Human protein-based porous scaffolds as platforms for xeno-free 3D cell culture

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Abstract

Extracellular matrix and protein-based biomaterials emerged as attractive sources to produce scaffolds due to their great properties regarding biocompatibility and bioactivity. In addition, there are concerns regarding the use of animal-derived supplements in cell culture not only due to risk of transmission of xenogeneic contaminants and antigens but also due to ethical issues associated with collection methods. Herein, we purpose novel human protein-derived porous scaffolds produced from platelet lysates (PL) as platforms for xeno-free three-dimensional (3D) cell culture. Human PL were chemically modified with methacryloyl groups (PLMA) to make them photocrosslinkable and used as precursor material to produce PLMA-based sponges. The herein reported human-based sponges have highly tunable morphology and mechanical properties, with an internal porous structure and Young's modulus dependent on the concentration of the polymer. Human adipose-derived stem cells (hASCs) were cultured on top of PLMA sponges to validate their use for 3D cell culture in xeno-free conditions. After 14 days hASCs remained viable, and results show that cells were able to proliferate during time even in the absence of animal-derived supplementation. Our study reveals for the first time that such scaffolds can be promising platforms for culture of human cells avoiding the use of any animal-derived supplement.

Introduction

The development of platforms for three-dimensional (3D) cell culture remains a challenge in tissue engineering (TE). An ideal scaffold requires specific biochemical and mechanical features close to those experienced by the cells in their native tissues. Mechanical strength, good porosity, biocompatibility and good cell attachment are important characteristics that should be taking into account during scaffold development process.[1–3] In this sense, natural and synthetic macromolecules have been explored to produce scaffolds for cell culture and TE. Despite all the advantages found in synthetic-based scaffolds regarding their mechanical properties, they usually lack bioactivity and have limited biodegradability. Natural-based polymers have emerged as promising materials due to their great properties regarding biocompatibility, low immunogenicity, and availability.[4] However, they usually suffer from poor mechanical properties and stability *in vitro*.[5] Therefore, strategies based on chemical modification of natural polymers, physical crosslinking methods or production of hybrid materials by conjugating natural and synthetic materials have been proposed.[6] Extracellular matrix (ECM) and protein-based biomaterials are particularly appealing for cell culture and TE strategies, since they are major components of the cellular native microenvironment.[7] In this sense, the fabrication of porous scaffolds using collagen[1,8], gelatin[9,10], decellularized ECM[11,12], albumin[13], keratin[14] and silk fibroin[15,16] has been widely reported.

Nowadays, there is a major concern regarding the use of animal-derived materials and serum supplements in cell culture procedures due to inherent risk of contamination related with their non-human origin. Besides that, there are also ethical issues related with collection methods of animal-derived products and potential limited availability.[17] Moreover, the use of animal-derived supplements is associated with a risk of transmission of xenogeneic infectious agents and immunization, making translation to clinical applications a more difficult process. Human-derived supplements such as platelet lysates (PL) have been proposed as a replacement of animal-derived supplements such as fetal bovine serum (FBS) with particular interest for the development of cellular products in clinical setting.[18,19]

Many of the concerns with using animal-derived supplements for cell culture extend to animal-derived materials for cell culture as well. There is a need to find alternatives to the current gold standards for cell culture, including Matrigel® and animal collagen. Their batch-to-batch variability, and animal-derived nature led to experimental uncertainty and a lack of reproducibility.

PL have been used for TE and regenerative medicine purposes as an autologous source of growth factors and other bioactive proteins involved in tissue healing process.[17] By taking advantage of the clotting properties of PL due to their richness in fibrinogen, scaffolds have been developed by mixing PL with calcium and/or thrombin that will trigger gels formation by the conversion of fibrinogen to fibrin.[20,21] However, PL-derived biomaterials usually suffer from poor mechanical properties and poor stability *in vitro*.[17,22] Recently, human photocrosslinkable PL-derived hydrogels were produced based on the chemical modification of human PL with methacryloyl groups (PLMA).[23] The addition of such groups allowed the creation of covalent bounds after exposure to light leading to the formation of hydrogels with increased and tunable mechanical properties, dependent on PL degree of modification or PLMA concentration, and increased stability *in vitro*. Moreover, PLMA-based hydrogels showed great potential to be used not only as platform for human adipose-derived stem cell culture (hASCs)[23] but also to study tumor invasion behavior of cancer cell lines spheroids (MG-63, SaOS-2 and A549) and human bone marrow mesenchymal stem cell (hBM-MSCs) spheroids.[24] In addition, Monteiro *et al* were also able to demonstrate the ability of such materials to be used to bioengineer a 3D tri-culture osteosarcoma model.[25] In another approach, Tavares *et al*[26] developed a PLMA-based nanocomposite – PLMA hydrogels loaded with bioactive silica nanoparticles (MSNCaPDex) – able to support the culture and induce osteogenic differentiation of hBM-MSCs without osteogenic supplementation.

In this work we explored such biomaterial to develop a completely novel platform to be used for 3D cell culture in xeno-free conditions that will facilitate GMP validation for cell therapies and regenerative medicine purposes. PLMA-based porous scaffolds were produced by a simple freeze-drying methodology and their ability to accommodate stem cell cultures in the absence of animal-derived serum supplementation was evaluated. (Figure 1). Our hypothesis is that PLMA-based scaffolds, due to their richness in bioactive proteins and growth factors involved in cell maintenance and proliferation, will be able to support human cell culture avoiding the use of animal-derived serum supplements such as FBS. This would constitute a first attempt of developing a scaffold that besides providing the necessary 3D support for cells (including the necessary surface biochemical landscape for cells to adhere) it could also offer a unique environment exempting the need of such culture media.

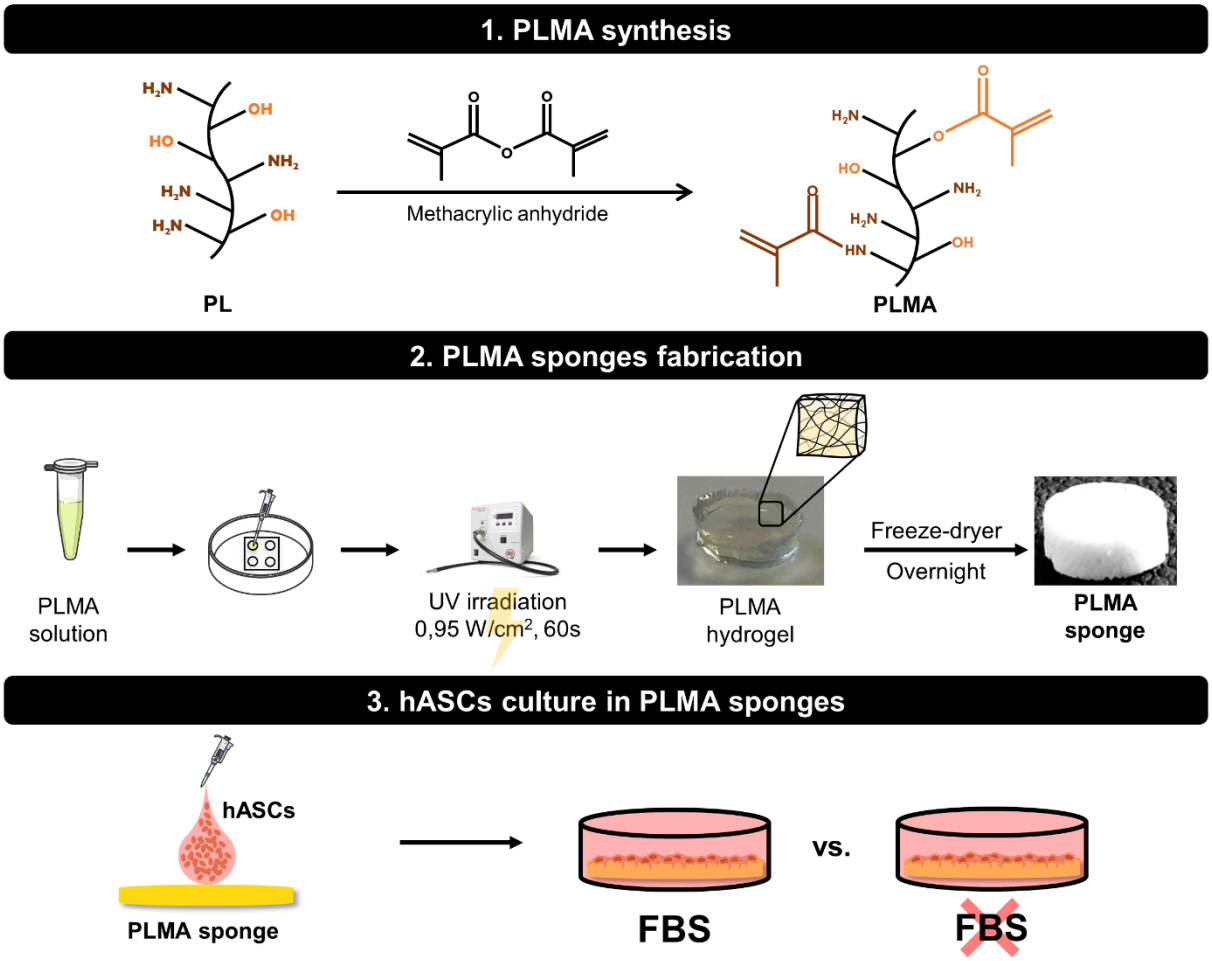

Figure 1: Schematic representation of PL modification, PLMA sponges production and hASCs culture. PLMA was synthesized by reaction of PL with methacrylic anhydride. PLMA sponges were produced by a simple freeze-drying technique and afterwards hASCs were cultured on top of PLMA sponges in medium with or without FBS supplementation.

Results and discussion

1. PLMA sponges characterization

a. CryoSEM analysis and pore size measurements

PLMA sponges internal structure was assessed by cryoSEM. CryoSEM has the advantage to access the native structure of the protein scaffolds after sponge re-hydration. Figure 2A shows representative images obtained by cryoSEM for the three conditions under analysis in this work – PLMA10, PLMA15 and PLMA20. Differences in pore dimension and connectivity between PLMA10 and PLMA15 conditions when compared to PLMA20 are evident. As it can be seen, increasing the concentration of PLMA led to tighter porosity, suggesting that PLMA

sponges internal structure is affected by changing the concentration of the polymer in the precursor solution used to produce the scaffold. Using the ImageJ software, pore size measurements for all conditions were performed and are presented in figure 2B. Results confirm the slight decrease in the size of the pores when the concentration of PLMA increases from 10% to 15% (w/v) and a significant difference in comparison to 20% (w/v) sponges. PLMA20 shows a porous network with smaller diameter as well as less variation regarding the pore size when compared to PLMA10 or PLMA15. In the case of these last conditions, besides the fact that both have a network with larger pores than PLMA20, PLMA10 and PLMA15 present a more heterogeneous porous network, while from these three conditions, PLMA20 present the most homogeneous structure. As such, PLMA sponges have a porous internal structure that can be easily adjusted by varying the concentration of polymer used in the precursor solution to fabricate the final scaffold.

b. Mechanical properties assessment

Mechanical properties of PLMA sponges were assessed through compression tests. Young's modulus of scaffolds was calculated, and results are shown in Figure 2C. The Young's modulus of the scaffolds increases as the polymer concentration increases. This trend was expected because with the increase of the PLMA concentration of the precursor solution the density in the final scaffolds will increase. The results confirmed that the mechanical properties of PLMA scaffolds can be easily adjusted by changing PLMA concentration with the Young's modulus varying from approximately 15 kPa (PLMA10) to approximately 25 kPa (PLMA20).

c. Swelling rate

Herein, the swelling ability of PLMA sponges was studied. Figure 2C shows a representation of the swelling rate (%) curve for all three conditions in analysis – PLMA10, PLMA15 and PLMA20. For all three conditions, after approximately 5 minutes in PBS the PLMA sponges achieve the swelling equilibrium. Significant differences can be noticed between all three conditions. PLMA10 present a swelling rate curve slightly higher than the curve represented for the other two conditions, being that PLMA20 present the lowest swelling rate profile. This fact can also be related with the porous network previously analyzed. Pore measurements showed that PLMA10 present larger porous network and interconnectivity than for the other two conditions under analysis, being a reason for the differences that can be found in the swelling rate curve. Regarding the mechanical properties results, an increase in the Young's

modulus is related with a decrease in the swelling rate and therefore from all studied conditions, PLMA20 present the lowest swelling rate profile and also the higher Young's modulus.

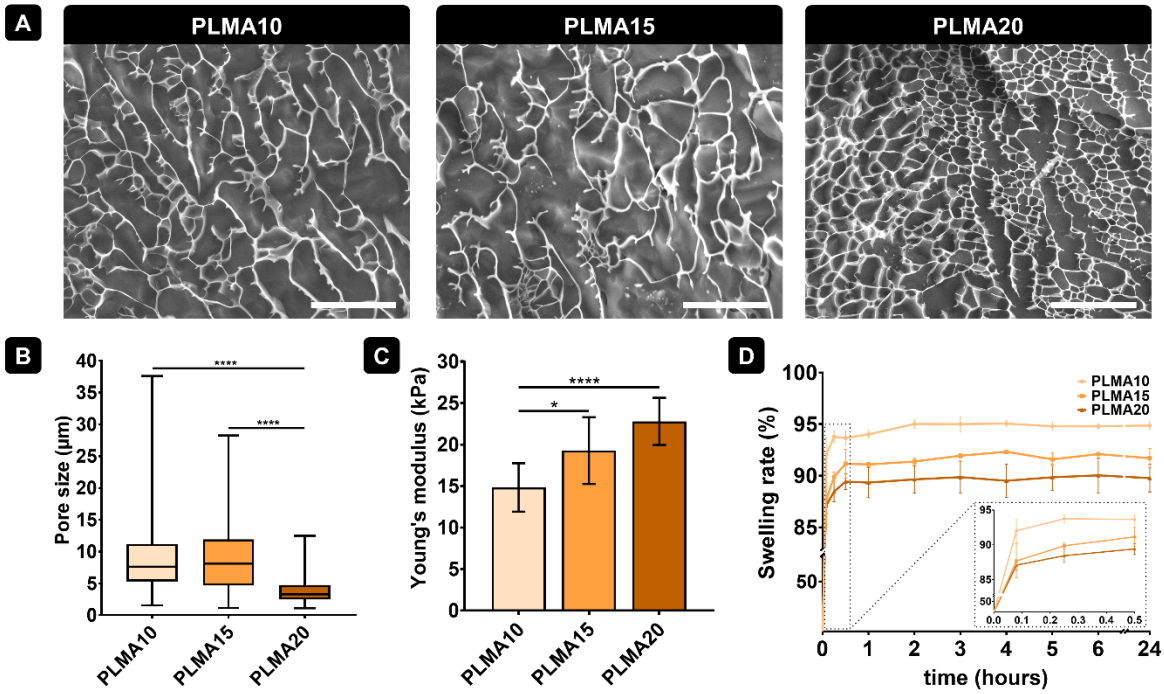

Figure 2: PLMA-based sponges characterization. (A) CryoSEM representative images of PLMA scaffolds internal structure. Scale bar: 30 µm (B) Pore size measurements analysis from cryoSEM images by ImageJ, represented by median, maximum and minimum values of the measured pore sizes. (C) Young's modulus (kPa) of the studied scaffolds previously hydrated in PBS. (D) Swelling ratio (%) of PLMA sponges after distinct immersion times in PBS at 37ºC. For all analysis PLMA10, PLMA15 and PLMA20 were used. Statistical analysis through *one-way ANOVA* showed significant differences (*$p < 0.05$, ****$p < 0.0001$) between the analyzed groups. (n=3)

2. Stem cell culture in PLMA and collagen-based sponges

The development of animal-free 3D cell culture platforms remains a challenge in TE. The use of animal-derived serum supplements is part of the standard procedures for cell culture nowadays, however the search for human-based alternatives has become a major goal. The reasons behind this are risks of xenogeneic contaminants transmission due to the animal origin as well as ethical issues regarding the collection methods. In addition, the use of such supplements make the translation to the clinic process more difficult. As such, PL and PL-based materials have been explored in an attempt to diminish or even replace the use of animal-

derived supplements like FBS.[17] Herein, customized PLMA-based sponges were studied as platforms for xeno-free 3D cell culture. PL-based materials are highly rich in growth factors and bioactive proteins involved in cell growth and proliferation[17,22], still, the use of fully human protein-based materials was never explored to culture cells in xeno-free conditions.

hASCs were cultured on the top of both PLMA-based sponges (PLMA15) and SpongeCol®, a commercially available Type I collagen-based sponge which supports cell attachment and proliferation. The cells in both PLMA and collagen scaffolds were cultured for 14 days in medium with and without animal-derived serum supplementation.

In order to assess cell viability during time, live/dead staining was performed at 3, 7 and 14 days of culture. As shown in figure 3, hASCs were able to adhere to PLMA sponges and remain viable for up to 14 days even when cultured in medium without animal-derived serum supplement. Figure 3 also shows live/dead representative images of hASCs cultured in SpongeCol®. As it was expected, in the absence of FBS cells barely attach and do not spread in SpongeCol® contrary to what happened when cells were cultured in full media. Therefore, such results support the idea that PLMA-based sponges have high potential to be used as platforms to support 3D xeno-free cell culture.

The morphological features of cells cultured in PLMA and collagen sponges was assessed by staining the F-actin filaments (phalloidin, red) and nuclei (DAPI, blue). It can be observed that hASCs in the PLMA sponges are perfectly spread and they are able to create networks among themselves both cultured with and without FBS. Even in the absence of any animal supplement, hASCs were able to adhere, proliferate and maintain their viability until 14 days of culture. In the case of collagen-based sponges, cells are only able to spread and form networks when cultured in media with animal-derived supplement.

Cross-section images of hASCs with DAPI/Phalloidin staining after 14 days of culture are also represented in figure 3. As it can be seen, hASCs were able to penetrate the PLMA sponge porous network, and after 14 days of culture the cells are perfectly spread inside the scaffold allowing the formation of a cell network essential for tissue development. It is also important to emphasize once again that this phenomenon happens for both conditions, i.e., it happens even without animal-derived serum supplementation. Penetration of cells within these 3D scaffolds causes cells to experience a 3D microenvironment, thus mimicking what happens in native tissues. The use of 3D approaches for cell culture instead of 2D cultures allows better cell-cell interaction/communication, more realistic proliferation rates and more accurate representation of responses to different stimuli.[27,28]

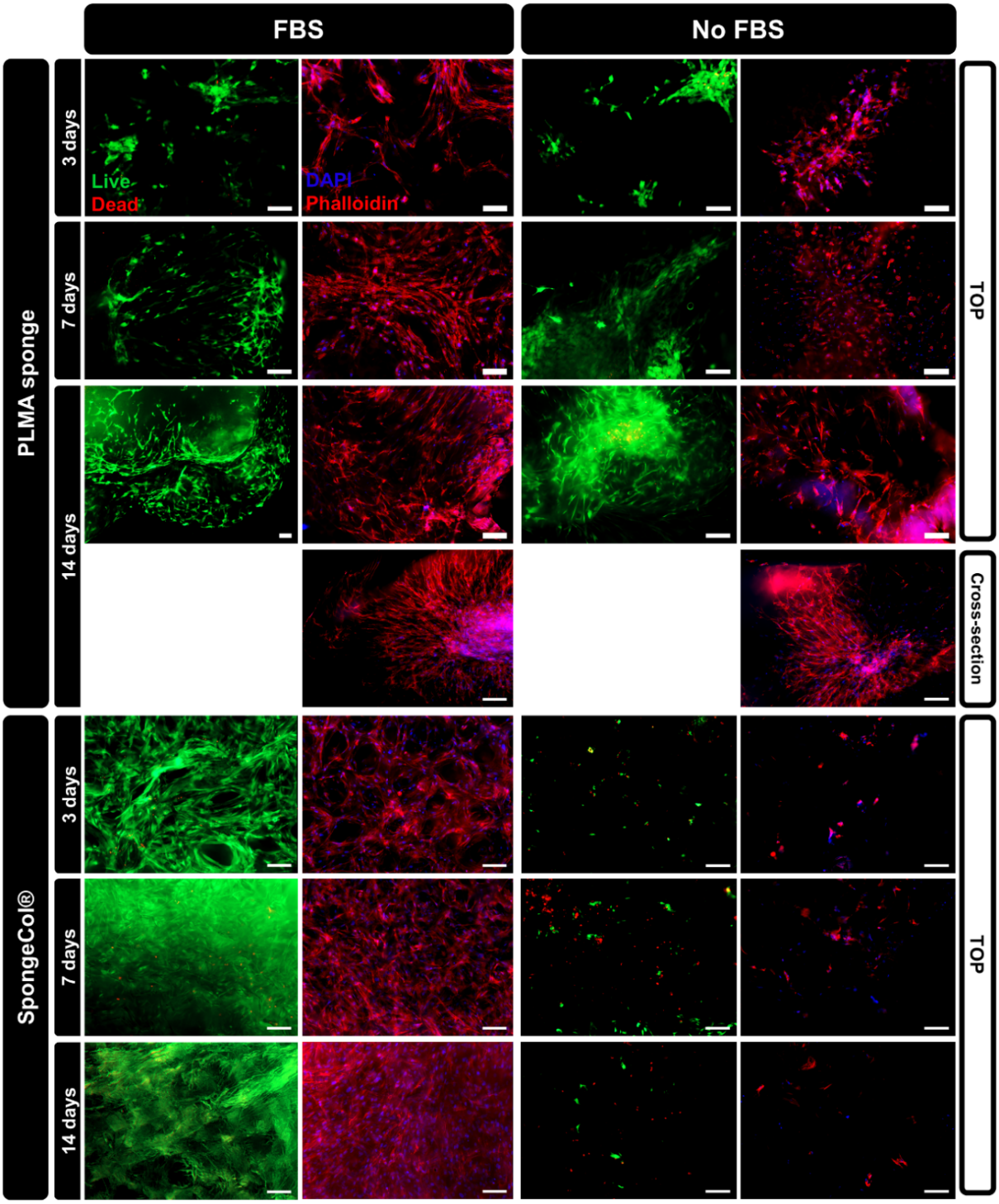

Figure 3. Live/Dead assay and DAPI/Phalloidin staining. Representative images of Live/Dead and DAPI/Phalloidin staining for hASCs cultured on top of PLMA15 sponges and SpongeCol® at 3, 7 and 14 days of cell culture with and without animal-derived serum supplement. DAPI/Phalloidin staining cross-section representative images of hASCs cultured in PLMA sponges at 14 days. Scale bar: 100 µm.

Cell proliferation and metabolic activity of cells cultured in PLMA-based sponges were assessed by DNA quantification and MTS assay, respectively. From day 3 until day 14 there is an increase in both DNA content and metabolic activity – see Figure 4A and 4B. Therefore, as it was already demonstrated by the live/dead and DAPI/Phalloidin fluorescence images, hASCs are able to proliferate when cultured in PLMA scaffolds. The tendency in hASCs proliferation rate and metabolic activity is the same in both culture media, suggesting that PLMA sponges are self-sustainable as platforms for cell culture and do not affect normal cell function. Regarding the control (SpongeCol®) results show an increase in both DNA and MTS from 3 to 14 days of culture when the medium was supplemented with FBS. However, when no FBS was added to the medium used in cell culture, the MTS is decreasing over time and no changes are observed in the DNA quantification among all time-points.

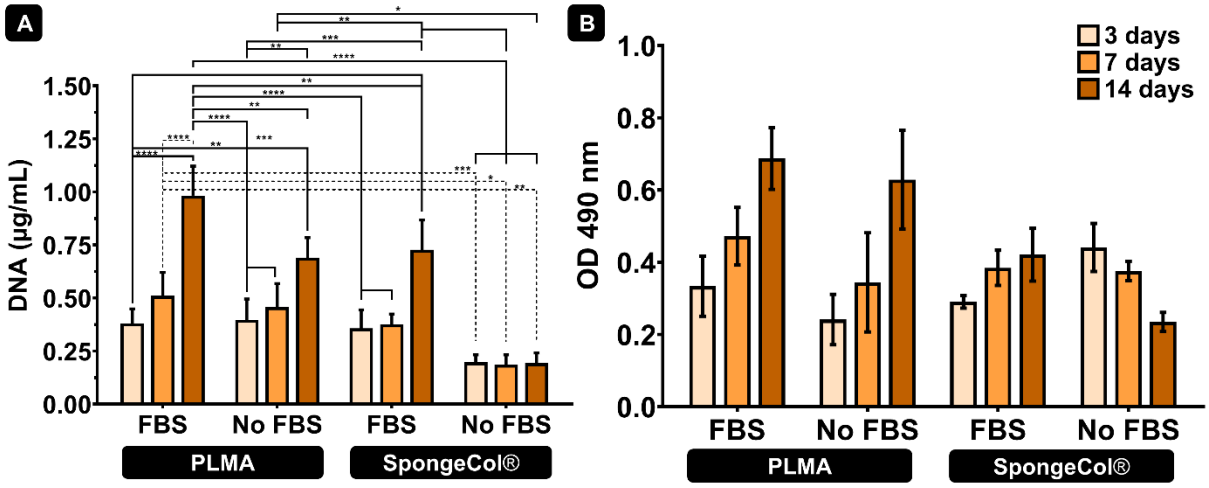

Figure 4. Cell proliferation and viability assessment. (A) DNA and (B) MTS results for hASCs after 3, 7 and 14 days of cell culture with and without animal-derived serum supplement. Statistical analysis through *two-way ANOVA* combined with Tukey's multiple comparisons test showed significant differences between the analyzed groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. PLMA growth factors quantification and PLMA sponges protein/growth factors release profile

PL are a pool of growth factors and bioactive proteins involved in cell proliferation and growth and therefore they have been explored in cell culture studies as a way to replace animal-derived suplements.[17] Indeed, when compared with FBS, PL are richer in growth factors that

increase cell growth and proliferation and due to their human nature, they are ideal candidates for human cell maintenance.[29–31] The quantification of growth factors in PLMA was performed by LEGENDplex™ Human Growth Factor Panel – see figure 4A. Results show that PLMA have several growth factors in their composition known to improve cell proliferation and maintenance. These findings corroborate the hypothesis that PLMA-based materials can be ideal candidates to engineer xeno-free cell culture platforms.

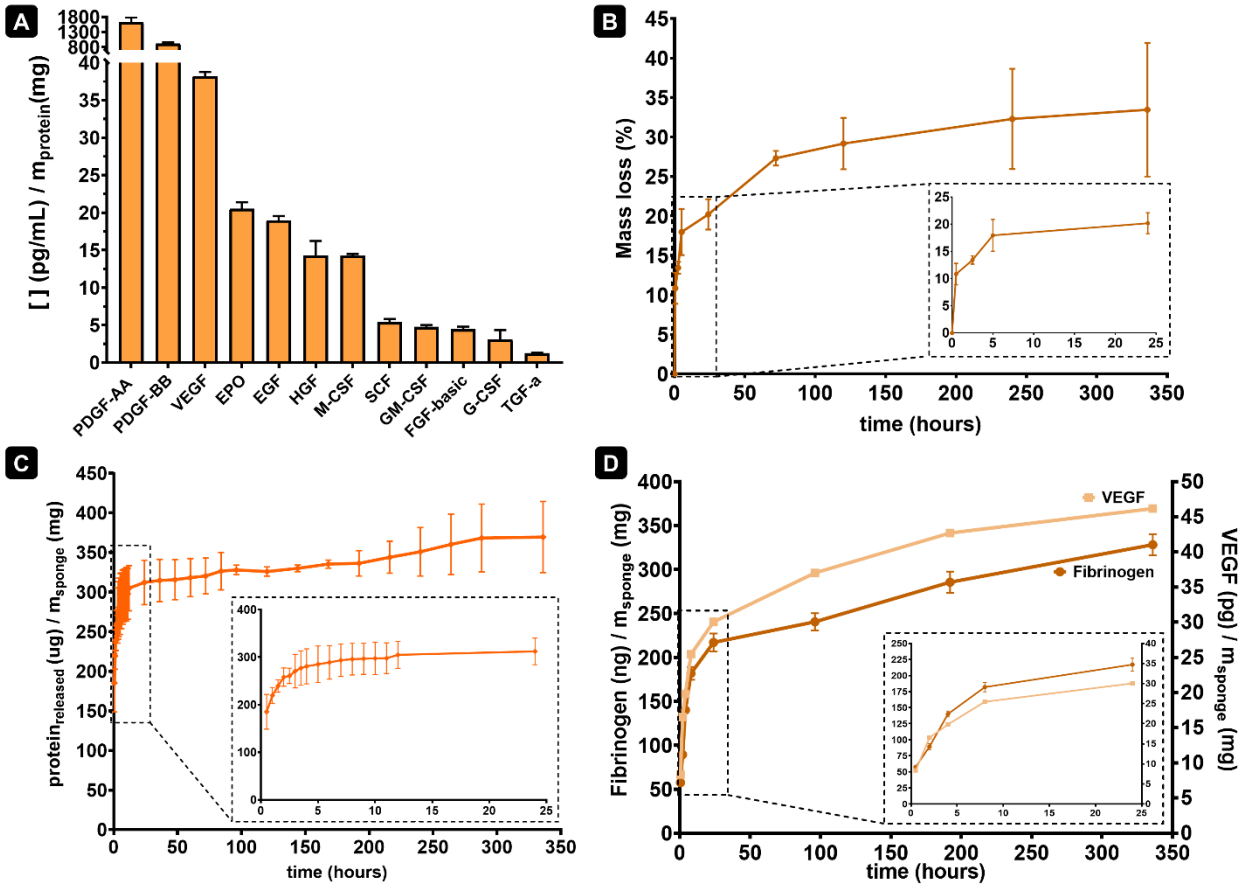

Figure 5: PLMA growth factors quantification and protein release profile of PLMA-based sponges. (A) Quantification of PLMA growth factors by LEGENDplex™ Human Growth Factor Panel. (B) Mass loss (%) of PLMA15 sponges. (C) Total protein release quantification of PLMA15 sponges. (D) Quantification of specific proteins and growth factors (Fibrinogen and VEGF) released by PLMA15 sponges. Protein release assays were performed with PLMA15 during 14 days.

As previously mentioned, hASCs were able to maintain their viability and proliferate when cultured on PLMA sponges in xeno-free conditions. We hypothesize that soluble factors could be gradually released from the human-protein based scaffolds, helping to maintain the culture

of the cells. Therefore, protein release assays were performed during 14 days, in order to verify the total protein amount released by PLMA15 sponges, as well as to quantify proteins and growth factors that are involved in the maintenance of cells viability and proliferation. Figure 5C shows the total protein release profile of PLMA sponges. Results show an overall sustained release of proteins from PLMA-based sponge, being slightly faster in the first 24 hours. Such results can corroborate the mass loss assays (Figure 5B), where it can be observed that PLMA scaffold lose about 30% of their initial mass after 14 days.

It is well known that human PL are a pool of bioactive proteins and growth factors essential for cell proliferation and growth. In this work we assume that PLMA-based sponges will provide the essential proteins and growth factors for the normal function of cells during the *in vitro* culture. To confirm that assumption, the quantification of the total amount of proteins released from PLMA sponges as well as the analysis of specific proteins, namely fibrinogen and vascular endothelial growth factor (VEGF) was performed. Fibrinogen is one of the main components in human PL and plays a key role in the fabrication of PL/fibrin-based platforms already reported.[17] VEGF is an important growth factor essentially involved in angiogenesis process, a mandatory step during tissue repair.[32] Taking all of this into account, ELISA quantification of fibrinogen and VEGF was performed to explore their release profile from PLMA sponges. Results show an overall sustained release of such growth factors during the 14 days (see figure 5D) and thus contributing to cell maintenance and growth.

Altogether, such results emphasize the idea that PLMA-based materials, in this case PLMA-based sponges, are promising platforms for the culture of cells in xeno-free conditions. In fact, by applying a simple freeze-drying process to PLMA-based hydrogels previously reported[23], we were able to obtain PLMA-based scaffolds with improved mechanical properties and larger porous networks, key factors to promote cell maintenance and proliferation.[3,33] PL and PLMA richness in growth factors and bioactive proteins allows the development of novel platforms to be used for the culture of cells avoiding the use of animal-derived serum supplements, which is often a barrier to clinical translation.

Conclusions

The development of efficient strategies for 3D cell culture in xeno-free conditions is still a challenge in TE. Taking advantage of the great potential of human based PLMA materials recently proposed as platforms for 3D cell culture, here PLMA-based sponges were explored as highly porous scaffolds to support human-derived stem cell culture in the absence of animal-

derived substances. These scaffolds showed an internal porous structure reliant on the concentration of the polymer in the precursor solution, permitting the invasion and colonization of cells within their volume. PLMA sponges were able to support stem cell culture up to 14 days with non-significative differences in cell proliferation between the cultures with animal-derived serum and xeno-free conditions. Moreover, studies on the protein release profile of PLMA-based sponges exhibited an overall sustained release of proteins during time, that most probably are playing an important role in cell maintenance during cell culture time.

PLMA-based scaffolds here developed offer a new possibility to culture cells in a 3D environment avoiding the use of animal-derived serum supplements, thus open new routes for clinically compliant protocols for GMP *in vitro* cell culture. It is also important to highlight that this platform can have an autologous origin, being adequate to produce personalized matrices, combined with cells from the patient for *in vitro* or *in vivo* applications with no risk of cross-reactivity, immune reaction, or disease transmission.

Materials and methods

1. Methacryloyl platelet lysates synthesis

Methacryloyl platelet lysates (PLMA) were synthesized as previously reported.[23] Briefly, human PL (STEMCELL Technologies, Canada) were reacted with methacrylic anhydride 94% (MA) (Sigma-Aldrich, Germany) in a 100:1 ratio under constant stirring at room temperature. Afterwards, PLMA were dialyzed against deionized water to remove the excess of MA. The PLMA solution was filtered with a 0.2 µm filter in order to sterilize it, frozen, lyophilized and stored at +4ºC until further use.

2. PLMA-based sponges production

PLMA was dissolved in phosphate-buffered saline (PBS) (Sigma-Aldrich) with 0.5% (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) to final concentrations of 10%, 15% and 20% (w/v) of PLMA. Hydrogels were formed by pipetting the hydrogel precursor solution to 96-well plates (50 uL) followed by ultraviolet (UV) irradiation (95 mW cm^{-2}) during 60s. The obtained PLMA hydrogels were frozen at -80ºC and freeze-dried overnight in order to produce PLMA sponges with a diameter of 6mm and 2mm height.

3. PLMA-based sponges characterization

3.1. Structural properties by Cryo Scanning Electron Microscopy (CryoSEM)

PLMA sponges from 10% (PLMA10), 15% (PLMA15) and 20% (PLMA20) (w/v) of precursor PLMA solution were produced as described in Section 2. For CryoSEM analysis, the samples were re-hydrated 24 hours prior to image acquisition in water at room temperature. The SEM exam was performed using a High-resolution Scanning Electron Microscope with X-Ray Microanalysis and CryoSEM experimental facilities: JEOL JSM 6301F/Oxford INCA Energy 350/Gatan Alto 2500. The specimen was rapidly cooled and transferred under vacuum to the cold stage of the preparation chamber. The specimen was fractured, sublimated for 120 seconds at -90°C, and coated with Au/Pd by sputtering for 45 seconds with a 120mA current. The sample was then transferred into the SEM chamber. The sample was studied at a temperature of -150°C. CryoSEM images were analyzed by ImageJ software for pore size measurements.

3.2. Mechanical properties

The mechanical behavior of PLMA sponges was assessed by compression tests employing a Instron Universal Mechanical Testing Machine 3343 (Instron, USA) equipped with a load cell of 50 N. To this end, compression assays were performed at room temperature on as-prepared cylindrical sponges with a diameter of 6 mm and height of 2 mm re-hydrated in PBS overnight prior to mechanical testing. The compressive modulus was defined as the slope of the linear region of the compressive strain/compressive stress curve, corresponding to 0–5% compressive strain. Young's modulus values are expressed as means ± standard deviations ($n \geq 3$).

3.3. Swelling rate

PLMA10, PLMA15 and PLMA20 sponges were prepared as described in section 2, pre-weighted (W_0) and immersed in PBS at 37ºC. Swollen sponges were weighed at pre-determined time-points (W_1). The swelling rate was calculated as follows:

$$Swelling\ rate\ (\%) = [(W_1 - W_0)/W_1] \times 100 \quad (1)$$

Swelling rate values are expressed as means ± standard deviations ($n \geq 3$).

4. *In vitro* 3D cell culture

4.1. Stem cell isolation from adipose tissue

Human adipose derived stem cells (hASCs) were isolated from human adipose tissue following a previously reported protocol.[34] The liposuction tissue was obtained under a cooperation agreement between University of Aveiro and Hospital da Luz (Aveiro, Portugal).

Informed consent was obtained from the donor (52 years old female). The sample was transported in PBS supplemented with 1% (v/v) antibiotic/antimycotic (Thermo Fisher Scientific, USA) and stored at +4ºC until isolation. Lipoaspirate samples were washed with PBS and incubated at 37ºC in a shaking water bath for 45 minutes with a 0.1% (w/v) collagenase type I (MP Biomedicals, USA) solution. After incubation, the solution was centrifuged at 1200 rpm for 10 minutes. The supernatant was removed, and the pellet was resuspended in PBS and centrifuged at 1200 rpm for 10 minutes. The supernatant was then removed and the pellet containing the isolated hASCs was resuspended in Minimum Essential Alpha Medium (α-MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% antibiotic/antimycotic. Cells were cultured in a humidified 5% CO_2 atmosphere incubator at 37ºC. Culture medium was changed after 24h and then every 2-3 days.

4.2. *In vitro* hASCs culture

hASCs isolated from lipoaspirate samples were seeded on top of PLMA15 sponges (6mm diameter, 2mm height) at a density of 7×10^5 cells cm^{-2}. To each PLMA sponges, 10 µL of cell suspension were carefully placed on top of the scaffold. After 4 hours, α-MEM medium supplemented with 10% FBS and 1% antibiotic/antimycotic or α-MEM medium supplemented with 1% antibiotic/antimycotic was added. Cells were cultured during 14 days in a humidified 5% CO_2 atmosphere incubator at 37ºC. The culture medium was changed every 2-3 days. As a control, SpongeCol® (Advanced BioMatrix, USA) was used. hASCs were cultured on the top of SpongeCol® discs following the same procedure used for PLMA sponges.

4.2.1. Live/Dead assay

At pre-determined time-points (3, 7 and 14 days of cell culture) a Live/Dead assay was performed. PLMA and collagen sponges were incubated in a solution of 2 µL Calcein AM 4 mM solution in DMSO (Thermo Fisher Scientific) and 1 µL Propidium iodide (PI) 1 mg mL^{-1} (Thermo Fisher Scientific) in 1000µL of PBS during 30 minutes at 37ºC. After washing with PBS, sponges were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany). Image processing was performed using ZEN v2.3 blue edition software (Carl Zeiss Microscopy GmbH).

4.2.2. Cell morphology analysis

For cell morphology assessment, a DAPI/Phalloidin staining was performed. At pre-determined time-points PLMA and collagen sponges were washed with PBS and fixed with a

4% paraformaldehyde (PFA, Sigma-Aldrich) solution during at least 2 hours. A phalloidin solution (Flash Phalloidin™ Red 594, 300U, Biolegend, USA) 1:40 in PBS was prepared and the sponges were incubated at room temperature during 45 minutes. After washing with PBS, a DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Thermo Fisher Scientific) solution was diluted 1:1000 in PBS and the sponges were incubated during 5 minutes at room temperature. After washing with PBS, sponges were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss). Image processing was performed using ZEN v2.3 blue edition software (Carl Zeiss Microscopy GmbH).

4.2.3. Cell metabolic activity quantification

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) (MTS) was used to quantify cell metabolic activity. At pre-determined time-points, sponges were washed with PBS and incubated in a solution of MTS reagent diluted in PBS following manufacturer instructions. Samples were then incubated overnight at 37ºC, protected from light. The quantification was achieved by measuring absorbance at 490 nm (Microplate Reader - Synergy HTX with luminescence, fluorescence and absorbance, Biotek, USA). Triplicates were made for each sample and per culturing time. MTS values are expressed as means ± standard deviations (n = 5).

4.2.4. DNA quantification

Total DNA quantification was performed after cell lysis using a Quant-iT PicoGreen dsDNA kit (Thermo Fisher Scientific). At pre-determined time-points, sponges were washed with PBS, incubated in sterile deionized water and frozen at -80ºC. In order to induce disruption of the cells, the samples were thawed at 37ºC and placed in an ultrasounds bath at 37ºC during approximately 30 minutes. Samples were processed according to the specifications of the kit and DNA standards were prepared with concentrations ranging between 0 and 2 $\mu g\ mL^{-1}$ from the dsDNA solution provided in the kit. After 10 min of incubation in the dark at room temperature, fluorescence was measured using an excitation wavelength of 480 nm and an emission wavelength of 528 nm (Microplate Reader - Synergy HTX with luminescence, fluorescence and absorbance, Biotek). Triplicates were made for each sample and per culturing time. DNA values are expressed as means ± standard deviations (n = 5).

5. PLMA sponges mass loss quantification

Mass loss assays were carried out for PLMA15 sponges prepared as described in section 2. Pre-weighted (W0) scaffolds were immersed in PBS at 37ºC and PBS was replaced every 2 days. At pre-determined time points, PBS was removed, and the samples were freeze-dried. Afterwards, scaffolds were weighed (W_1) and mass loss was calculated as follows:

$$Mass\ loss\ (\%) = [(W_0 - W_1)/W_0] \times 100 \quad (2)$$

Mass loss values are expressed as means ± standard deviations (n = 5).

6. PLMA growth factors quantification

PLMA growth factors content was quantified by LEGENDplex™ Human Growth Factor Panel (13-plex) (Biolegend, USA) according to the manufacturer's instructions. This kit is a bead-based multiplex assay that allows the quantification of 13 different growth factors simultaneously: angiopoietin-2, epidermal growth factor (EGF), erythropoietin (EPO), basic fibroblast growth factor (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-SCF), hepatocyte growth factor (HGF), macrophage colony-stimulating factor (M-CSF), platelet-derived growth factor AA (PDGF-AA), platelet-derived growth factor BB (PDGF-BB), stem cell factor (SCF), transforming growth factor alpha (TGF-α), and vascular endothelial growth factor (VEGF). PLMA growth factors concentration values are expressed as means ± standard deviations (n = 3).

7. PLMA sponges protein release assays and quantification

For protein release assays, PLMA15 sponges were immersed in 5 mL of PBS and incubated in a bath at 37 °C with constant agitation (60 rpm). At each time point, an aliquot of 600 µL was taken from each sample and 600 µL of fresh PBS was added. Collected samples were stored at −20 °C until further use. Total protein quantification was performed with Micro BCA Protein Assay Kit (Thermo Fisher Scientific, USA). ELISA quantification of fibrinogen (Human Fibrinogen ELISA Kit, Abcam, UK) and VEGF (Human VEGF ELISA Kit, Abcam, UK) was also performed. Total protein and ELISA values are expressed as means ± standard deviations (n ≥ 3).

8. Statistical analysis

All data were subjected to statistical analysis and were reported as a mean ± standard deviation. Statistical differences between the analyzed groups were determine by *one-way ANOVA*. *Two-way ANOVA* was used for statistical analysis between two groups where $p < 0.05$

were considered as significant. All statistical analysis were performed using GraphPad Prism 8 software.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table of contents (ToC):

Protein-based materials, due to inherent biocompatibility/bioactivity, have emerged as alternatives for the fabrication of scaffolds. Moreover, concerns are raised regarding the use of animal-derived supplements due to risks of contaminants/antigens transmission and also due to ethical issues associated with collection methods. Therefore, we purpose a novel human protein-derived porous scaffold to be used as platform for xeno-free cell culture.