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Platelet Lysate-Loaded Photo-cross-linkable Hyaluronic Acid Hydrogels for Periodontal Endogenous Regenerative Technology

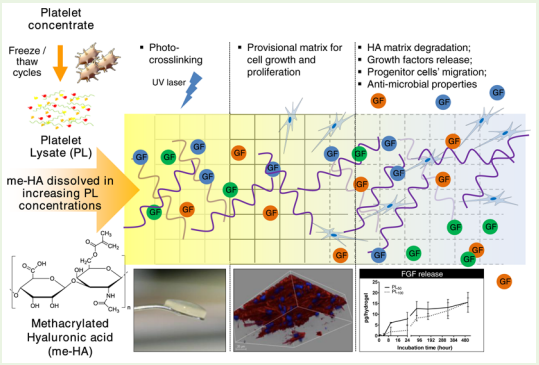
Pedro S. Babo,[†,‡] Ricardo L. Pires,[†,‡] Lívia Santos,[†,‡] Albina Franco,[†,‡] Fernando Rodrigues,[‡,§] Isabel Leonor,[†,‡] Rui L. Reis,[†,‡] and Manuela E. Gomes*[,†,‡]

[†]3B's Research Group—Biomaterials, Biodegradables and Biomimetics, University of Minho, Avepark—Zona Industrial da Gandra, 4806-017 Barco GMR, Portugal

[‡]ICVS/3B's—PT Government Associate Laboratory, University of Minho, Braga/Guimarães 4710-057, Portugal

[§]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga 4710-057, Portugal

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. Herein is proposed the development of an injectable hydrogel system able to release a growth factors and cells to the periodontal defect. This injectable system is based on a photo-cross-linkable hydrogel, prepared from methacrylated hyaluronic acid (me-HA) and incorporating platelet lysate (PL). The delivery of growth factors and cells in situ is expected to enhance regeneration of the periodontium. Various formulations of me-HA containing increasing PL concentrations were studied for achieving the formation of stable photo-cross-linkable hydrogels. The produced hydrogels were subsequently characterized to assess mechanical properties, degradation, protein/growth factor release profile, antimicrobial activity and response toward human Periodontal Ligament fibroblasts (hPDLFs). The results demonstrated that it was possible to obtain stable photo-cross-linkable hydrogels incorporating different amounts of PL that can be released in a sustained manner. Furthermore, the incorporation of PL improved ($p < 0.02$) the viscoelastic properties of the hydrogels and enhanced their resilience to the degradation by hyaluronidase (HAase). Additionally, the PL was shown to provide antimicrobial properties. Finally, hPDLFs, either seeded or encapsulated into the developed hydrogels, showed enhanced proliferation over time ($p < 0.05$), proportionally to the increasing amounts of PL present in the hydrogel formulations.



KEYWORDS: *photo-cross-linkable hydrogels, platelet lysate, hyaluronic acid, periodontal ligament, endogenous regenerative technology*

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 process.[1] 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 loss.[1,2]

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 cues.[3] These signals are expected to encourage homing of stem and progenitor cells, leading to the formation of new periodontal ligament and cementum.[3]

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 ingrowth.[4] In this research work we propose the development of photo-cross-linkable hyaluronic acid hydrogels enriched with platelet lysate as a stable system

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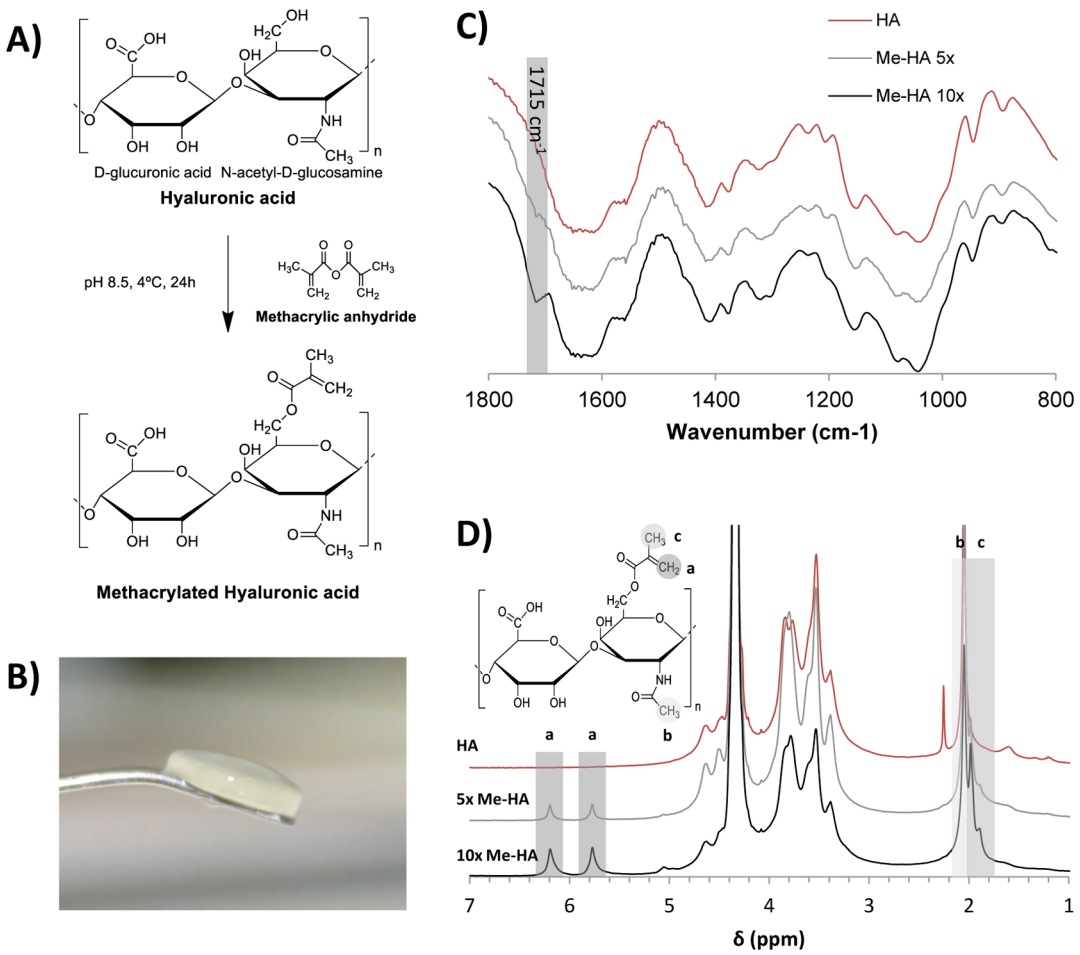

Figure 1. (A) Scheme of the methacrylation process of Hyaluronic acid using methacrylic anhydride. (B) Representative image depicting a typical me-HA/PL hydrogel obtained by photopolimerization. (C) FTIR Spectra of HA and me-HA produced with 5 and 10× molar excess of (5× and 10× me-HA). (D) ^{1}HNMR spectra of HA, 5× me-HA and 10× 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).

for the delivery of endogenous GFs, directed for periodontal ERT.

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 material.[3] 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-β1 and -β2, insulin-like growth factor, epidermal growth factor, epithelial cell growth factor, hepatocyte growth factor, and bone morphogenetic proteins[7−9] are known to be involved in essential stages of wound healing and regenerative processes such as chemotaxis, cell proliferation and differentiation.[10,11] Moreover, platelets release numerous cell adhesion molecules (fibrin, fibronectin, and vitronectin) which can provide a provisional matrix for the adhesion and migration of cells.[10] In addition, platelet concentrates (PCs) have also been reported to exhibit antimicrobial properties[12] 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 of standardizing the production process and the higher consistency in GF content between batches,[5] that is expected to yield more predictable clinical outcomes.

Because PL is obtained as a liquid solution, it was incorporated in a photo-cross-linkable 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 repair.[13] HA-based biomaterials have demonstrated positive results for several potential applications in the regeneration of hard or soft tissues.[14] Moreover, given HA anti-inflammatory, antiedematous, and antibacterial effects, it has been also proposed for the treatment of lesions caused by periodontal diseases.[15−17]

The aim is to characterize these PL-rich scaffolds with regard to mechanical properties, release of proteins, periodontal cell response, and antimicrobial action against dental plaque bacteria. This new ERT scaffold offers a new and promising periodontal treatment modality that should encourage tissue regeneration through the release of PL-derived GFs while providing concomitant antimicrobial action. Furthermore, functionalization of HA with methacrylic groups allows the production in situ of stable photopolymerizable hydrogels, enabling the application in periodontal defects in a clinical scenario.

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MATERIALS AND METHODS

Materials. HA obtained from *Streptococcus equi* (M_w = 1.5 to 1.8 MDa), methacrylic anhydride Irgacure 2959 (2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone), hyaluronidase type IV from bovine origin (HAase), phosphate buffered saline (PBS), phalloidin-tetramethylrhodamine B isothiocyanate 4,6-diamidino-2-phenylindole, dilactate (DAPI) and the dialysis tubing cellulose membrane were all purchased from Sigma (Sigma-Aldrich, USA). Sodium hydroxide (NaOH) and hydrochloride acid (HCl) were purchased from VWR Chemicals (BDH, Prolabo - international, USA). Alpha MEM (α-MEM) culture medium and fetal bovine serum (FBS) were purchased from Gibco (Life Technologies, UK). Deuterium oxide (^2H_2O) was purchased from LaborSpirit lda (PT) and the polydimethylsiloxane (PDMS) from Down Corning (USA). The Muller−Hinton agar plate was obtained from Oxoid (UK).

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 described.[18,19] 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. Afterward, the lysis product was centrifuged at 1400 rcf for 10 min and the supernatant stored at −20 °C until further use.[18,19]

Methacrylation of Hyaluronic Acid (HA). The method followed for the methacrylation of HA was based on a previously described protocol,[20] (depicted in Figure 1A), consisting in the addition between 5- to 10-fold molar excess (5× and 10×) 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 h 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 dialyzed using a membrane with a cutoff of 14 000 kDa for a week against mili-Q water, replaced 3 times a day, to remove the unreacted reagents and byproducts. Finally, the solution was filtered, frozen at −80 °C and the methacrylated HA (me-HA) recovered upon lyophilization.

Characterization of the me-HA. 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. ^{1}HNMR 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 ^2H_2O. The degree of methacrylation (D*met*) 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 Photo-cross-linkable me-HA Hydrogels Incorporating PL. The development of the photo-cross-linkable 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 (5 mm diameter) PDMS mold and exposed to a UV light (Omnicure series 2000 EXFO S2000-XLA, Omnicure, Canada) to trigger the photo-cross-linking, 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)[a]

me-HA (wt/v%)	Irgacure (wt/v%)	PL (v/v%)
1	0.10	0
		50
		100
	0.20	0
		50
		100
2	0.10	0
		50
		100
	0.20	0
		50
		100

[a]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 were prepared using both the batches of me-HA (5× and 10× molar excess).

Characterization of the HAPL Hydrogels. Only the 10× 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 Igacure 2959 presents some cytotoxicity,[21] 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 (PL_0, PL_{50}, and PL_{100} with 1% or 2% of me-HA and with 0.1% of Irgacure) were evaluated by dynamic mechanical analysis (DMA) (TRITEC8000B, Triton Technology, UK), equipped with the compressive mode. DMA spectra were obtained during a frequency scan ranging between 0.1 and 15 Hz for all time points. The experiments were performed under constant strain amplitude, corresponding to approximately 1% of the original height of the sample. Samples were tested while immersed in PBS and at 37 °C, to simulate the physiological conditions.

Swelling and Weight Loss. The results obtained from the DMA analysis revealed better mechanical properties for the 2% me-HA formulation and thus this was selected for the subsequent studies, namely degradation, protein release and cell response. Thus, formulations of hydrogels with increasing concentrations of PL (PL_0, PL_{50} and PL_{100}), were prepared into disc-shaped samples of 5 mm in diameter and 1 mm thickness, as above-described, and placed in 24 wells plate.

Periodontal ligament fibroblasts express hyaluronidase (HAase) and generate HAase activity that regulates extracellular hyaluronan metabolism.[22] Given the presence of this enzyme in the periodontium, the degradation promoted by a HAase was investigated. Similar assay was conducted in PBS. Each sample was incubated in 1.6 mL of PBS at 37 °C, pH 7.4. For the enzymatic degradation assays, the same formulations were incubated at 37 °C in 1.6 mL of a HAase solution of 100 U/mL in PBS.

The assays were carried out using 4 samples of each formulation immersed in each of the solutions. The samples were retrieved after 1, 3, 7, 14, and 21 days of incubation.

The wet weight of the samples was registered (PI-214 analytical balance, Denver Instrument Company, USA) at each predetermined time point. The dry weight of the samples was also registered after allowing samples to dry overnight at 37 °C. The percentage of weight loss was calculated according to eq 1

$$\text{weight loss} = \frac{(m_i - m_f)}{m_i} 100 \quad (1)$$

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where m_i is the initial weight and m_f the final weight.

The water uptake ratio was also calculated following eq 2 by dividing each sample wet mass (m_{wet}) by the final dry hydrogel mass (m_{dry}).

$$\text{water uptake ratio} = \frac{m_{wet}}{m_{dry}} 100 \quad (2)$$

Quantification of Protein Release. Protein release from PL_0, PL_{50} and PL_{100} was quantified after 30 min, 1, 4, and 8 h, and 1, 7, 14, and 21 days of incubation in PBS at 37 °C. For this purpose, at each time point, a volume of supernatant was collected and stored at −20 °C. The total protein content was quantified using a micro BCA protein assay (Thermo Fischer Scientific, USA), following the manufacturer's instructions. Additionally, the release of fibroblast growth factor-2 (FGF-2), present in the PL, was also quantified using an enzyme-linked immunosorbent assay kit (Human FGF-basic, ELISA Development Kit, by PeproTech, USA), according to manufacturer's instructions.

Evaluation of the Response of Human Periodontal Ligament Fibroblasts (hPDLFs). The response of hPDLFs to the photo-cross-linked me-HA/PL hydrogels was assessed upon either encapsulation or seeding of the cells onto the hydrogels surface and further cultured for up to 14 days.

The hPDLFs (ScienCell Research Laboratories) at passage 3 were seeded on disc-shaped (5 mm diameter) samples of the formulations PL_0, PL_{50}, and PL_{100} produced as previously described, at a cell density of 5×10^4 cm^{-2}. A 50 μL drop of a cellular suspension containing 1×10^4 cells was seeded on the surface of each sample, previously placed in a 24 wells plate, and allowed to adhere for 1 h. After this period, 450 μL of α-MEM basal medium (supplemented with 10% of FBS and 1% antibiotic-antimycotic) were added to each well. The 24 wells plates containing the cell-seeded hydrogels were further incubated at 37 °C, 5% CO_2 for 1, 4, 7, and 14 days, renewing the culture medium every 3 days. Cells cultured on polystyrene coverslips (Sarstedt) were employed as positive control.

For the encapsulation, hPDLFs cells were resuspended in 2% me-HA solutions containing 0, 50 and 100% PL to obtain a final cell density of 4×10^6 cells mL^{-1}. Then, 25 μL (1×10^5 cells) of the cellular suspension in each hydrogel solution formulation was injected into circular molds (5 mm diameter) and exposed to UV light, as previously described to obtain the hydrogel samples. The cell-laden hydrogels were subsequently transferred to individual wells of 24-well plates, each one containing 500 μL of basal medium. The 24-wells plates were incubated at 37 °C, 5% CO_2 for 1, 4, 7, and 14 days renewing the culture medium every 3 days.

The metabolic activity of the cells seeded/encapsulated in the hydrogels and further cultured was evaluated using the Alamar blue assay (AbDseroTec, USA), following the manufacturer's instructions. Briefly, at each time point, the culture medium was discarded, the samples were washed twice with PBS and then incubated in a 10% Alamar blue solution in basal medium (450 μL of basal medium, and 50 μL of Alamar blue) at 37 °C, 5% CO_2 for 150 min. The fluorescence of the supernatant solution was read in triplicates in a microplate reader (Synergy HT, Biotek, USA) at 560 nm of excitation and 590 nm of emission.

The cellular proliferation was also evaluated as a function of the dsDNA quantification using the PicoGreen dsDNA quantification kit, according to manufacturer's specifications (Life Technologies, USA).

Finally, the morphology and the migration of the cells either encapsulated or seeded on the surface of the hydrogels were investigated by confocal microscopy, upon staining with DAPI and phalloidin. For this purpose, samples retrieved after each of the preset culturing times were fixed with 10% formalin (in PBS) for 30 min at room temperature. Afterward, the samples were washed 2 times with PBS to remove the formalin and 300 μL of phalloidin solution (1:100 in PBS) were added per well and incubated 1 h at room temperature. Then phalloidin solution was discarded and the samples were washed 3 times with PBS. A DAPI solution (1:1000 in PBS) was prepared and 300 μL were added per well and incubated 5 min. The samples were washed 3 times and the prepared for visualization under a confocal microscopy (TCS SP8 from Leica Mycrosystems CMS GmbH) with Vectashield mounting medium.

Antimicrobial Assay. The antimicrobial activity of PL soluble factors released form from the HA hydrogels was evaluated using the radial diffusion assay, according to Kirby-Bauer method.[23] Five different bacteria species were used: the Gram-positive bacteria *Bacillus megaterium* (Internal collection), Methicillin Resistant *Staphylococcus aureus* (MRSA) (Internal collection), and Vancomycin Resistant *Staphylococcus aureus* (VRSA) (internal collection) and the Gram-negative species *Pseudomonas aeruginosa* T6BT12, Escherichia *coli* DH5α) and the fungus *Candida albicans* (Internal collection). With the exception of *P. aeruginosa,* which was isolated from environmental samples, all the other microorganisms were isolated from clinical samples. Prior to the antimicrobial activity testing, these microorganisms were cultured aerobically in Luria−Bertani broth at 37 °C overnight with agitation (150 rpm). Afterward, they were centrifuged at 8000 rpm for 5 min, and washed three times with PBS. Microbial cultures were adjusted to a concentration corresponding to ca. 1×10^7 CFU mL^{-1}, and pipetted with 0.4% agar into a Petri dish containing 5 mL of Muller-Hinton (MH) Agar plate.

The PL_0, PL_{50} and PL_{100} hydrogels and the negative control (PBS) were placed on MH−agar plates and cultured with each of microbial strain at 37 °C for 16 h, upon which the inhibition halo measure and the general macroscopic response was recorded. Experiments were performed in triplicate.

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

RESULTS

Development of the Photo-cross-linkable me-HA Hydrogels. *HA methacrylation.* In this study, unmodified hyaluronan was methacrylated Reacting a 1% HA aqueous solution at pH 8, with 5× and 10× 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 ^{1}HNMR 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 nonmodified HA spectrum.

The degree of methacrylation 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 the methyl protons of N-acetyl-D-glucosamine (∼1.98 ppm). A D*met* of 14% was obtained for the me-HA batch produced with 5× excess of MA (5× me-HA), while the batch produced with 10× excess MA (10× me-HA) presented a D*met* of 24%.

Mechanical Properties of the Developed Hydrogels. Dynamic mechanical analysis (DMA) experiments were performed in a hydrated environment at 37 °C, in an array of biologically relevant frequencies, in order to assess the viscoelastic properties of the samples in a physiological-like environment. Both storage (elastic) modulus, E', and the loss factor, tan δ, were obtained at different frequencies. E′ is a measure of the materials stiffness. The loss factor is the ratio of the amount of energy dissipated (viscous component) relative to energy stored (elastic component); tan $\delta = E'/E''$.

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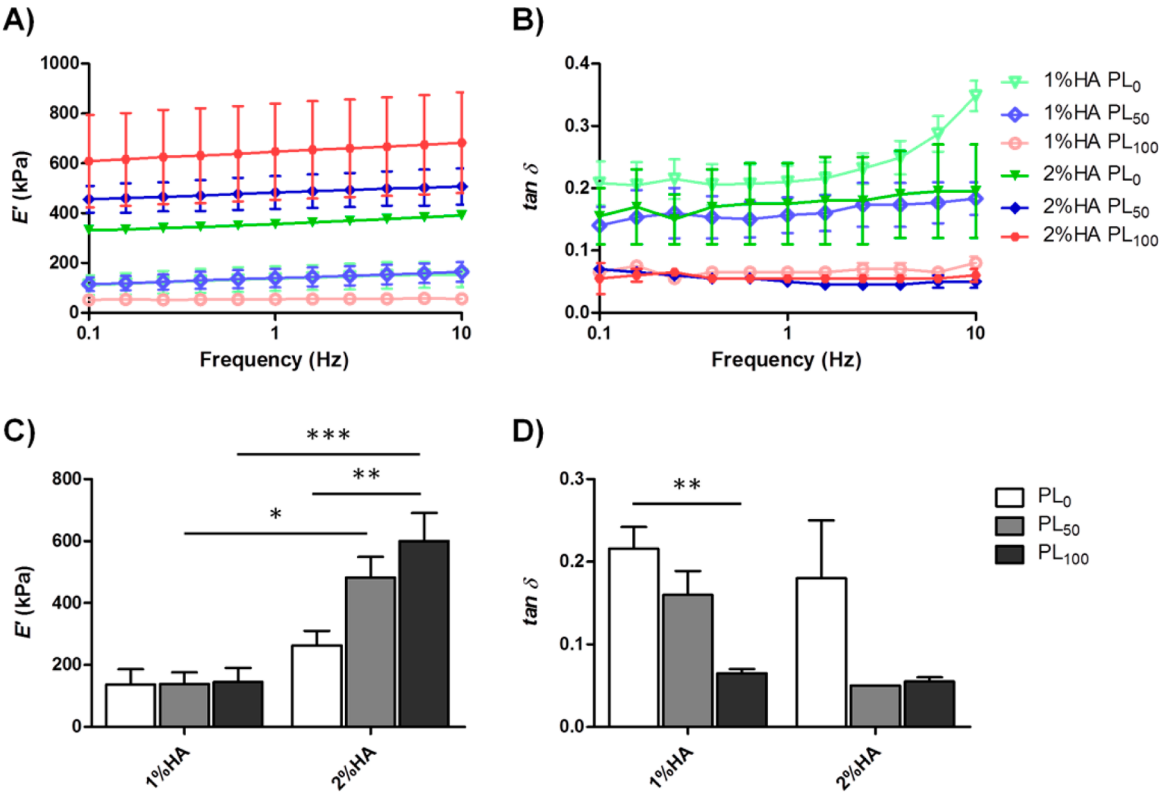

Figure 2. Variation in (A) elastic modulus (E') and (B) loss factor (tan δ) with frequency of 1% and 2% HA hydrogels incorporating 0, 50, and 100% v/v PL (PL_0, PL_{50}, and PL_{100}) measured by dynamic mechanical analysis. Differences observed on (C) elastic modulus (E') and (D) loss factor (tan δ) at 1 Hz. * $p < 0.05$, ** $p < 0.02$; *** $p < 0.001$.

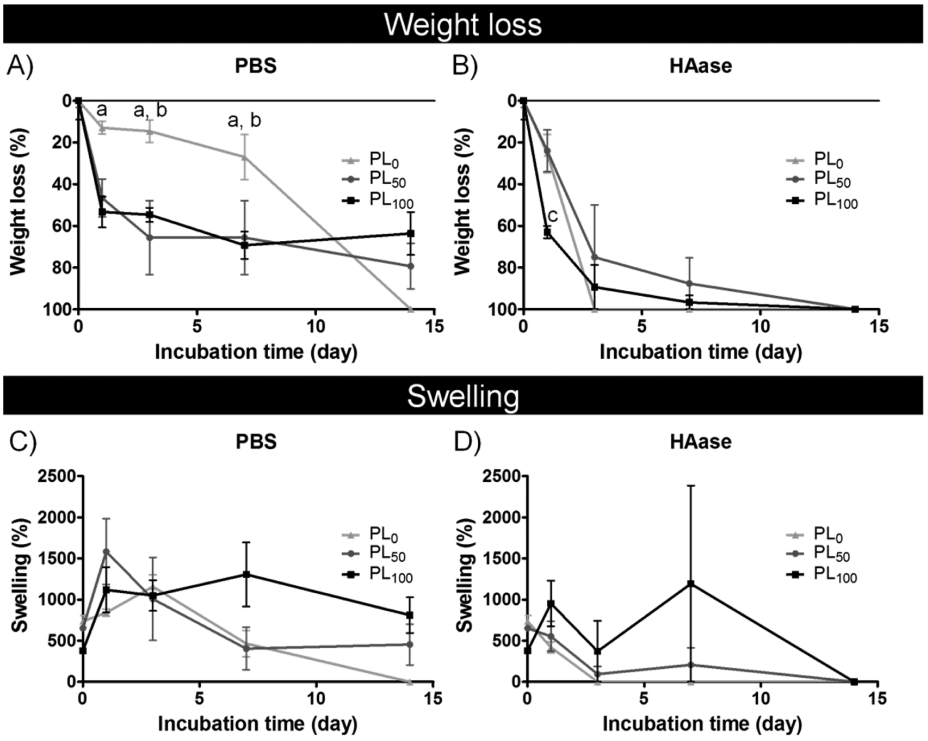

Figure 3. Weight loss (A, B) and (C, D) swelling ratio profile of PL_0, PL_{50}, and PL_{100} hydrogels in (A, C) PBS and (B, D) HAase solution (100 U/mL). (a) Statistically different ($p < 0.05$) from PL_{100}; (b) statistically different ($p < 0.05$) from PL_{50}; (c) statistically different ($p < 0.05$) from PL_0 and PL_{50}.

The obtained results (Figure 2) showed the effect of different concentrations of me-HA and/or PL on the stiffness of the developed hydrogels. When the concentration of me-HA was increased from 1% to 2% the elastic storage modulus of the

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hydrogels also increased above three to four times, from approximately 100 kPa to 428−600 kPa, in formulations incorporating PL (PL_{50} and PL_{100}). The concentration of PL in the hydrogels also showed to influence the elastic modulus that was found to increase proportionally with the amount of PL. The formulation that exhibited the highest elastic modulus corresponds to the formulation containing 2% of me-HA dissolved in 100% PL.

Degradation Behavior. The weight loss and swelling ratio profiles of the PL_0, PL_{50} and PL_{100} 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.

Weight Loss. Overall, the results obtained showed that the incorporation of PL in me-HA hydrogels influences its stability. Although the PL_0 hydrogels showed lower weight loss until the seventh day of immersion in PBS, they were completely degraded after 14 days (Figure 3A). On the other hand, despite the weight loss profile of the formulations incorporating PL is characterized by an initial loss of around 70% of the dry weight in the first 3 days, the PL_{50} and PL_{100} hydrogels tend to be more stable along immersion time in PBS.

The weight loss results obtained upon immersion in HAase, revealed that PL_{100} formulation displays higher degradability, upon the first day. Nevertheless, it was found that samples containing PL were only completely degraded after 14 days, while all the hydrogels of the PL_0 formulation were completely degraded after only 3 day of immersion in the enzymatic solution.

Swelling Ratio. In the beginning of the assay, the swelling of freshly produced PL_{100} hydrogels was significantly lower than the formulations with lower PL concentration. When immersed in the PBS solution the PL_0 and PL_{50} hydrogels, did not show significant statistical differences among them for all the time points studied. Accordingly, both hydrogels formulations presented a similar profile characterized by a peak around day 1 (1500% for PL_{50}) and day 3 (1000% for PL_0), followed by a decrease of swelling until the end of the assay, because of the total degradation of the material. On the other hand, PL_{100} hydrogels had a later peak at day 7, reaching near 1400% of swelling.

Regarding the swelling in HAase solution, the values were statistically similar for PL_0, PL_{50} and PL_{100} hydrogels. Nevertheless, while the formulations PL_0 and PL_{50} depicted a similar behavior, presenting a constant decrease in the swelling values from the beginning of the assay, the PL_{100} formulation reached an average swelling of 1400% at day 7, before starting to decrease.

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

Both PL_{50} and PL_{100} hydrogels displayed a similar release profile that is characterized by an initial "burst" of protein released during the first hour, that represents around 15% for PL_{100} hydrogels and 25% for PL_{50} hydrogels of the total protein contained, followed by a sustained release up to 14 days. Although no statistically significant differences were observed between the formulations during the first day of release, there was a substantial difference in the amount of protein released by the PL_{100} formulation, which is proportional with the amount of protein incorporated in the formulations.

To evaluate the release of PL-specific GFs from the developed HA hydrogels, and the interaction of the GFs with the HA mesh, hydrogels were incubated either in PBS or in 100 U/mL HAase solution and the release products were quantified by ELISA.

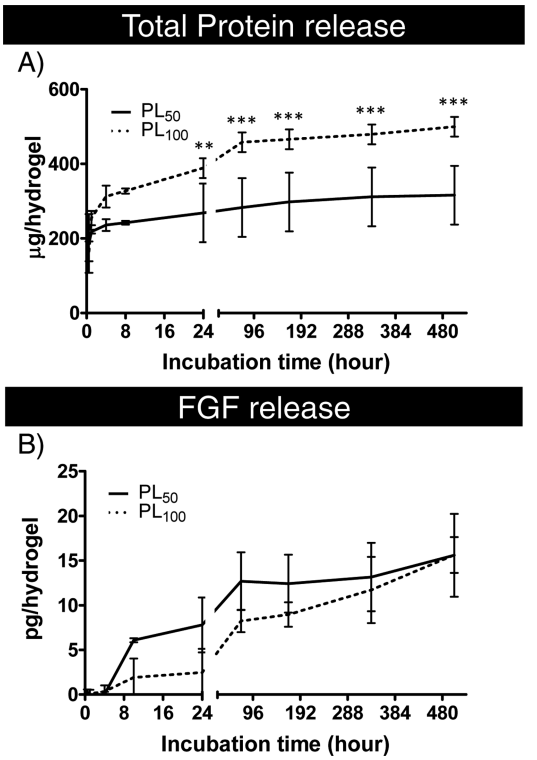

Figure 4. Total protein released from the hydrogels containing PL, (A) assessed using the Pierce BCA protein assay kit incubated in PBS. Fibroblasts growth factor (FGF) release, (B) assessed using the PeproTech ELISA development kit incubated in HAase solution (100 U/mL). ** $p < 0.02$; *** $p < 0.001$.

The results for the release of FGF-2, depicted in Figure 4B, showed that the PL_{50} and PL_{100} had a different profile for FGF-2 release. The FGF-2 released by PL_{50} was characterized by an initial burst of release up to day 3, as observed. After day 3, the release kinetics reached an apparent plateau, and a slow sustained delivery remained up to day 21. On the other hand, PL_{100} hydrogels showed a sustained release, progressing in a linear way, during all the duration of the assay, without signs of deceleration. Nevertheless, despite the PL_{100} hydrogels have higher amount of total protein incorporated, they depicted a FGF-2 release similar to the PL_{50} hydrogel.

Cell Response to the Developed Hydrogels. The response of hPDLFs, either surface seeded or encapsulated onto the PL_0, PL_{50} and PL_{100} 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.

The results presented in Figure 5A show that there were no significant differences between the PL_0 and PL_{50} hydrogels with respect to proliferation and metabolic activity of encapsulated cells. Remarkably, PL_{100} hydrogels exhibited higher cell growth and metabolic activity than PL_0 and PL_{50} hydrogels. Regarding the morphology of the encapsulated cells, Figure 5B shows that hPDLFs dispersed and stretched inside of the hydrogels, following the alignments of the fibrous structures observed macroscopically in the hydrogels.

The Figure 5C shows the behavior of the hPDLFs cells when seeded at the surface of the PL_0, PL_{50} and PL_{100} hydrogels. No

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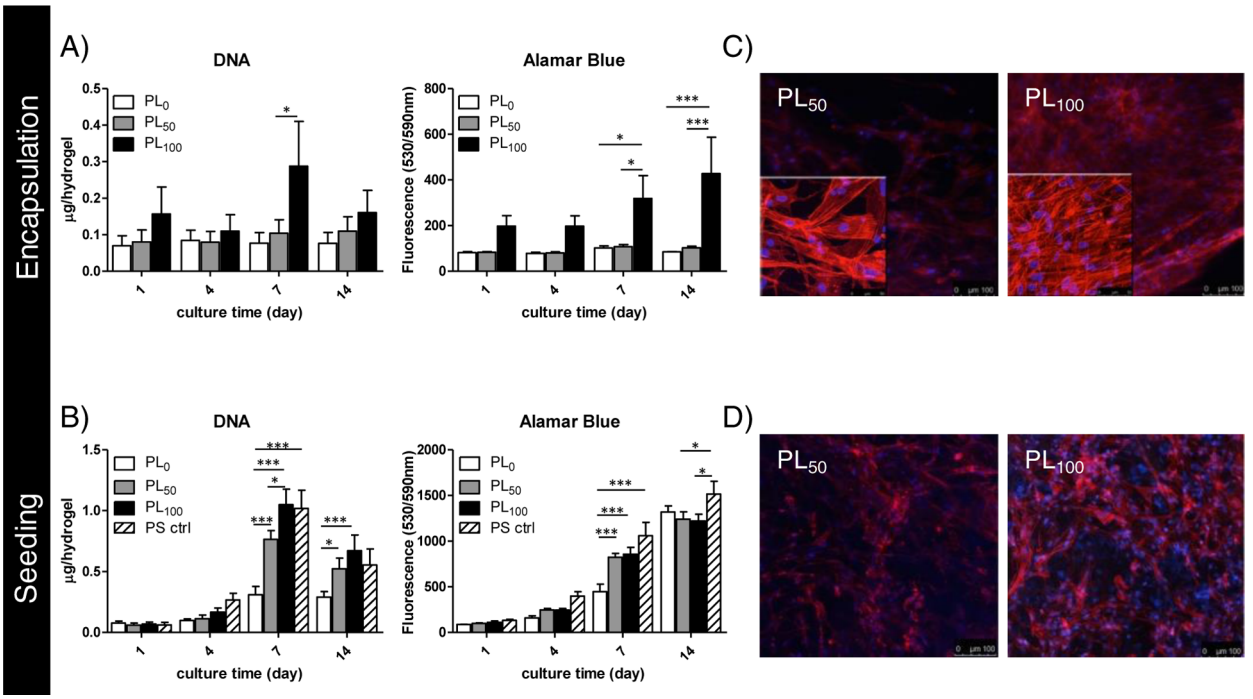

Figure 5. Response of hPDLFs seeded/encapsulated on the hydrogels with the formulations PL_0, PL_{50}, PL_{100}. (A) DNA quantification and metabolic activity of encapsulated cells. (B) DNA quantification and metabolic activity of seeded cell. (C) Representative pictures of hPDLFs encapsulated in PL_{50} and PL_{100} hydrogels, stained with DAPI (blue) and phalloidin (red). for 21 days. The small micrographs on the bottom left depict the spindlelike shape morphology of the hPDLFs encapsulated into the hydrogels. (D) hPDLFs seeded on PL_{50} and PL_{100} hydrogels and cultured for 21 days, stained with DAPI (blue) and Phalloidin (red).

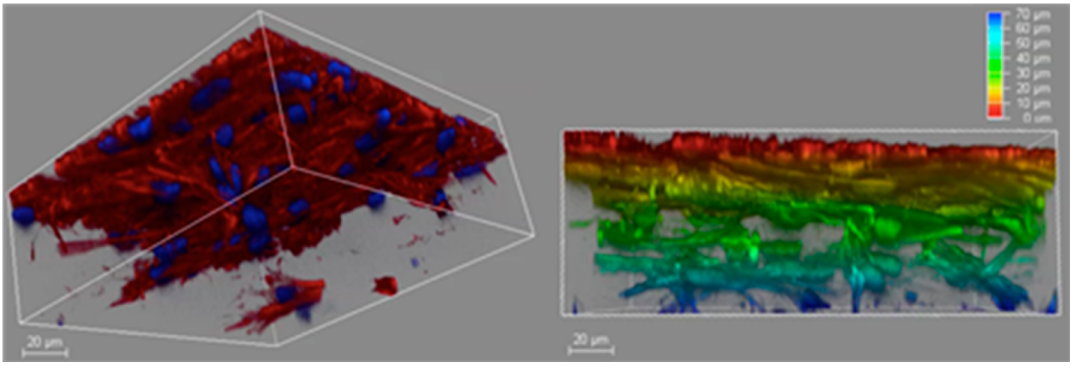

Figure 6. Three-dimensional reconstruction obtained by confocal microscopy of hPDLFs distribution on PL_{100} hydrogels at day 21.

significant differences were seen in terms of seeding efficiency on the hydrogels and on the PS positive control.

The analysis of hPDLFs distribution throughout the PL-enriched hydrogels, obtained by confocal microscopy from PL_{100} hydrogels 21 days after being seeded on the surface, is represented in Figure 6. This picture shows that hPDLFs seeded in the surface of the hydrogels migrated up to 70 μm deep into to the hydrogel after 21 days in culture.

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, *Bacillus megaterium*, vancomycin-resistant *Staphylococcus aureus* (VRSA), and 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 PL_{100} hydrogel (Figure 7F). Moreover, it is dependent on the PL content, because no inhibition hallo was observed for the formulations with lower amounts of PL incorporated (PL_0 and PL_{50}). 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.

DISCUSSION

The present work describes the development of novel photo-cross-linkable hydrogels incorporating allogenic platelet lysate, a platelet rich hemoderivative (PRHd), aimed at endogenous

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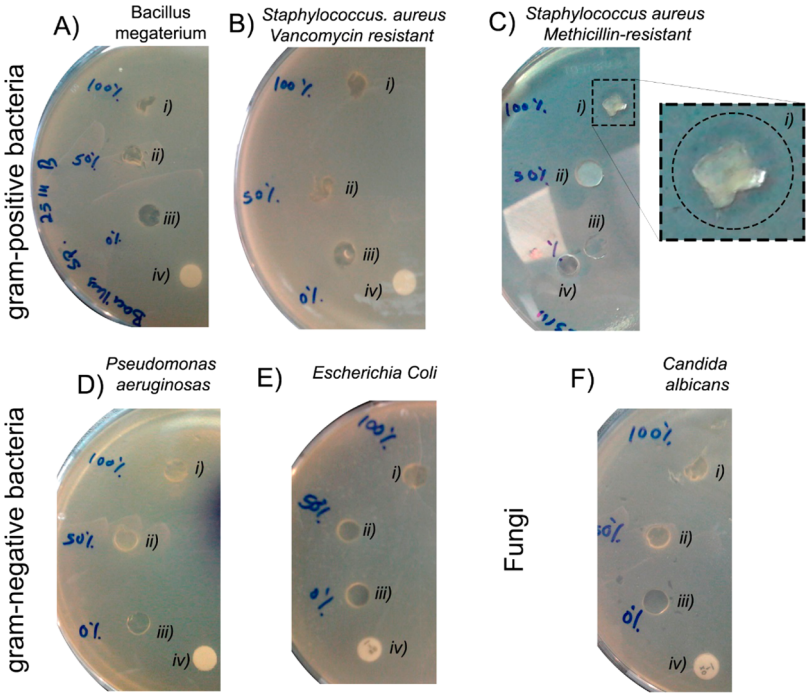

Figure 7. Antimicrobial assay for (i) PL_{100}, (ii) PL_{50}, and (iii) PL_0 formulations where control is (iv) PBS using (A) *Pseudomonas aeruginosa*, (B) *Candida albicans*, (C) *Escherichia coli* (E. coli), (D) *Bacillus megaterium*, (E) vancomycin-resistant *Staphylococcus aureus* (VRSA), and (F) methicillin-resistant *Staphylococcus aureus* (MRSA).

regenerative technology (ERT) being used for the regeneration of periodontal ligament. PL can be used in clinical applications as an autologous therapy. However, several authors[5,24] have reported high donor-to-donor variability in PRHds batches, which could correlate with the high variability associated with the clinical outcomes of PRHds treatments.[25] On the other hand, Crespo-Diaz et al.[5] reported lower variability in PL batches produced from outdated platelet concentrates obtained by plasma apheresis from different donors; therefore more predictable therapeutic outcomes could be anticipated. Furthermore, these PL batches were shown to be safe of standard pathogens and infectious diseases. In the present work, were used outdated (>5 days old) platelet concentrates obtained by plasma apheresis and biologically qualified according to Portuguese legislation (Decreto-Lei No. 100/2011) for blood products collection, transport and therapeutic administration. Therefore, these PL batches are expected to be as safe as any other blood component aimed for therapeutic administration and used in allogenic PL-based strategy as proposed. The combination of me-HA with PL, as herein proposed, produced a photo-cross-linkable system with several advantages for tissue engineering applications. Being injectable, these biomaterials can be implanted using minimally invasive techniques without requiring surgical interventions. Moreover, the system can fit perfectly to irregular-shaped defects, deeply interacting with the preserved tissue margins, before being photo-cross-linked to produce a stable matrix.

With regard to viscoelastic properties, DMA analysis revealed that these hydrogels exhibit elastic modulus ranging from 264 ± 81 kPa for the PL_0 formulation to 600 ± 186 kPa to the PL_{100} formulation (at 1 Hz), comparable to other HA hydrogels incorporating fibrin described for artificial cartilage implantation (445 kPa),[26] which support the use of our photo-cross-linkable hydrogels for soft tissue reconstruction. Moreover, periodontal tissue is continuously subjected to very dynamic forces, acting the periodontal ligament as a damper.[27,28] Therefore, the viscoelastic properties displayed by the hydrogels herein developed are of paramount importance for periodontal therapy approaches.

Regarding the degradation of HA hydrogels, it was faster in the presence of the HAase, the specific enzymes that degrade the HA in vivo,[29] than in saline solution, as previously reported.[13,30] Remarkably, the PL-enriched hydrogels remained stable for longer periods. The time to total degradation of PL_{100} was even longer when compared with other HA hydrogels exposed to similar conditions.[13] It should be noted that in this study we used a supra-physiologic concentration of HAase (100 U/mL), which in human plasma ranges from 0.0028 ± 0.0004 U/L to 3.8 ± 0.7 U/L depending on patient health condition.[31] Therefore, these findings suggest that PL-enriched photo-cross-linkable HA hydrogels, may maintain the necessary space stability in vivo for new tissue ingrowth.[4] Such reinforcement is attributed to the presence of fibrinogen in the PL,[1,18] as this protein is capable of cross-linking, forming a fibrin mesh which is not susceptible to degradation by the HAase. The fibrin/fibrinogen interact specifically with HA for the formation of ECM either during wound healing or in normal tissues.[32] This result is in line with previous studies in which HA hydrogels incorporating fibrin were proposed for cartilage repair[26] given their improved biomechanical properties and the ability to provide an adequate environment for cell encapsulation.

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

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physical degradation of the hydrogel. Since the PL proteins have different isoeletric points (pI), the electrostatic interactions and probability of remaining adsorbed to the HA mesh, which are negatively charged at physiologic pH, will vary. In this way, the albumin, which is the main soluble protein in PL,[33] with an acidic pI (at pH 4.7), is expected to be easily washed out from the HA mesh. On the other hand, most of the GFs present in PL with therapeutic interest have 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; FGF-2 at pH 9.6). So, they are expected to bind electrostatically to the HA matrix and to the insoluble PL proteins to be further released by ion exchange or by the degradation of the HA mesh promoted by HAses released for the ECM remodeling promoted during the wound healing process. In fact, the release of PL-specific GFs from the photo-cross-linkable hydrogels, namely FGF-2, was detected only after degradation of the hydrogels in HAase (Figure 4B), whereas no detectable traces of GFs were detected after incubation of the hydrogels in PBS. Studies with FGF-2 have shown that this GF upregulate the migration and proliferation of PDL cells.[34] In fact, to fully regenerate functional of periodontal tissues, several GFs and cytokines should interplay in a temporal as spatial controlled manner.[10] Therefore, the controlled release of growth factors is a real asset to our hydrogels.

In line with what has been reported in literature, our findings show that the encapsulation of hPDLFs in nonsupplemented HA hydrogels (PL_0) affects cell proliferation and metabolic activity. The biological performance of cells encapsulated in me-HA hydrogels is affected by the concentration of the macromer,[13,35] as well as by the concentration of photo-initiator.[35] Furthermore, the exposure to UV radiation was also reported to have adverse effects on viability and cell cycle progression, whereas the differentiation potential remains unchanged.[35] Remarkably, the adverse effects of photo-encapsulation were overcome by the incorporation of PL into the hydrogels. The viability and metabolic activity of the encapsulated hPDLFs increased proportionally with the incorporation of PL. Previous works have reported the positive effect of PL in the proliferation and maintenance of stemness phenotype of human periodontal ligament stem cells.[36] In the same line, we observed, in previous works that (hPDLFs) adhere and proliferate in genipin-cross-linked PL membranes.[37] It is known that platelets release several growth factors, namely PDGF and FGF-2, which have a mitogenic effect over human periodontal ligament cells.[38,39] Moreover, PDGF and FGF-2 have been reported to have chemotactic properties over hPDLFs,[34,40] while the adhesion sites provided by the clot-forming proteins present in PL should facilitated the inward cell migration observed (Figure 6). Therefore, a strategy that can recruit progenitor cells from the preserved periodontal tissue and promote their proliferation and maintenance of stemness to colonize the periodontal defect with cells with great potential to regenerate periodontal tissue would be a valuable asset for periodontal ERT. Hereupon, the first intentional repair promoted by cells originated from periodontal tissues could partially restore the primitive anatomy and function of the periodontium.[4]

Finally, we have studied the antimicrobial properties of the developed hydrogels, a very important aspect considering the target application. It is known that the main cause of periodontal disease, as well as the main factor of rejection for some of the GTR techniques, is bacterial infections.[41,42] The HA was previously described to have bacteriostatic properties against oral and nonoral bacteria.[43] Carlson et al.[43] suggested that the bacteriostatic effect of HA may be due to the saturation of the bacterial hyaluronate lyase by the excess HA, which prevents the bacteria from maintaining elevated levels of tissue permeability and penetrating the physical defenses of the host. This would enhance the ability of the host's immune system to eradicate pathogens. HA molecules in the hydrogels also form a random network of chains that may act as a sieve preventing the spread of the bacteria. Platelet concentrate (PC) was previously reported to have antimicrobial properties[12] signifi-cantly reducing the growth of methicillin-sensitive or -resistant *Staphylococcus aureus*, Group A *Streptococcus*, and *Neisseria gonorrhea*, among others. As PL is a product of PC activation by freeze/thaw cycles, the same would be expected for this hemoderivative. The obtained results in this study meet with the antimicrobial properties already described in the literature for platelet concentrates.[12] Here, the methicillin resistant *Staphylococcus aureus* (MRSA) was more susceptible to the hydrogels containing PL_{100} than the other microbial strains tested. Yeaman and Bayer proposed that the bactericidal activity against MRSA involved β-lysin, which is responsible for blood clotting found after platelets activation.[44,45] β-lysin, which is one of the most abundant compound found in PL after activation[46] has been described to act against bacteria cell-wall, rapidly killing and stopping bacteria reproduction,[44,45] which could explain the results from this study. In addition, other PL-derived molecules with antibacterial properties against Gram+ bacteria could be involved in this response, such as neutrophil activating protein-2 demonstrated capacity to kill Gram-positive and Gram-negative bacteria.[47,48] Although no effect was observed against Gram bacteria and fungus, other factors can be found in PL with bactericidal and fungicidal activity. For instance, Platelet factor-4 can bind to Gram-negative bacteria because it has an affinity for the lipopolysaccharide from these bacteria, facilitating their clearance.[49,50] Nevertheless, further investigation is needed in order to fully understand PL antimicrobial properties against microbial pathogens, especially whether the molecules that demonstrate antimicrobial potential interact alone or together when supplemented as PL and not from induced platelets.

CONCLUSIONS

Overall, our findings demonstrate that is possible to obtain versatile photo-cross-linkable 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.

AUTHOR INFORMATION

Corresponding Author

*E-mail: megomes@dep.uminho.pt.

ORCID

Pedro S. Babo: 0000-0003-4347-599X

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PL, platelet lysate
hPDLFs, human periodontal ligament fibroblasts
GTR, guided tissue regeneration
HAase, hyaluronidase
ERT, endogenous regenerative technology
MRSA, methicillin -resistant *Staphylococcus aureus*
VRSA, vancomycin-resistant *Staphylococcus aureus*
HA, hyaluronic acid
me-HA, methacrylated hyaluronic acid
GFs, growth factors
α-MEM, minimum essential medium Eagle alpha modification
PDMS, polydimethylsiloxane
MA, methacrylic anhydride
dH_2O, distilled water
FTIR, Fourier transform infrared spectroscopy
^{1}HNMR, proton nuclear magnetic resonance
Dmet, degree of methacrylation
PL_0, hydrogel incorporating 0 v/v% PL
PL_{50}, hydrogel incorporating 50 v/v% PL
PL_{100}, hydrogel incorporating 100 v/v% PL
HAPL, hyaluronic acid hydrogels incorporating PL
m_{wet}, hydrogel wet mass
m_{dry}, dry hydrogel mass
m_i, initial weight
m_f, final weight
ELISA, 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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