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Original Article

Platelet lysate membranes as new autologous templates for tissue engineering applications

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Platelet lysate (PL) has been increasingly explored in tissue engineering and regenerative medicine fields as a natural source of growth factors and/or as a support for cell growth and differentiation. Despite its tremendous potential, when used as tridimensional scaffolding systems, PL presents an inherent fragility and fast dissolution which hinders its application. The main objective of this work was to develop novel and robust PL-based membranes, crosslinked with genipin, for tissue engineering purposes. The membranes prepared present unique physical and mechanical properties, which indicate that these structures might be used in a vast range of applications. These PL-based structures present a high water uptake and stability over time. Overall, the developed membranes demonstrated a positive *in vitro* biological response as they were able to support and promote the adhesion and proliferation of human adipose derived stem cells. In addition to their role as supportive matrices for cell culture, the proposed membranes may simultaneously act as a growth factor controlled delivery systems, and thus hold great potential in several approaches for the regeneration of tissues.

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Introduction

Growth factors (GFs) and extracellular matrix (ECM) molecular cues are the signals that cells use to communicate and modulate their microenvironment. GFs are critical for the cell activities occurring during wound healing, being essential to direct tissue regeneration. Current tissue engineering challenges have focused on the development of systems that not only provide the adequate architecture and mechanical properties for tissue regeneration, but also exhibit biochemical cues to actively guide and control cell attachment, migration, proliferation and differentiation^{1, 2}. In the last decades, several efforts have been made to develop biomaterials that somehow mimic the “wound healing cascade” which orchestrate the regenerative process^{3, 4}.

The advent of new drug delivery systems, for the sequestration of multiple GFs and their adequate dose and timely release, brought new hope to the development of improved therapies for the repair and regeneration of damage tissues as well as for the treatment of some medical disorders². Nevertheless, several concerns about the safety, cost, and effectiveness of these systems have overshadowed their attractiveness². Moreover, the release of single GF is not expected to induce the desired regenerative process as native tissue healing requires the interplay and synergistic action of distinct bioactive factors⁵.

Platelet-based products (Platelet rich plasma- PRP; Platelet Lysate— PL) hold an enormous potential for regenerative therapy as an alternative source of GFs⁶ as they can be obtained using simple and cost-effective procedures, and used in autologous approaches⁷. The autologous application of endogenous GFs largely reduces the risks of disease transmission and simultaneously allows the induction of the “wound healing cascade” in a physiological manner. The release of GFs stored in platelets can be triggered through different activation mechanisms, namely contact with thrombin⁸, thromboplastin⁹, calcium salts⁸⁻¹¹ and collagen¹² or just by platelets lysis, caused by physical disruption through thermal and osmotic shock to produce PL^{11, 13, 14}. Platelets play a fundamental role in hemostasis and constitute a natural source of GFs including platelet-derived growth factor (PDGF), Transforming Growth Factors (TGF)- β 1 and - β 2, Insulin-like Growth Factor (IGF), Epidermal Growth Factor (EGF), Epithelial Cell Growth Factor (ECGF), Hepatocyte Growth Factor (HGF), Bone Morphogenetic Proteins (BMPs)-2, -4, and -6, Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth

Factor (FGF)^{11, 15-17}. The GFs derived from platelets are involved in essential stages of wound healing and regenerative processes such as chemotaxis¹⁸, cell proliferation¹⁹ and differentiation, including angiogenesis^{11, 17}, chondrogenesis²⁰ and osteogenesis^{13, 21, 22}. In addition to GFs, platelets release numerous other molecules²³, including cell adhesion molecules (fibrin, fibronectin and vitronectin) that are important for the formation of ECM and for the adhesion and migration of cells²⁴.

The use of platelet derived GFs for the regeneration of soft and hard tissues has been attempted, namely for tendon^{20, 25, 26}, bone¹⁰ and cartilage²⁰. Moreover, PL has been suggested as a safer alternative to animal origin media supplements for cell propagation, aiming for tissue engineering applications¹⁹.

PL can be applied externally, added to implanted material or injected directly in a lesion and used as either a GF-releasing agent alone or as a scaffold for simultaneous cell delivery. In fact, the conjugation of Platelet-based concentrates with calcium, which induces the release of GFs by platelets, allowed the sustained release of GFs and new bone formation in heterotopic and orthotopic sites¹⁰. Nevertheless, the hydrogels formed upon the activation of Platelet-based concentrates typically present poor mechanical properties and are consequently difficult to handle. Moreover, they tend to dissolve too quickly, thus failing in acting as temporary support system for tissue regeneration.

In order to overcome these limitations, we propose an alternative PL-based membrane with significantly enhanced stability and mechanical properties, developed by cross-linking PL proteins with genipin aiming for a sustained release of multiple GFs. Genipin is a natural crosslinker for proteins and presents a low acute toxicity, with LD50i.v. 382 mg/kg in mice, therefore, being much less toxic than other commonly used synthetic crosslinking reagents²⁷. For this reason, genipin has been used in biomaterials research as a crosslinker to produce membranes²⁸ and micro-²⁹ and nanospheres³⁰ for protein and drug delivery.

The sustained release of proteins from the PL-based membranes crosslinked with genipin is expected to stimulate the migration and proliferation of seeded cells during culture and/or endogenous cells, upon implantation. Moreover, we can take advantage of the fact that PLs proteins present in the membrane will have a multifunctional role as a connectivity agent for the matrix as well as providing GFs in physiologic dosages for tissue regeneration.



Table 1 Summary of conditions used for the development of PL membranes crosslinked with genipin

Formulation designation	Volume of PL	Concentration of genipin (wt%)
PL		0
0.10% gPL	1mL (rectangular molds) / 50 μ L (circular molds)	0.10
0.18% gPL		0.18
0.25% gPL		0.25

Materials and Methods

1)Preparation of PL

PL was collected from different platelet concentrates provided by Instituto Português do Sangue (IPS, Porto, Portugal), under a previously established cooperation protocol. All the products were biologically qualified according to Portuguese legislation. The platelet count was performed at the IPS and the sample volume was adjusted to one million platelets per ml. Platelet concentrate was processed as previously described³⁰, to obtain PL. Briefly, initial samples with a platelet count of 10⁶/mL from three different donors were mixed and subjected to three repeated freezing and melting cycles (frozen with liquid nitrogen at -196 °C and thawed in a 37°C water bath), lysing the platelets and releasing their protein content. The cellular debris was removed by centrifugation at 1400g for 10 min and the supernatant was stored at -20°C until further use.

2)Development of PL membranes

PL membranes were produced by crosslinking PL proteins with genipin (Wako Pure Chemical Industries, Japan), followed by solvent casting. Briefly, PL was thawed at room temperature and filtered using a 70 μ m sieve to remove platelet clumps, in aseptic conditions. Then, PL suspensions mixed with genipin at increasing concentrations (0.10, 0.18, and 0.25% w/v) were injected into either circular (5mm diameter) or rectangular (30 x 8mm) Polydimethylsiloxane (PDMS) molds to obtain the membranes and were allowed to dry overnight. Table 1 summarizes the different conditions used to develop the membranes, as well the abbreviations used throughout the manuscript.

3)Characterization of the developed membranes

(1)Fourier Transform Infra Red (FTIR) spectroscopy

Fourier transform infrared spectroscopy (IR-Prestige-21, Shimadzu) was used to analyze PL and PL-crosslinked

membranes. For this purpose, the samples were powdered, 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.

(2)Dynamic Mechanical Analysis (DMA)

The viscoelastic measurements were performed using a TRITEC8000B DMA from Triton Technology (UK), equipped with the tensile mode. The experiments were performed in wet state, in PBS, and at 37°C. The distance between the clamps was 5 mm and the membranes samples were cut with a width of about 4 mm. Samples were always analyzed immersed in a liquid bath placed in a Teflon[®] reservoir. Membranes were previously immersed in a PBS solution until equilibrium was reached. The geometry of the samples was then measured and the samples were clamped in the DMA apparatus and immersed in the PBS bath. After equilibration at 37°C, the DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz. The experiments were performed under constant strain amplitude (30 μ m). A static pre-load of 1 N was applied during the tests to keep the sample tight.

(3)Determination of weight loss and water uptake properties of the PL membranes

PL-genipin membranes were prepared into disc-shaped samples of 5mm in diameter and 20 μ m thickness, as described above, and its initial weight measured (PI-214 analytical balance, Denver Instrument Company, USA). The samples were incubated in 3 mL of PBS at 37°C, pH 7.4, under a 60 rpm constant agitation for 1, 3, 7, 14, 21 and 30 days. For each time point, three samples of each formulation were collected and rinsed with distilled water (dH₂O) and the wet weight of the samples was measured. After dried at 37°C overnight, the dry weight of the samples was also registered. The percentage of weight loss was calculated according to equation (1):

$$\frac{(m_i - m_f)}{m_i} \times 100 \quad (\text{equation 1})$$

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

The water uptake ratio was also calculated following equation (2) by the ratio between each sample wet mass after immersion in dH₂O (pH=7.4, T=37°C, under stirring) and the final dry membrane mass.

$$\frac{m_{wet}}{m_{dry}} \times 100 \quad (\text{equation 2})$$



(4) Quantification of protein release by micro-BCA

The protein released from the 0.10% gPL, 0.18% gPL, and 0.25% gPL formulations was assessed up to 14 days. For this purpose, the developed membranes were immersed in PBS at 37°C, pH 7.4, under a 60 rpm constant agitation. At the determined time points, aliquots of 1 mL were collected and stored at -20°C and an equal volume of fresh PBS was added to the suspension to replace the sample. The amount of released protein in the supernatant was quantified using a total protein assay (micro-BCA, Thermo Fisher Scientific, USA), following the manufacturer's instructions. The absorbance was read at 562 nm on a multiwell microplate reader (Synergy HT, Bio-Tek Instruments).

(5) Quantification of GFs release by Enzyme-Linked Immunosorbent Assay (ELISA)

The release of basic-FGF (bFGF) from PL membranes was assessed by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's specifications. Optical density was read at 450 nm ($n=3$) on a multiwell microplate reader (Synergy HY, Bio-Tek Instruments, USA).

4) *In vitro* biological studies

(1) Human Adipose derived Stem Cells seeding and culture

The *in vitro* biological response of PL-based membranes was assessed by direct contact with seeded human adipose derived stem cells (hASCs). These cells were isolated from adipose tissue samples obtained under a protocol established with Hospital da Prelada (Porto, Portugal), as previously described³⁰ and resulting from lipoaspiration procedures performed on women aged 35-50 years

For these experiments, disk shaped PL membranes of 5 mm were used, obtained by crosslinking with different amounts of genipin (0.10% gPL, 0.18% gPL, and 0.25% gPL formulations). All the samples were sterilized with ethylene oxide before cell culture studies. Adherent polystyrene cover slips were selected as positive control.

The samples were incubated in 500 L of PBS for 1h at 37°C in order to hydrate the samples. Human ASCs were grown in basal culture medium consisting of α -Minimum essential medium (α -MEM) (Invitrogen, USA), 10% fetal bovine serum (FBS) (Biochrom AG, Germany) and 1% antibiotic-antimycotic (A/B) solution (Gibco, Spain). Cells were expanded until achieving the necessary number for the envisioned experiments and detached from the flasks with trypsin-EDTA.

A cellular suspension of 5×10^4 cells (in 50 μ L of medium) was seeded in each sample and positive polystyrene control. The cells were then allowed to adhere on the membranes for 1h in the incubator at 37°C and 5% CO₂. After this period, 450 L of basal medium were added to each well. The constructs were cultured at 37°C and 5% CO₂, for 1, 3, and 7 days, and the metabolic activity and cellular proliferation were analyzed. The media was replaced each 2-3 days. The negative controls of each formulation consisted of samples incubated in basal medium without cells for the same time periods.

(2) Determination of metabolic activity by Alamar Blue assay

The metabolic activity of the cells seeded on the samples was assessed by Alamar Blue assay, according to the manufacturer's recommendation (AbDseroTec). Briefly, after each incubation time, the culture medium was discarded, the samples were washed twice with PBS and transferred to a new non-adherent 48 well-plate. The samples were incubated in a 10% Alamar blue solution in basal medium (450 μ L of basal medium, and 50 μ L of Alamar Blue) at 37°C, 5% CO₂ for 150 min. The fluorescence of the supernatant solution was read in triplicate in a microplate reader using the filter parameters: 560 nm of excitation, and 590 nm of emission.

The samples were then washed twice with PBS, transferred to eppendorf tubes containing 1 mL of ultrapure water and stored at -80°C for further DNA extraction and quantification.

(3) Determination of cell proliferation by DNA quantification

Proliferation of hASCs in PL-genipin membranes was evaluated by quantifying the total amount of double-stranded DNA present at different culture times (day 1, 3 and 7 days). Quantification was performed using the Quanti-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Invitrogen), according to the manufacturer's instructions. Before the analysis, the samples, previously washed with PBS, were immersed in 1 mL ultrapure water, stored at -80°C and finally were thawed and sonicated for 15 min. The fluorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, on a multiwell microplate reader (Synergy HT, Bio-Tek Instruments). The DNA concentration for each sample was calculated using a standard calibration curve.

(4) Scanning Electron Microscopy (SEM)

The distribution of hASCs on the surface of the PL-genipin membranes was analyzed by Scanning Electron Micro-

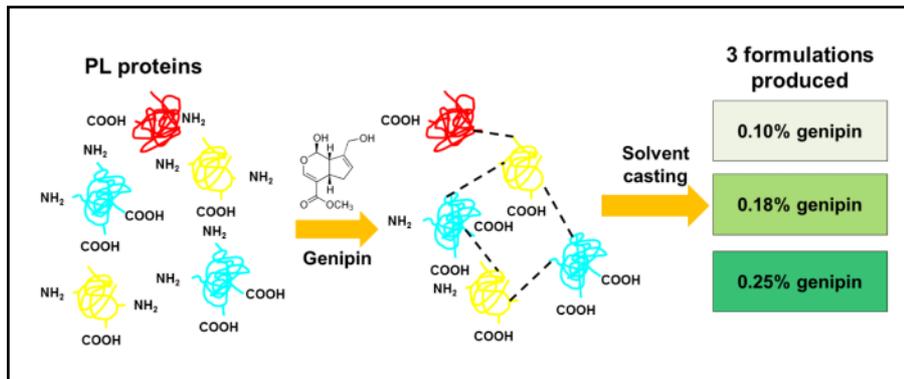


Fig.1 Experimental scheme for the production of PL-membranes by crosslinking with genipin

scope (SEM). Samples were collected for each time point, washed with PBS and fixed in 10% formalin (BIO OPTICA, Milan, Italy) overnight, at 4°C. After fixation, the constructs were dehydrated using a graded series of ethanol for 15 min, twice (30, 50, 70, 90, 100%) and were dried at room temperature. Finally, the constructs were sputter coated with gold and analyzed using a Nova NanoSEM 200 scanning electron microscope (Nova NanoSEM 200).

5) Statistical analysis

All the experiments were performed with at least three replicates. Results are expressed as mean ± standard deviation (SD). Statistical analysis of the data was conducted using IBM SPSS Statistics version 20 software. Normality of the measurements was tested using Shapiro-Wilk normality test. Differences between the experimental results were analyzed according to Kruskal-Wallis non-parametric test as the measurements do not follow a normal distribution. Differences between the groups with $p < 0.05$ were considered to be statistically significant.

Results and Discussion

1) PL-based membranes by crosslinking with genipin

PL membranes were produced by a simultaneous process of protein crosslinking with genipin and solvent casting. The crosslinking reaction is depicted in Figure 1. Genipin is a naturally occurring crosslinking reagent with the ability to spontaneously react with amino groups and form, by polymerization, a crosslinked network³¹. Crosslinking with genipin is a moderate reaction that occurs in an extended period of time³¹.

The reaction of genipin with primary amines originates a deep blue coloration, as observed in chitosan membranes crosslinked with genipin³¹. This color acquisition allowed

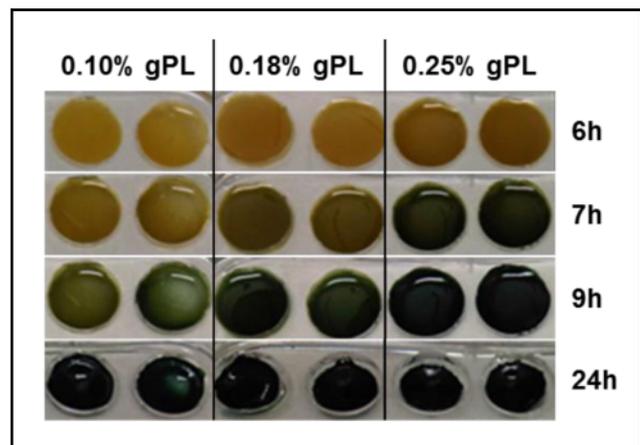


Fig.2 Evolution of crosslinking reaction for PL-genipin membranes over time

to follow up the evolution of crosslinking reaction since the color intensity can be correlated with crosslinking degree³².

Figure 2 presents the crosslinking evolution of PL-genipin membranes for three different crosslinking ratios (0.10, 0.18 and 0.25 % w/v genipin), during time. The reaction between PL and genipin occurred gradually in a period of approximately 24h. For all the tested conditions, an initial colorless solution turned dark green over time. These membranes were produced with different sizes and shapes, preserving their characteristics.

2) Chemical characterization of the membranes

Chemical characterization of PL-genipin membranes was performed by FTIR (Fig.3). PLs contain a high protein content rich in crosslinking sites available to interact with genipin. The regions of the spectra that are normally affected by genipin crosslinking with amines were identified

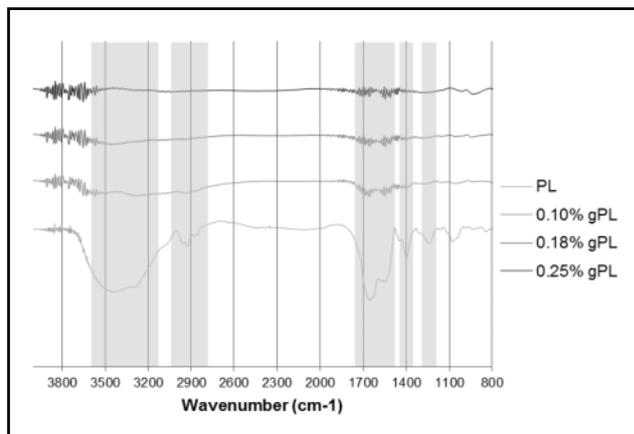


Fig.3 FTIR spectra of PL-genipin membranes prepared at different concentrations of genipin (0.10, 0.18 and 0.25% w/v). Non-crosslinked PL powder was used as a control.

in Figure 3 and correspond to amide I, II and II, respectively, $1600\text{-}1690\text{ cm}^{-1}$ (correspondent to amides I, C=O stretching), $1480\text{-}1575\text{ cm}^{-1}$ (correspondent to amides II, CN stretching, NH bending), and $1229\text{-}1301\text{ cm}^{-1}$ (correspondent to amides III, CN stretching, NH bending)³³. Clear differences can be observed between the spectra of PL and PL crosslinked with genipin in these regions of the spectra. Furthermore amine bands at 3300 and 2950 cm^{-1} significantly decreased after the crosslinking reaction, indicating that the amount of -NH_2 groups available in the proteins decreased and most likely was converted into C=N bonds³¹. Samples produced with different genipin concentrations exhibited a similar spectra, which may indicate that increasing the concentration of genipin did not lead to reaction of different functional groups of proteins present in the PL.

3) Determination of mechanical properties of the membranes

The mechanical properties of the developed membranes were assessed by Dynamic Mechanical Analysis (DMA). This technique allows the evaluation of mechanical response of these PL-based hydrogel membranes in the wet state and at 37°C , thus representing more closely the *in situ* environment to which a material is submitted when it is implanted in the human body. Figure 4 presents the viscoelastic behavior of the membranes, particularly the storage (elastic) modulus, E' , and the loss factor, $\tan \delta$, as a function of frequency.

The results obtained demonstrated the effect of the crosslinking on the stiffness of the membranes. A higher percentage of genipin used in the processing of the mem-

brane led to higher elastic storage modulus. Nevertheless, the trend of all the curves for E' was similar, exhibiting a slight increase with increasing frequency. Although the modulus was generally lower than that reported for other membranes aiming tissue engineering applications described in the literature^{34, 35}, PL-based membranes have the advantage to contain biomolecules that are able to provide cues for cell adhesion and proliferation¹⁴. The loss factor is defined as the ratio between the amount of energy dissipated by viscous mechanisms and the energy stored in the elastic component, providing information about the damping properties of the material. The membranes prepared with $0.10\text{ wt}\%$ genipin had a more elastic behavior than the ones prepared with 0.15 or $0.18\text{ wt}\%$ genipin, which was observed in Figure 6b. For all formulations, $\tan \delta$ tended to increase with increasing frequency, indicating that the materials became less elastic.

4) Total weight loss and water uptake

The weight loss of the PL-genipin membranes was followed upon immersion of the materials in PBS for 30 days and it is presented in Figure 5. The data shown in Figure 5 suggests that, after a fast initial weight loss, the membranes remained stable for a long period of time. The initial weight loss might be explained by the initial release of proteins not involved in the crosslinking reaction and non-reacted genipin present on PL-based membranes. The different crosslinking degree did not significantly affect the degradation profile of the samples.

The long term stability of PL membranes crosslinked with genipin herein shown represents one the main features of this novel biomaterial, thus overcoming the fast shrinkage

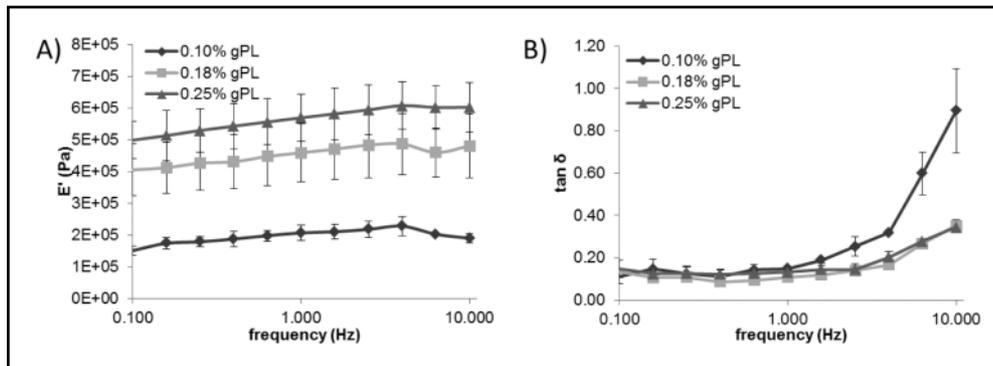


Fig.4 Dynamic mechanical analysis of the membranes prepared with different crosslinking degrees of genipin. (A) elastic storage modulus. (B) loss factor. Error bars represent standard deviation (n=3).

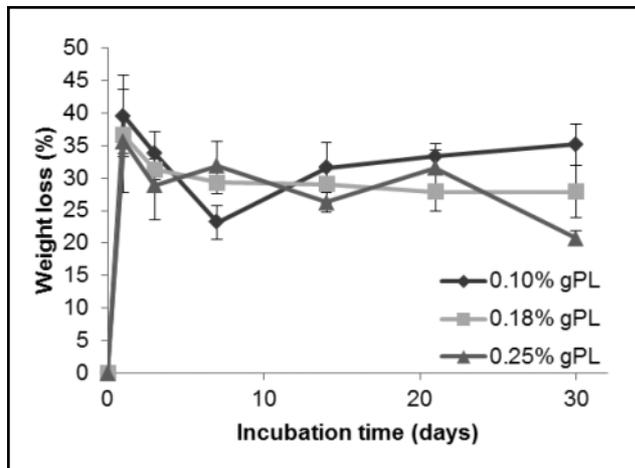


Fig.5 Weight loss profile of PL-genipin membranes over 30 days of incubation in PBS. Error bars represent standard deviation (n=3).

typically associated with PL-based hydrogels. This advantage offers the possibility to apply these membranes in tissue engineering and regenerative medicine strategies as these materials appear to provide the mechanical support to withstand the initial stages of tissue regeneration. Other tissue engineering approaches, aiming the controlled release of platelet GF showed that the scaffolds derived from PRP are not stable and degrade almost completely in a short period of time^{10, 17}. Therefore, the PL-based membranes might be a suitable alternative biomaterial for distinct tissue engineering approaches, namely for periodontal ligament and periosteum tissues.

PL-crosslinked membranes are hydrophilic and present high water uptake ability. The water uptake profile over time was followed up to 30 days of immersion in PBS solution. The membranes reached a maximum water uptake value few hours after immersion in an aqueous solution, which is nearly 400%. Despite their large water uptake ability, their shape and stability was not compromised. After hydration, the membranes presented a gel-like structure, which makes

it easy to handle without damage of the matrix.

5)Protein release from PL membranes

Cumulative release of PL proteins in total mass is presented in Figure 6a. A controlled release profile of proteins from the three tested formulations was followed for up to 14 days. No significant differences were observed among the formulations studied, being, therefore, independent from the crosslinker content. The release profile of PL proteins from the membranes followed a typical controlled release pattern that can be explained in three stages: i) “burst” of protein release during the first hour, representing 50% of the total protein released during the 14 days of the assay; ii) transient sustained release of protein in the first three days of the assay, with a release peak centered between the day 1 and 2; iii) slower sustained release up to 14 days.

Protein release rate depends upon solubility, diffusion, size and biodegradation of the matrix materials. The release of proteins from materials produced via genipin-mediated crosslinking has been reported to be dependent on

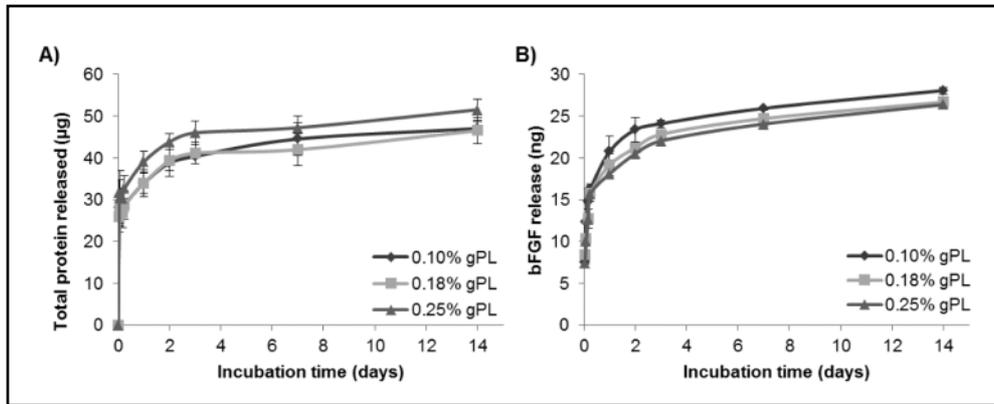


Fig.6 Cumulative protein elution from PL-genipin membranes prepared at increased concentrations of genipin (0.10, 0.18 and 0.25% (w/v)) by incubation in PBS for over 14 days. (A) Total protein release quantified by micro-BCA, (B) ELISA quantification of basic-FGF release. Error bars represent standard deviation (n=3).

the crosslinking degree²⁹). Nonetheless, our results on protein release did not follow this trend, which might be explained by the presence of an excess genipin in the formulations, which led to self-polymerization of genipin monomers, as described by Mi and co-workers³¹). This way, the initial burst of protein release could be due to the presence of free proteins dispersed in the matrix of crosslinked proteins which could be easily washed from the surface of the membranes. The first release stage might represent the delivery of non-crosslinked proteins.

Crosslinking with genipin is a reaction that occurs in aqueous solution and is extended in time³¹). The removal of the water during the solvent casting step could be arresting the reaction of genipin with the amino groups of PL proteins, leaving unreacted PL proteins and genipin free to be washed out. The proteins deeply entrapped in the membranes or weakly linked to other proteins by electrostatic forces, represent the proteins released in a sustained manner during all the assay.

6) Release of bFGF

PL is a rich source of a plethora of GFs and cytokines which are prompt to induce wound healing and the recruitment of cells for autologous tissue regeneration³⁶). The direct entrapment of proteins, namely GFs, may be inefficient due to loss of protein activity³⁷). In order to confirm the integrity of the entrapped GFs, the release of specific GFs with known function related with wound regeneration was confirmed by ELISA. The release profile of bFGF is represented in Figure 6b, and was characterized by an initial

“burst” of GF release during the first hours, followed by a sustained delivery up to 14 days, in all the tested formulations, similar to the kinetic observed to the total protein release. The sustained release of GFs is a requisite and plays an important role in the creation of correct biological functions for inducing tissue regeneration²). In case of bFGF, its controlled release has been shown to be effective in the enhancement of angiogenesis¹¹) and new bone formation³⁸), and, together with PDGF, is responsible for the mitogenic effect of PL¹⁴).

The use of PL in tissue engineering, instead of PRP or platelet concentrates, was previously defended by the potential for the standardization of the PL production process¹³), which would yield more reproducible results for tissue engineering applications. PRP is typically contaminated with red blood cells unlike the lysates used for the development of the membranes described in this study. Matsui and Tabata¹¹) reported a higher PDGF- $\beta\beta$ content in PL than in PRP activated with CaCl₂. Thus, the PL processing, which involves the release of all the platelet cytoplasmatic content, could be more efficient in the liberation of platelet proteins. Future studies should focus in the analysis of the fine composition of PL, namely, the presence of chaperones and chaperonins specific of GFs. The chaperonins are small proteins which assure the correct folding and stability of the large proteins in the “crowded” cytosol of eukaryotic and prokaryotic cells³⁹). The presence of this group of molecules in PL is expected to extend the life span and activity of platelet derived GFs.

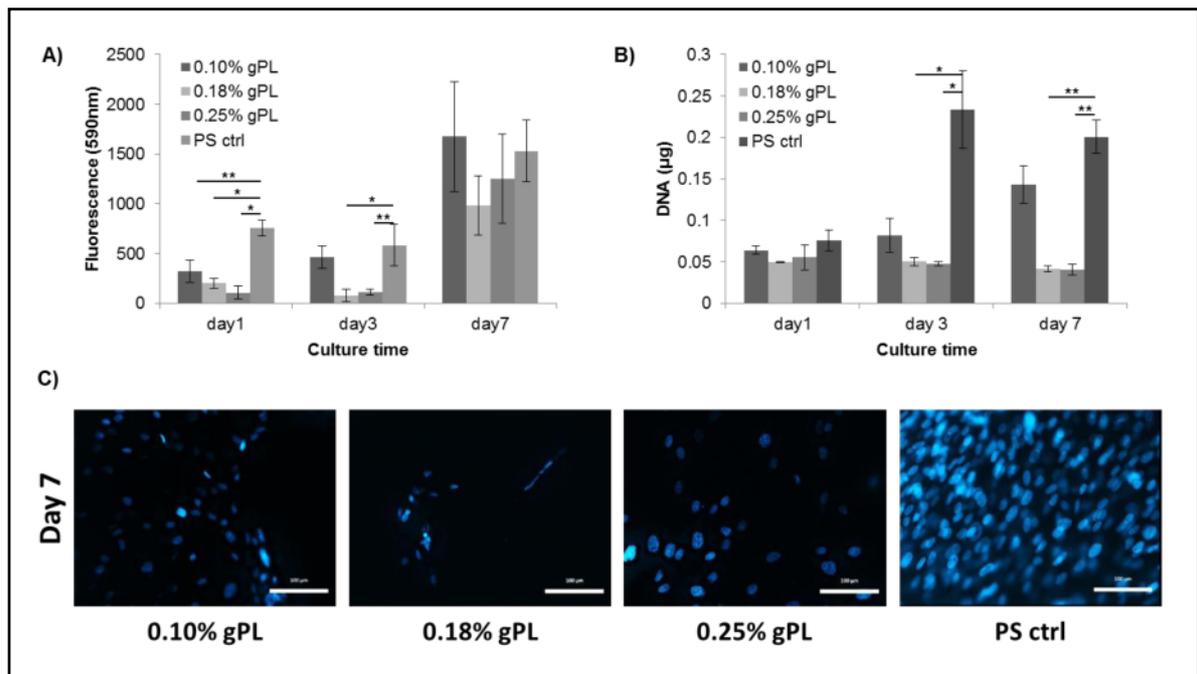


Fig.7 *In vitro* direct contact assay of hASCs cultured in PL membranes crosslinked with 0.10, 0.18 and 0.25% w/v of genipin. Human ASCs cultured in polystyrene disks (PS ctrl) were used as positive control. (A) Metabolic activity measured by Alamar Blue assay at 1, 3 and 7 days of incubation. (B) Quantification of DNA content at 1, 3 and 7 days of incubation. (C) Fluorescence microscopy of hASCs stained with DAPI after 7 days in culture. The statistical significance of the results was studied using the Kruskal-Wallis test for $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$). Error bars represent standard deviation ($n=4$ for Metabolic activity, $n=3$ for DNA quantification).

7) *In vitro* biological response of PL-membranes

The analysis of *in vitro* performance of hASCs seeded on the PL membranes was assessed by measuring the metabolic activity by alamar blue, and the proliferation of the cells, as a function of the DNA content measured at different time points. The results, graphically represented in Figure 7, shows that, while the 0.10% gPL formulation did not induce a significant decrease of cells' metabolic activity over the periods of the study, the cells grown in the 0.18% gPL and 0.25% gPL formulations presented a reduced metabolic activity and proliferation until the third day of culture. Nevertheless, by the 7th day of culture there is an apparent adaptation of the cells, reflected by an increase in the metabolic activity, which closely resemble the values obtained for the 0.10% gPL formulation and the positive control. We hypothesize that the genipin is in excess in these formulations and the non-reacted genipin is toxic to the cells. The accumulation of non-reacted genipin might also be proportional to the initial amount of genipin used for the preparation of the membranes, as crosslinking saturation and evaporation of the aqueous solvent might block

the reaction. During the incubation time, the proteins present in culture medium could be reacting with genipin, inactivating its toxicity, enabling cells to recover their activity. Moreover the reported ability of PL proteins to enhance the proliferation of hASCs *in vitro*³⁰ would have benefited the recovery of cells activity.

In spite of the observed influence of genipin content in the cytotoxicity of the membranes, the unreacted genipin could facilitate the integration of the PL membranes crosslinked with genipin in the tissues. The reaction of genipin with free reactive amino-groups of molecules present in ECM might allow the adhesion of the membranes to the native tissue⁴⁰.

Overall, the data obtained suggests that the most suitable membranes for future tissue engineering applications are those corresponding to the 0.10%gPL formulation, as they presents similar physico-chemical properties in comparison with the remaining formulations while simultaneously providing a better *in vitro* biological response and lower genipin content.



Conclusions

PL has been widely used in tissue engineering research for applications in which a reliable and inexpensive source of GFs was needed. Nevertheless, so far, it was challenging to obtain a long-term stable hydrogel for controlled delivery of GFs from PL. The PL-based membranes developed in this work presented adequate mechanical properties, particularly high stiffness and elasticity and most importantly a long-term stability, quite uncommon for PL-based biomaterials. Consequently, these membranes present a group of properties that favor their application for the regeneration of elastic and mechanically active tissues *in vivo*. Moreover, these membranes demonstrated the potential to act as a valuable substrate for stem cell attachment and growth in 2D conditions and provide an environment rich in GFs with a major role in wound healing. Our findings suggest that it is possible to produce stable PL-based membranes crosslinked with genipin for different tissue engineering applications. The use of the PL membranes herein studied as a substrate for cell sheet engineering would allow the development of strategies envisioning the regeneration of membrane-like tissues, such as the periodontal ligament or the periosteum.

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Conflict of Interest

The authors have no conflicting financial interests.

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