hydrogels
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31 migrated up to 70 μm deep into to the hydrogel after 21 days in culture.
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50 **Figure 6.** Three-dimensional reconstruction obtained by confocal microscopy of hPDLFs
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52 distribution on PL₁₀₀ hydrogels at day 21
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Antimicrobial activity

The antimicrobial effect of PL soluble factors against *Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia Coli*, *Bacillus megaterium*, *Staphylococcus* (VRSA), and *Staphylococcus* (MRSA), was evaluated.

The antimicrobial properties of the developed hydrogels containing PL were assessed using the agar well diffusion method, adapted from the Kirby-Bauer original method for testing microbial resistance to antibiotic drugs. The Figure 7 shows the effect of the hydrogels incorporating increasing amounts of PL in the *Pseudomonas aeruginosa*, *Candida albicans*, and *Escherichia coli* and e, *Bacillus megaterium*, vancomycin resistant *Staphylococcus aureus* (VRSA), methicillin resistant *Staphylococcus aureus* (MRSA).

The release of PL provides antimicrobial action against methicillin resistant *Staphylococcus aureus*, as shown by the inhibition of growth in the space occupied by the PL₁₀₀ hydrogel (Figure 7F). Moreover, it is dependent on the PL content, since no inhibition halo was observed for the formulations with lower amounts of PL incorporated (PL₀ and PL₅₀). Nevertheless, despite no inhibition halo was observed in the rest of the species for the formulations investigated, no degradation or bacterial growth on the hydrogel surface was reported.

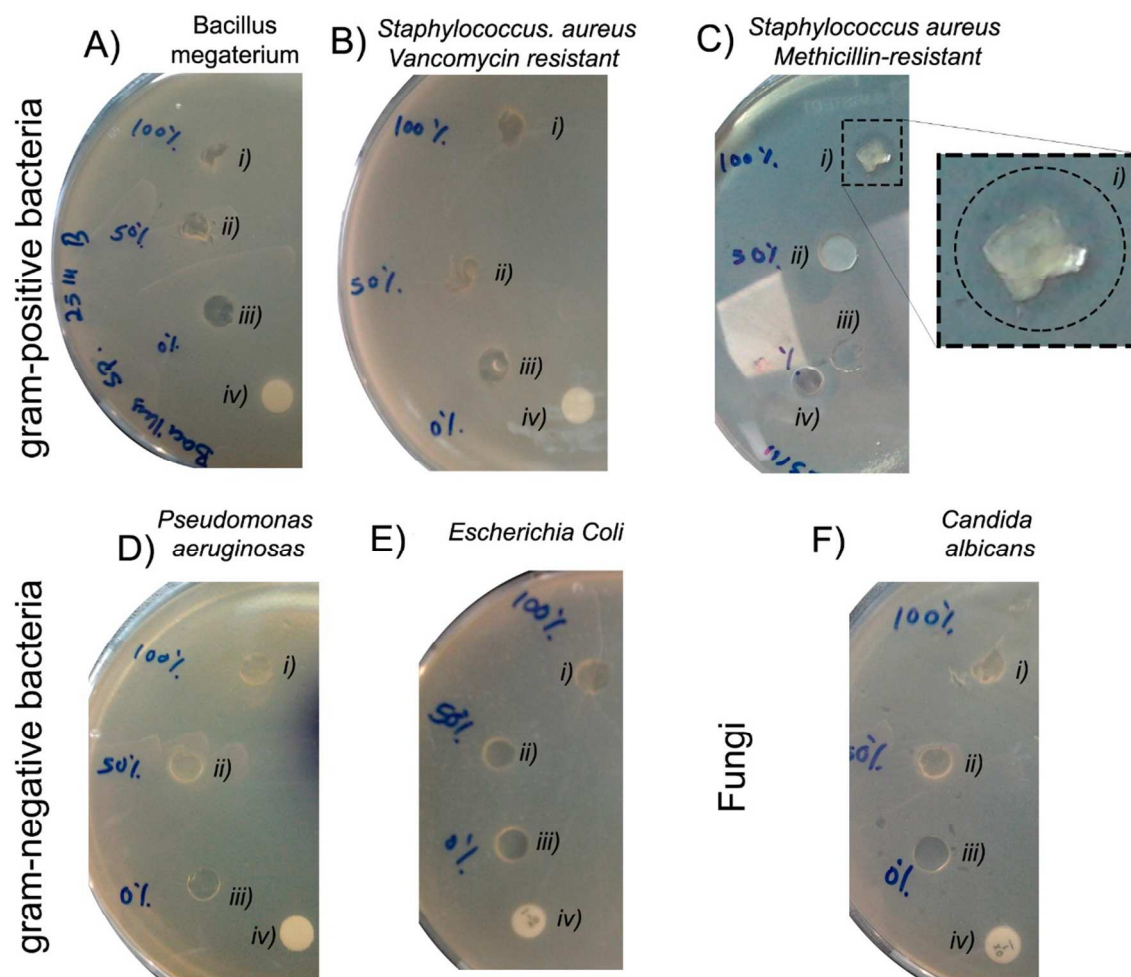


Figure 7. Antimicrobial assay for PL₁₀₀ (i) PL₅₀ (ii) and PL₀ (iii) formulations where control is PBS (iv) using A) *Pseudomonas aeruginosa*, B) *Candida albicans* C) *Escherichia Coli* (E.Coli) D) *Bacillus megaterium* E) Vancomycin resistant *Staphylococcus aureus* (VRSA) F) Methicillin resistant *Staphylococcus aureus* (MRSA).

DISCUSSION

The present work describes the development of novel photocrosslinkable hydrogels incorporating allogenic platelet lysate, a platelet rich hemoderivative (PRHd), aimed at endogenous regenerative technology (ERT) being used for the regeneration of periodontal

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3 ligament. PL can be used in clinical applications as an autologous therapy. However, several
4 authors ^{5, 24} have reported high donor-to-donor variability in PRHds batches, which could
5 correlate with the high variability associated with the clinical outcomes of PRHds treatments ²⁵.
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8 On the other hand, Crespo-Diaz et al. ⁵ reported lower variability in PL batches produced from
9 outdated platelet concentrates obtained by plasma apheresis from different donors; therefore
10 more predictable therapeutic outcomes could be anticipated. Furthermore, these PL batches were
11 shown to be safe of standard pathogens and infectious diseases. In the present work, were used
12 outdated (> 5 days old) platelet concentrates obtained by plasma apheresis and biologically
13 qualified according to Portuguese legislation (Decreto-Lei No. 100/2011) for blood products
14 collection, transport and therapeutic administration. Therefore, these PL batches are expected to
15 be as save as any other blood component aimed for therapeutic administration and used in
16 allogenic PL-based strategy as proposed. The combination of me-HA with PL, as herein
17 proposed, produced a photocrosslinkable system with several advantages for tissue engineering
18 applications. Being injectable, these biomaterials can be implanted using minimally invasive
19 techniques without requiring surgical interventions. Moreover, the system can fit perfectly to
20 irregular shaped defects, deeply interacting with the preserved tissue margins, before being
21 photocrosslinked to produce a stable matrix.
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43 With regard to viscoelastic properties, DMA analysis revealed that these hydrogels exhibit
44 elastic modulus ranging from 264±81 kPa for the PL₀ formulation to 600±186kPa to the PL₁₀₀
45 formulation (at 1 Hz), comparable to other HA hydrogels incorporating fibrin described for
46 artificial cartilage implantation (445kPa)²⁶, which support the use of our photocrosslinkable
47 hydrogels for soft tissue reconstruction. Moreover, periodontal tissue is continuously subjected
48 to very dynamic forces, acting the periodontal ligament as a damper²⁷⁻²⁸. Therefore, the
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3 viscoelastic properties displayed by the hydrogels herein developed are of paramount importance
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5 for periodontal therapy approaches.
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8 Regarding the degradation of HA hydrogels, it was faster in the presence of the HAase, the
9 specific enzymes that degrade the HA *in vivo*²⁹, than in saline solution, as previously reported¹³,
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13³⁰. Remarkably, the PL enriched hydrogels remained stable for longer periods. The time to total
14 degradation of PL₁₀₀ was even longer when compared with other HA hydrogels exposed to
15 similar conditions¹³. It should be noted that in this study was used a supra-physiologic
16 concentration of HAase (100 U/mL), that in human plasma range from 0.0028±0.0004 U/L to
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The total PL-proteins release kinetics from the HA hydrogels herein developed was
characterized by an initial “burst”, followed by a sustained release over time. The release profile
observed can be explained by two different processes: 1) the fast elution of large amount of the
soluble proteins that are not physically interacting with the HA mesh, facilitated by the strong
initial swelling of roughly two times the hydrogel initial weight; 2) a slow release of the proteins
entrapped in the hydrogel mesh or adherent to the mesh, that are released by the physical

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3 degradation of the hydrogel. Since the PL proteins have different isoelectric points (pI), the
4 electrostatic interactions and probability of remaining adsorbed to the HA mesh, which are
5 negatively charged at physiologic pH, will vary. In this way, the albumin, which is the main
6 soluble protein in PL³³, with an acidic pI (at pH 4.7), is expected to be easily washed out from
7 the HA mesh. On the other hand, most of the GFs present in PL with therapeutic interest have
8 basic pI (TGF- β at pH 8.90; PDGF-A at pH 9.52; PDGF-B at pH 9.39; VEGF-1 at pH 8.66;
9 FGF-2 at pH 9.6). So, they are expected to bind electrostatically to the HA matrix and to the
10 insoluble PL proteins to be further released by ion exchange or by the degradation of the HA
11 mesh promoted by HAs released for the ECM remodeling promoted during the wound healing
12 process. In fact, the release of PL-specific GFs from the photocrosslinkable hydrogels, namely
13 FGF-2, was only detected after degradation of the hydrogels in HAase (Figure 4B), while no
14 detectable traces of GFs were detected after incubation of the hydrogels in PBS. Studies with
15 FGF-2 have shown that this GF upregulate the migration and proliferation of PDL cells³⁴. In fact,
16 in order to fully regenerate functional of periodontal tissues, several GFs and cytokines should
17 interplay in a temporal as spatial controlled manner¹⁰. Therefore, the controlled release of growth
18 factors is a real asset to our hydrogels.

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41 In line with what has been reported in literature, our findings show that the encapsulation of
42 hPDLFs in non-supplemented HA hydrogels (PL₀) affects cell proliferation and metabolic
43 activity. The biological performance of cells encapsulated in me-HA hydrogels is affected by the
44 concentration of the macromer^{13, 35}, as well as by the concentration of photoinitiator³⁵.
45 Furthermore, the exposure to UV radiation was also reported to have adverse effects on viability
46 and cell cycle progression, while the differentiation potential remains unchanged³⁵. Remarkably,
47 the adverse effects of photo-encapsulation were overcome by the incorporation of PL into the
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3 hydrogels. The viability and metabolic activity of the encapsulated hPDLFs increased
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5 proportionally with the incorporation of PL. Previous works have reported the positive effect of
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7 PL in the proliferation and maintenance of stemness phenotype of human periodontal ligament
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9 stem cells³⁶. In the same line, we observed, in previous works that (hPDLFs) adhere and
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11 proliferate in genipin-crosslinked PL membranes³⁷. It is known that platelets release several
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13 growth factors, namely PDGF and FGF-2, which have a mitogenic effect over human
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15 periodontal ligament cells³⁸⁻³⁹. Moreover, PDGF and FGF-2 have been reported to have
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17 chemotactic properties over hPDLFs^{34, 40}, while the adhesion sites provided by the clot-forming
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19 proteins present in PL should facilitated the inward cell migration observed (Figure 6).
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21 Therefore, a strategy that can recruit progenitor cells from the preserved periodontal tissue and
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23 promote their proliferation and maintenance of stemness to colonize the periodontal defect with
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25 cells with great potential to regenerate periodontal tissue would be a valuable asset for
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27 periodontal ERT. Hereupon, the first intentional repair promoted by cells originated from
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29 periodontal tissues could partially restore the primitive anatomy and function of the
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31 periodontium⁴.

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34 Finally, we have studied the antimicrobial properties of the developed hydrogels, a very
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36 important aspect considering the target application. It is known that the main cause of
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38 periodontal disease, as well as the main factor of rejection for some of the GTR techniques, is
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40 bacterial infections⁴¹⁻⁴². The HA was previously described to have bacteriostatic properties
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42 against oral and non-oral bacteria⁴³. Carlson et al.⁴³ suggested that the bacteriostatic effect of HA
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44 may be due to the saturation of the bacterial hyaluronate lyase by the excess HA, which prevents
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46 the bacteria from maintaining elevated levels of tissue permeability and penetrating the physical
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48 defenses of the host. This would enhance the ability of the host's immune system to eradicate
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3 pathogens. HA molecules in the hydrogels also form a random network of chains that may act as
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5 a sieve preventing the spread of the bacteria. Platelet concentrate (PC) was previously reported to
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7 have antimicrobial properties¹² significantly reducing the growth of methicillin -sensitive or -
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9 resistant *Staphylococcus aureus*, Group A *Streptococcus*, and *Neisseria gonorrhoea*, among
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11 others. Being PL a product of PC activation by freeze/thaw cycles, the same would be expected
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13 for this hemoderivative. The obtained results in this study meet with the antimicrobial properties
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15 already described in the literature for platelet concentrates¹². Here, the methicillin resistant
16
17 *Staphylococcus aureus* (MRSA) was more susceptible to the hydrogels containing PL₁₀₀ than the
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19 other microbial strains tested. Yeaman and Bayer proposed that the bactericidal activity against
20
21 MRSA involved β -lysin, which is responsible for blood clotting found after platelets activation⁴⁴⁻
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23 ⁴⁵. β -lysin, which is one of the most abundant compound found in PL after activation⁴⁶ has been
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25 described to act against bacteria cell-wall, rapidly killing and stopping bacteria reproduction⁴⁴⁻⁴⁵,
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27 which could explain the results from this study. In addition, other PL-derived molecules with
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29 antibacterial properties against Gram+ bacteria could be involved in this response, such as
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31 neutrophil activating protein-2 demonstrated capacity to kill Gram-positive and Gram-negative
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33 bacteria⁴⁷⁻⁴⁸. Although no effect was observed against Gram- bacteria and fungus, other factors
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35 can be found in PL with bactericidal and fungicidal activity. For instance, Platelet factor-4 can
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37 bind to Gram-negative bacteria since it has an affinity for the lipopolysaccharide from these
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39 bacteria, facilitating their clearance⁴⁹⁻⁵⁰. Nevertheless, further investigation is needed in order to
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41 fully understand PL antimicrobial properties against microbial pathogens, especially whether the
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43 molecules that demonstrate antimicrobial potential interact alone or together when supplemented
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45 as PL and not from induced platelets.
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CONCLUSIONS

Overall, our findings demonstrate that is possible to obtain versatile photocrosslinkable HA-PL hydrogels that provide adequate substrates for hPDLFs attachment and growth while enabling the sustained release of PL and inhibit bacterial growth. Besides providing adequate space and stability, as well as biochemical cues for the regeneration of the lost tissues the hydrogels developed in this study present antimicrobial properties, which can contribute for the prophylaxis, preventing recurrent microbiotic colonization of the periodontal wound. These results suggest the great potential of these materials as cell and/or autologous growth factors carriers for endogenous regenerative technology (ERT) envisioning tissue engineering approaches targeting various tissues, namely the periodontal ligament.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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2
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17
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26 grant SFRH/BPD/100760/2014.
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31 32 ABBREVIATIONS

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34 PL, Platelet Lysate; hPDLFs, human Periodontal Ligament fibroblasts; GTR, guided tissue
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36 regeneration; HAase, hyaluronidase; ERT, endogenous regenerative technology; MRSA,
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38 Methicillin Resistant *Staphylococcus aureus*; VRSA, Vancomycin Resistant *Staphylococcus*
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40 *aureus*; HA, hyaluronic acid; me-HA, methacrylated hyaluronic acid; GFs, growth factors; α -
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42 MEM, minimum essential medium eagle alpha modification; PDMS, polydimethylsiloxane; MA,
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44 methacrylic anhydride; dH₂O, distilled water; FTIR, fourier transform infrared spectroscopy;
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46 ¹HNMR, Proton nuclear magnetic resonance; Dmet, degree of methacrylation; PL₀, hydrogel
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48 incorporating 0 v/v% of PL; PL₅₀, hydrogel incorporating 50 v/v% of PL; PL₁₀₀, hydrogel
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50 incorporating 100 v/v% of PL; HAPL, hyaluronic acid hydrogels incorporating PL; *mwet*,
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52 hydrogel wet mass; *mdry*, dry hydrogel mass; *m_i*, initial weight; *m_f*, final weight; ELISA,
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Enzyme-Linked Immunosorbent Assay; FGF-2, fibroblast growth factor-2; MH, Muller-Hinton (agar); DMA, dynamic mechanical analysis, pI, isoelectric point.

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35 36 **Table of Contents Graphic**

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