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A PLA/calcium phosphate degradable composite material for bone tissue engineering: an in vitro study

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Abstract Biodegradable polymers reinforced with an inorganic phase such as calcium phosphate glasses may be a promising approach to fulfil the challenging requirements presented by 3D porous scaffolds for tissue engineering. Scaffolds' success depends mainly on their biological behaviour. This work is aimed to the in vitro study of polylactic acid (PLA)/CaP glass 3D porous constructs for bone regeneration. The scaffolds were elaborated using two different techniques, namely solvent-casting and phase-separation. The effect of scaffolds' micro and macro-structure on the biological response of these scaffolds was assayed. Cell proliferation, differentiation and morphology within the scaffolds were studied. Furthermore, polymer/glass scaffolds were seeded under dynamic conditions in a custom-made perfusion bioreactor. Results indicate that the final architecture of the solvent-cast or phase separated scaffolds have a significant effect on cells' behaviour. Solvent-cast scaffolds seem to be the best candidates for bone tissue engineering. Besides, dynamic seeding yielded a higher seeding efficiency in comparison with the static method.

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1 Introduction

A tissue engineering scaffold's biological behaviour is, ultimately, its most critical property. The success of a tissue engineering implant, beyond issues such as availability of materials, ease of manufacture or costs, will always depend on it eliciting the appropriate biological reaction in vivo. The scaffold's porosity, pore interconnectivity, permeability, surface properties and chemistry will come into play to determine whether it can support cell attachment, growth and eventually cell differentiation into the appropriate tissue. In vitro studies must be performed with cell cultures in order to establish the scaffolds' basic biological interactions such as cytotoxicity, cell attachment behaviour, cell proliferation and cell differentiation.

In vitro cell cultures in two-dimensions (2D) are often used to assess material cytotoxicity or its influence on proliferation and differentiation. 2D configurations, however, have been shown to lead cells to completely different behaviours to those displayed in three-dimensions (3D) [1]. 3D cell cultures are complex to perform due to the difficulty in cell seeding, maintenance and monitoring. Indeed, cells can be either seeded throughout the scaffolds initially, or only on the exterior of the scaffold and allowed to migrate towards the interior during the culture. In both cases, cells growing in the interior of the scaffold must be able to receive nutrients and get rid of waste. Dynamic seeding or culture conditions can solve these issues, but they too involve a large degree of complexity and involve a larger risk of contamination [2–4].

This work is focused on the development of scaffolds for bone tissue engineering. Mature bone is produced by osteoblasts through a process of bone nodule formation or osteogenesis. This process has been subdivided into three

stages: proliferation, extracellular matrix (ECM) development and maturation, and matrix mineralisation. During the first two stages, cells undergo mitosis, increase in number, and produce proteins associated with the ECM such as type I collagen or fibronectin. After the down-regulation of proliferation, proteins associated with the osteoblastic phenotype can be detected. At the beginning of mineralisation all the cells in the culture produce alkaline phosphatase (ALP). Following the onset of mineralisation, other proteins such as bone sialoprotein, osteopontin (OP) and osteocalcin (OC) are induced [5].

ALP is an early differentiation marker associated with calcification. It provides localised enrichment of inorganic phosphate, one of the components of the mineral phase of bone [6]. Osteocalcin is a vitamin-K dependent protein, which, unlike osteopontin and other proteins, is mainly expressed post-proliferatively upon nodule formation. Due to the late expression of OC, it is considered a marker of osteoblast maturation and is believed to have the ability to chelate calcium ions to form bone minerals and play an important role in the bone formation—resorption sequence [7–9]. In the laboratory, osteogenesis can also be demonstrated by the expression of ALP, OP, OC, collagens or mineralisation nodules [10].

The choice of cell source has an enormous influence on the assessment of differentiation due to differences in cell behaviour such as marker expression or calcification [11]. Cells from stable osteosarcoma cell lines such as the MG63 and the SAOS-2, offer the advantages of stability, reproducibility and ease of comparison with other studies. Furthermore, their immortality allows almost unlimited passages and thus enables high flexibility for assay planning [12–14]. Their proliferative and differentiative properties, however, can be somewhat aberrant or distorted. Primary cell sources, on the other hand, offer real cell behaviour, although studies are subject to the variability and singularities of the individual source. These primary osteoblasts can be derived from different animal (rat, mouse, dog etc.) [15–18] or human sources [19–21].

Various studies have been performed seeding osteoblasts or osteoblast-like cells onto degradable polymeric scaffolds [20, 22, 23] in order to assess the scaffolds' potential to support cell growth and differentiation. These studies typically involve superficial cell seeding, culture in static conditions and the use of osteosarcoma cell lines, which simplify comparisons. The scaffold microstructure and surface roughness have been found to affect cell proliferation in vitro [23–26]. The microstructure in fact determines not only whether the cells can fit and attach in the structure, but also whether they have access to the nutrients in the medium. Scaffolds microstructure together with an even cell distribution and high cell density throughout the scaffold are also crucial to attain a functional tissue within a 3D construct. Dynamic culture conditions in a bioreactor under perfusion or low pressure have also been shown to improve and facilitate cell seeding and cell growth within porous scaffolds, and to tailor differentiation [27, 28].

The objective of this study was twofold, (a) to determine the biological properties of a polymer/glass composite scaffold by assessing the effect of its architecture and composition on cell behaviour, and (b) to evaluate a dynamic seeding system for further optimization of the seeding method. Solvent cast and phase-separated scaffolds, containing 0% or 50% wt% of calcium phosphate glass particles, were tested. A complete study using MG63 osteoblast-like cells was performed, assessing cell proliferation, differentiation and morphology within the scaffolds.

2 Materials and methods

2.1 Scaffold processing

The scaffolds are a composite of Poly-95L/5$_{DL}$-lactic acid (PLA) and a titania-stabilised, completely degradable, calcium phosphate glass [29]. They are manufactured with two processing techniques; a solvent-casting salt-leaching method and phase separation. A detailed description of scaffold processing has been described in a previous study [30].

Solvent Casting: A 5% (w/v%) PLA solution in chloroform was mixed with sieved sodium chloride (NaCl) measuring 80–210 μm, and if needed glass particles at 50 wt%, measuring <40 μm. The paste was cast into teflon moulds until complete chloroform evaporation. Cylindrical samples are punched out of the moulds and placed in distilled water for 48 h. After this time the NaCl particles have been leached out of the cylinders and leave behind a porous network.

Phase Separation: A 5 w/v% PLA solution in a mixture of 95% (v/v%) dioxane solution in water was prepared. If needed, glass particles at 50 wt% are added to the mixture and cast in cylindrical teflon moulds. The mixture was then quenched at −20°C. The dioxane was then eliminated by soaking the scaffolds in an ethanol bath. The composition and properties of the scaffolds are listed in Table 1.

2.2 Scaffold permeability

The permeability of the solvent cast scaffolds with 50 wt% of glass particles was measured at a constant water pressure

Table 1 Compositions and porosities of the scaffolds used in the MG63 cell culture assay

	0% glass solvent casting	50% glass solvent casting	0% glass phase separation	50% glass phase separation
PLA w/v%	5	5	5	5
Solvent used	Chloroform	Chloroform	Dioxane + H_2O	Dioxane + H_2O
Glass wt%	0	50	0	50
Glass particle size (μm)	<40	<40	<40	<40
NaCl particle size (μm)	[80–120]	[80–120]		
Approximate Pore size (μm)	[80–120]*	[80–120]*	[80–200]	[80–200]
Porosity (%)	94	95	89	90
Stiffness	300 kPa	190 kPa	4.72 MPa	7.10 MPa

* The pore size of the solvent cast scaffolds cannot be measured directly, due to the extremely high of the structure, adjacent pores often overlap and the real pore sizes are substantially higher

with an in-house constant head permeameter. Considering Darcy's law [31], samples were perfused with distilled water under a pressure gradient of 2.5 kPa. The fluid flow was measured over two hours and used to calculate the permeability coefficient k [m^2]. The scaffolds measured 6 mm in diameter and 12 mm in height. Samples were pre-wetted before each measurement; they were dipped in 30% solution of ethanol, rinsed in distilled water and kept in distilled water for at least 24 h. The pretreatment of the scaffolds has been validated by testing the permeability after pre-wetting for a period of 1, 3, 6, and 9 days. The different groups were analyzed by ANOVA to determine statistically significant differences.

2.3 MG63 Cell Culture

The cell culture study was performed with MG63 osteoblast-like cells on scaffolds with four different compositions and processing techniques: (a) solvent cast scaffold without glass (0%C), (b) solvent cast scaffold with glass (50%C), (c) phase-separated scaffold without glass (0%D) and (d) phase-separated scaffold with glass (50%D) (Table 1). The scaffolds measured 10 mm in diameter and 2 mm in height. They were sterilised prior to cell culture with gamma-radiation at 8 kGy.

2.3.1 Static cell seeding

200,000 MG63 osteblast-like cells were seeded per scaffold. The scaffolds were preconditioned by soaking in DMEM cell culture medium overnight. The cells were seeded in static conditions by injecting them with a syringe, loaded with 1,000 cells/μl at two points on the surface of the scaffolds. The scaffolds were then placed in multiwells in complete DMEM cell culture medium, and cultured for 21 days. The medium was changed every 3 or 4 days.

2.3.2 Cell proliferation

Cell proliferation was monitored by measuring Lactate Dehydrogenase (LDH) readings and by measuring the total protein content. The LDH readings were performed with a LDH Cytotoxicity Detection Kit (Roche). The scaffolds were transferred into new multiwells, and 500 μl of DMEM without pyruvate and with only 1% serum was added. (Both the pyruvate and the serum interfere with LDH readings). The cells were then frozen and thawed thrice in order to ensure they were all dead. The supernatant was then collected, centrifuged to remove debris which could hamper the spectrophotometric readings, and incubated. The absorbance of the incubated mixture was measured at 450 nm with a reference wavelength of 600 nm on a PowerWaveX Bio-Tek Instruments Spectrophotometer. Fresh medium and medium which had been incubated in the presence of scaffold without cells were used as controls. Cell proliferation was measured at days 1, 7, 14 and 21 of culture.

The total protein concentration was measured with a BCA Protein Assay kit (PIERCE). The reaction product of the assay is purple-coloured and can be read at 562 nm. The test was performed by adding 25 μl of Mammalian Protein Extraction Reagent (MPER) supernatant to 200 μl of the kit's Working Reagent. The mixture was incubated for 30 min at 37°C and read at 562 nm. The total protein concentration was measured at days 7, 14 and 21 of culture.

2.3.3 Cell differentiation

Cell differentiation was monitored by measuring the ALP and OC release from the cells. Both the ALP and the OC activity was normalised with the LDH readings. The ALP activity was measured with a Phosphatase, Alkaline Acid, Prostatic Acid assay kit (SIGMA Diagnostics). ALP was measured at days 7, 14 and 21 of culture in triplicate. Scaffolds were rinsed twice in phosphate buffered saline

(PBS), soaked in 500 μl of MPER, and cut with a scalpel in order to facilitate MPER penetration into the structure. The MPER is meant to sweep up the cells from the scaffolds. The readings were taken at 405 nm on a PowerWaveX Bio-Tek Instruments Spectrophotometer.

Osteocalcin concentration was measured by means of a Metra® Osteocalcin kit (Quidel Corporation), which is a competitive immunoassay. OC was measured on day 21 of culture in triplicate. 400 μl of supernatant of from the scaffold culture wells were centrifuged to avoid debris. The supernatant was then analysed and the results were normalised with the number of cells as given by the LDH readings.

Each composition was tested in triplicate for both the proliferation and the differentiation assays.

2.3.4 Cell-scaffold morphology

The morphology of the cell-scaffold construct was visualised using several techniques: stereomicroscopy, histological sections, confocal microscopy and SEM.

2.3.5 Stereomicroscopy

Scaffolds were harvested at days 1, 7, 14, and 21 of culture, rinsed in PBS and stained with ethylene bromide and acridine orange. They were then viewed under a MZ16F Leica Stereomicroscope.

2.3.6 Histological sections

Histological sections of scaffolds stained with methylene blue and included in paraffin were performed after 21 days of culture.

2.3.7 Confocal microscopy

Samples of scaffolds after 21 days of culture were viewed with a Leica TCS40 confocal miscroscope. The scaffolds were fixed with paraformaldehide 4% and were stained with phalloidin and Hoechst. Phalloidin stains the actin filaments of the cells in red and Hoechst stains the nuclei in blue.

2.3.8 Scanning electron microscopy

Samples of the scaffolds after 21 days of culture were viewed under a Strata BD235 High-Resolution SEM microscope (FEI). Samples were fixed with glutaraldehide 2.5% and critically point dried and gold-sputtered prior to visualisation.

2.3.9 Dynamic seeding

It was conducted by placing the solvent cast scaffolds in the perfusion system, where two chambers are located in one pump channel (ISMATEC IPC 8 channel peristaltic pump) holding one scaffold each. Using two channels, 4 scaffolds of 6 mm diameter and 12 mm height were perfused for every experiment. 10 ml of cell suspension containing 600,000 MG63 human osteoblast-like cells was inserted per channel (resulting in initial cell number of 300,000 cells/scaffold) and pneumatically forced to oscillate through the chambers. 465 cycles were completed for cell seeding velocities of 1 and 10 mm/s. Seeding time was 18 h for low velocity and 1.8 h for high velocity. The perfusion was conducted with frequencies of 0.007 and 0.07 Hz respectively, allowing to push the cell suspension in the bottom of the system through the scaffolds in every cycle. After perfusion, the scaffolds were incubated for 4 h and frozen with 1 ml assay medium in cryotubes. As control MG63 human osteoblast-like cells were injected with a syringe into the scaffolds. Cells were suspended to 600.000 cells/ml in assay medium (pyruvate-free DMEM with 1% FBS). 500 μl suspension was injected in every scaffold ($n = 4$), yielding an initial cell number of 300,000 cells/scaffold. After 4 h of incubation in assay medium scaffolds were frozen with 1 ml assay medium in cryotubes.

For cell quantification, samples were frozen and thawed three times to disrupt the cells' membrane. Concentration of liberated lactate dehydrogenase (LDH) was measured using a colorimetric test (Cytotoxicity Detection Kit/ROCHE). Afterwards, scaffolds were cut longitudinally with a scalpel. Cell distribution was observed by Acridine Orange staining of the cells (solution 100 mg/ml) and inspected by fluorescence microscopy (Nikon Eclipse 600A with FITC Filter, 4× magnification).

3 Results

3.1 Permeability

Prewetted sample groups (1, 3, 6 and 9 days) were measured over 2 h (see Fig. 1). ANOVA analysis that was conducted to compare each group ($n = 5$ for 1, 3, 6 and 9 days prewetting) at every time-point. No statistically significant differences were observed ($p < 0.05$). After reaching stable state at 2 h perfusion the permeability coefficient was calculated for each group (see Table 2). Using the data of 20 samples the permeability coefficient was calculated as

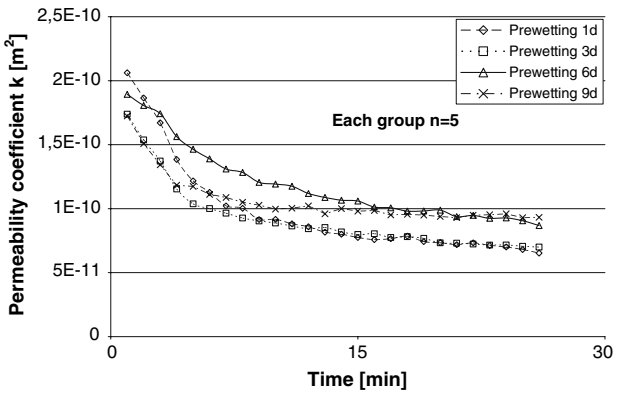

Fig. 1 Permeability measurements for 4 different pre-treatment periods for 5 samples per group over 2 h

Table 2 Permeability coefficient measured in stable state after 2 h for 4 different pre-treatment periods for 5 samples per group

Prewetting period (days)	Permeability Coefficient, k (m^2)	Standard deviation (m^2)
1	6.78E−011	5.59E−012
3	7.07E−011	2.15E−011
6	9.01E−011	2.63E−011
9	9.41E−011	2.76E−011

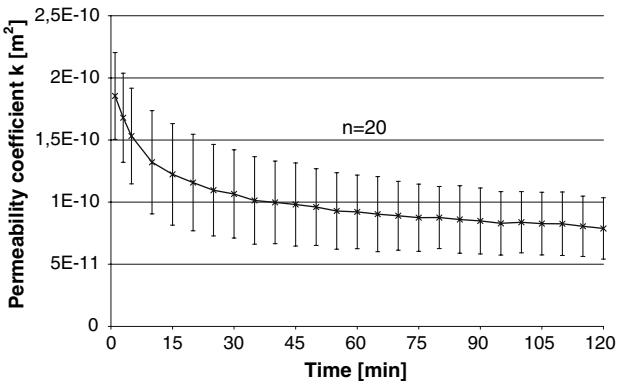

Fig. 2 Permeability measurements of all samples ($n = 20$). Permeability drops over 50% and reaches stable state after 2 h. Standard deviation for all time points is depicted

$8.1 \times 10^{-11} \pm 2.4 \times 10^{-11}$ m^2 (Fig. 2). It was observed that the mean values dropped more than 50% over 2 h showing a standard deviation of about 30% (Fig. 2).

3.2 Cell proliferation

The LDH readings indicate that the MG63 cells proliferated on the scaffolds during the 21 days of cell culture. For all compositions, cell proliferation increases from day 1 to 7 and then remains stable until day 21; the differences in proliferation between day 7, 14 and 21 are not statistically significant for any composition. In the case of the solvent cast scaffolds, the composition without glass, 0%C, sustained slightly higher cell proliferation than the 50%C (Fig. 4). For the phase-separated scaffolds, proliferation levels were similar with and without glass throughout the assay (Fig. 4). Comparing fabrication techniques, the phase-separated scaffolds induced less proliferation during the first week of cell culture, from then on proliferation levels are similar for both types of scaffold.

The total protein content results on all compositions peaked on day 14 and then decreased on day 21 (Fig. 5). The solvent cast scaffolds gave a higher protein concentration that the phase-separated ones. The differences between compositions with and without glass were not statistically significant for both types of scaffold.

3.3 Cell differentiation

The ALP results were normalised with the LDH readings in order to have a measure of cell differentiation during the cell culture period. Figure 6 shows the results of the ALP/LDH ratio for all compositions. The solvent cast and phase-separated scaffolds show markedly different trends. At 7 days of culture, the cells on the phase-separated scaffolds are at their maximum differentiation level and the levels of ALP activity decreases thereafter. The solvent cast scaffolds on the other hand, attain their maximum level of ALP activity at 14 days of cell culture and the level decreases at 21 days. There is a large difference between compositions with and without glass particles. Compositions with glass (50%C and 50%D) sustain much higher ALP activity values than those with only PLA (0%C and 0%D).

The osteocalcin concentration was measured at day 21 as a late differentiation marker. The OC concentration was normalised with the LDH readings on day 21. The results show no statistical differences between the compositions nor the scaffold types.

3.4 MG63 Cell–scaffold morphology

The stereomicroscope images of the MG63 cells stained with ethidium bromide and acridine orange reveal the distribution of the live cells within the scaffold structure (Fig. 7a). Qualitatively, the phase-separated scaffolds seem to have a higher density of cells seeded on their surface (images are brighter) than the solvent cast scaffolds. Close-up views of the surface of the scaffolds confirm this inference. In all cases, the cells adapt to the porosity of the scaffolds and have spread within the scaffold architecture (Fig. 8).

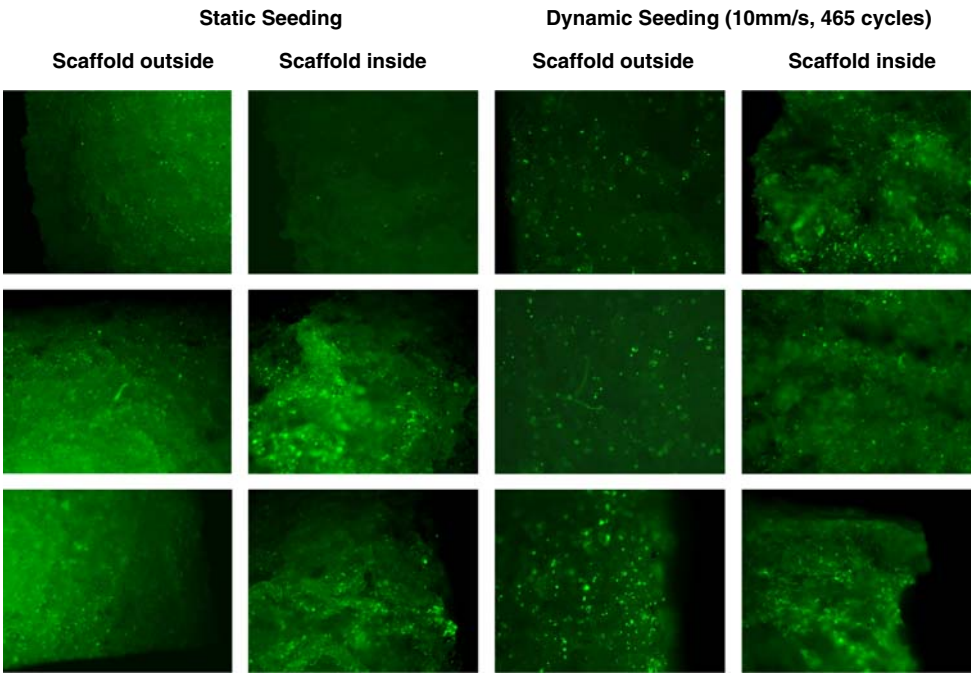

Fig. 3 MG63 cells seeded in the studied scaffolds using static and dynamic method. Acridine Orange staining shows cells located in the different scaffold areas marked as light green spots

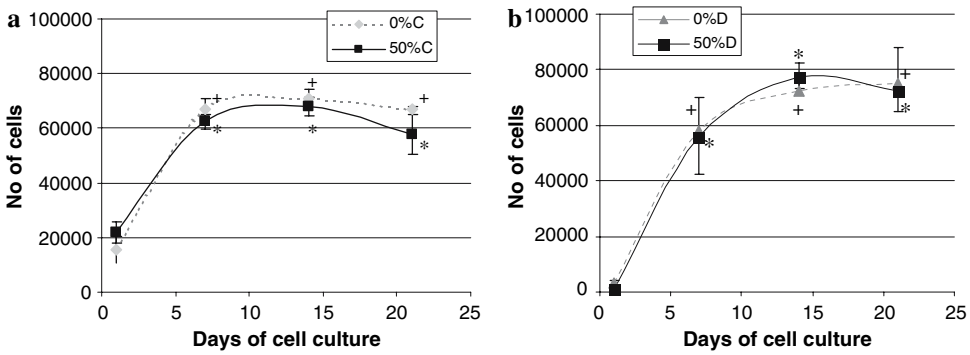

Fig. 4 MG63 proliferation measured by LDH on the solvent cast (**a**) and phase-separated (**b**) scaffolds with and without glass particles (50%C and 0%C, and 50%D 0%D respectively) during the 21 days of cell culture. *, +: the differences between readings on day 7, 14 and 21 are not statistically significant

The histological sections confirm that the cells are densely coated on the exterior of the phase-separated scaffolds (Fig. 7b). Indeed, compositions 0%D and 50%D exhibit a thick layer of cells on their surface which seem to be colonising the porosity close by. At higher magnifications cells can be seen to attach to the scaffold structure and specifically to glass particles (Fig. 9).

Confocal microscopy shows the cells growing on the scaffolds in 3D. The nuclei are stained in blue and the actin filaments of the cells appear red. The scaffold material was viewed in reflection and is grey in the images. Images of the surface of the scaffold reveal a dense layer of coated cells (Fig. 10).

SEM images complemented the previous qualitative analysis by stereomicroscope, confocal microscopy and histological sections. Figure 7c shows the MG63 cells growing on the surface of the scaffolds after the 21 days of cell culture. Despite the fact that either the sample preparation treatment (dehydration and critical point drying) or the high vacuum within the SEM broke or tore some of the cell structures, the original structure of the cells is clearly visible. As had been noted previously, the cells form a thick layer on the phase-separated scaffold surfaces. They seem to be growing more sparsely on the solvent cast scaffold surfaces, where the underlying porosity is still visible. Few cells were found in the interior of the phase-separated scaffolds during the SEM analysis, whereas the solvent cast scaffolds harboured large colonies of MG63 cells surrounded by their extracellular matrix (Figs. 7d and 11).

3.5 Dynamic seeding

Measurement of cell numbers resulted in 29,730 cells for control and 108,300 and 163,900 cells for dynamic seeding with 1 and 10 mm/s seeding velocity respectively. The seeding efficiency was 9.9 ± 2.2% for static, 36.1 ± 4.6 and 54.6 ± 12.5% for dynamic seeding compared to the

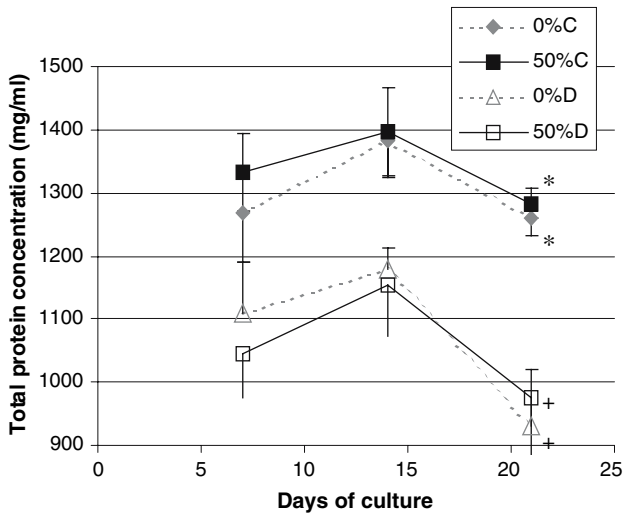

Fig. 5 Total protein content of the MG63 cell cultures on the solvent cast (C),and phase-separated (D), scaffolds with and without glass particles (50%D and 0%D respectively) during the 21 days of cell culture. *, +: the differences between 0%C and 50%C, and 0%D and 50%D are not statistically significant

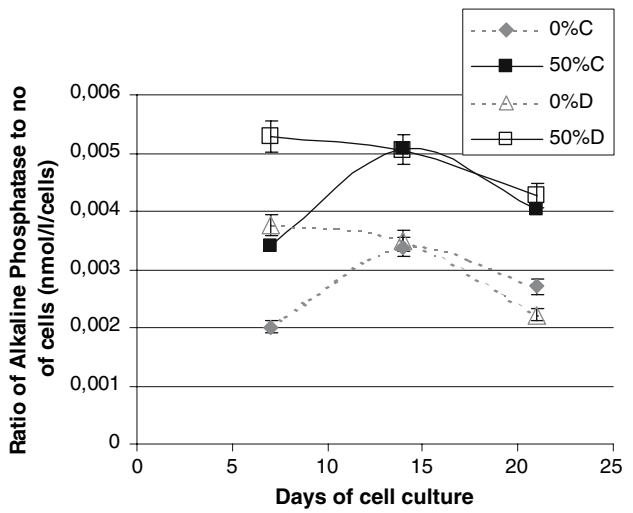

Fig. 6 MG63 differentiation results illustrated as the ratio between Alkaline Phosphatase concentration and the number of cells measured by LDH. The samples include solvent cast (C),and phaseseparated (D), scaffolds with and without glass particles (50%D and 0%D respectively) during the 21 days of cell culture

initial cell number. Cell distribution was assessed by fluorescence microscopy. Dynamic seeding showed a more homogenous distribution on the inside and outside of the scaffolds compared to static seeding (see Fig. 3).

4 Discussion

The cell culture study described in this study has characterised the properties of the scaffolds as supports for cell

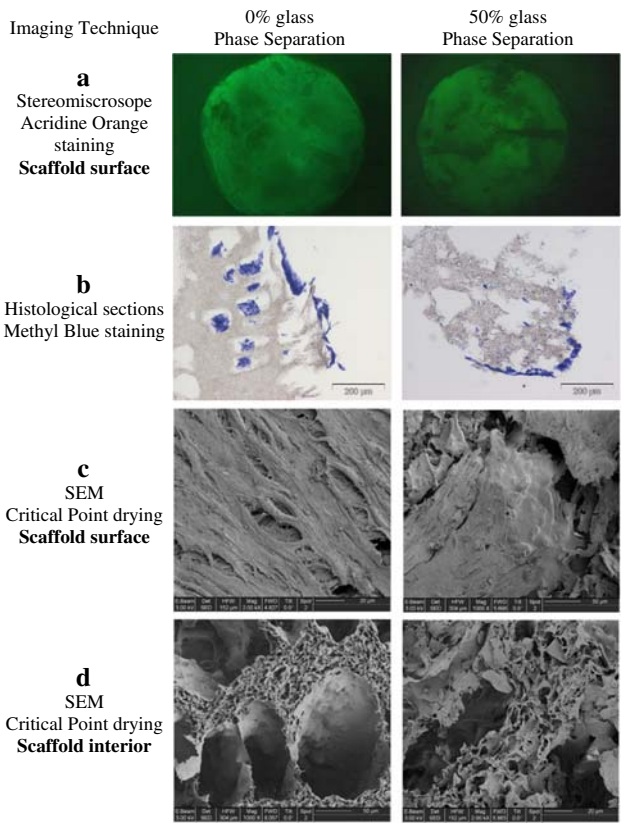

Fig. 7 Images of the MG63 cells seeded on the scaffolds viewed with different imaging techniques: (**a**) Stereomiscroscope, (**b**) Histological sections, (**c**) and (**d**) SEM. The MG63 cells proliferate on both scaffolds. In the case of the solvent cast scaffolds, they penetrate towards the interior. In the case of the phase-separated ones, they tend to form a thick crust on the scaffold surface

growth. The non-cytotoxic nature of the PLA and the calcium phosphate glass materials had been previously verified [30], and their surface properties had been characterised [32]. The objective of this study was to assess the scaffolds' potential as templates for cell attachment, migration, proliferation and differentiation. The results prove that cells are able to attach, migrate towards the interior, proliferate and differentiate on the scaffolds. Furthermore, the presence of glass particles seems to enhance cell differentiation.

The MG63 cell cultures were carried out during 21 days, and their morphology, proliferation and differentiation were characterised. Various microscopy techniques were used to perform a qualitative analysis of the cell-scaffold morphology. All imaging techniques seemed to indicate that the cells in the solvent cast scaffolds tended to spread towards their interior. In the case of the phase-separated scaffolds, the cells tended to remain on the surface scaffolds and form a thick layer there. Indeed, Fig. 7 shows a higher concentration of cells on the surface of the phase-separated scaffolds. This qualitative observation is logical if one takes into account the differences in scaffolds morphology.

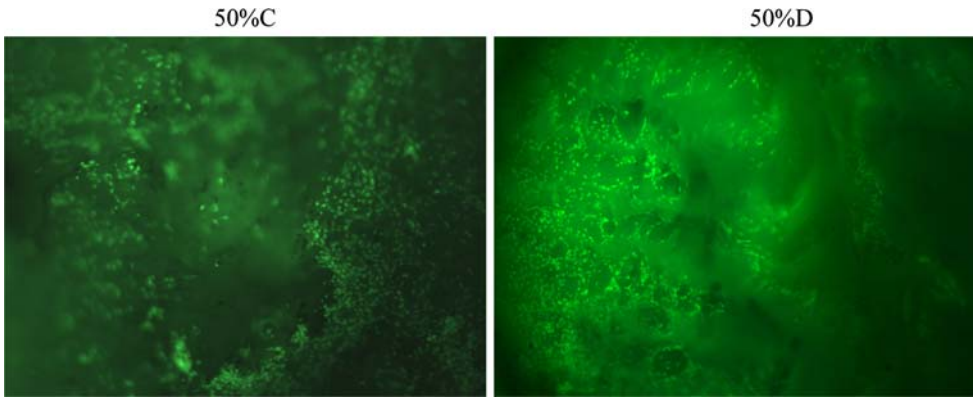

Fig. 8 Stereomicroscope images of live MG63 cells seeded on the scaffolds after 14 days of culture. The images show how the cells adapt to the porosity and architecture of each scaffold.

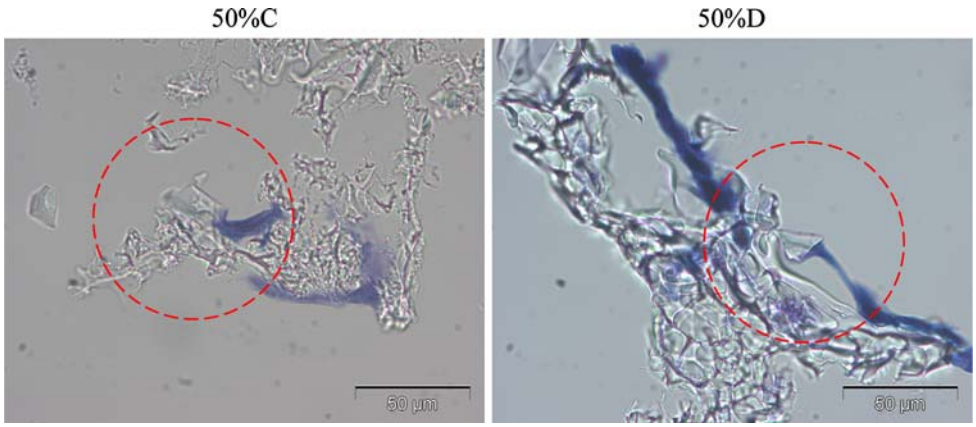

Fig. 9 Histological sections of the scaffolds with glass particles after 21 days of cell culture. The images show the MG63 cells attaching directly on the glass particles (red dotted circles)

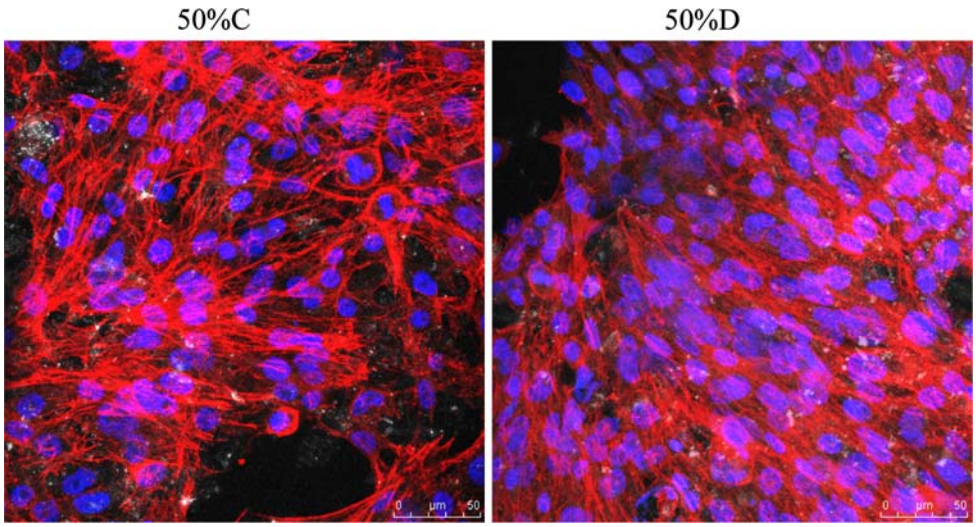

Fig. 10 Confocal microscope images of the MG63 cells on the surface of the scaffolds made of PLA and glass after 21 days of culture. The cell nuclei are stained blue and the actin filaments appear red. (Scale bars correspond to 50 µm)

The solvent cast scaffolds are more porous and much less stiff (Table 1) than the phase-separated scaffolds. This could influence the cell growth pattern in several ways. Firstly, cells may not penetrate the phase-separated structure well during static seeding, thus they remain on the surface of the scaffold, or in a localised area within the scaffold and proliferate there. Blaker et al. [33] also report higher presence of cells on the scaffold surface than in the deep interior after static seeding. Perhaps this is the case with the solvent cast scaffolds as well, but the solvent cast scaffold structure allows cells to easily colonise the interior of the scaffold by invading the porosity [34]. Davies et al. [10] postulate ideal pore sizes for cell invasion: pores measuring less than 200 µm become occluded by cells, and

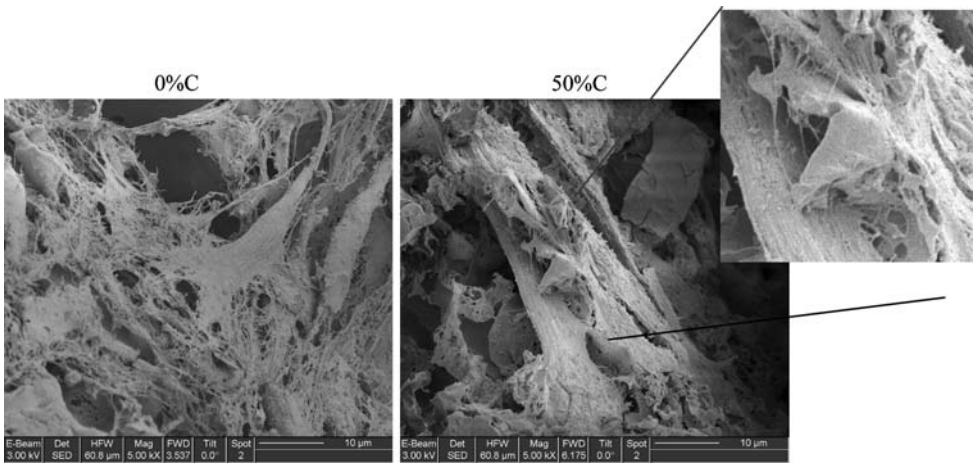

Fig. 11 SEM images of the interior of the solvent cast scaffolds. These images show details of how the cells stretch and adapt to the porosity of the scaffolds. In the case of the scaffold with glass (50%C), the cell processes attach directly onto the glass particles (see close-up in upper right-hand corner).

pores larger than 500 μm induce tissue in growth. The solvent cast scaffold pore-size cannot be calibrated exactly, but it can be assumed to be larger that 500 μm. Indeed, the high porosity and interconnectivity caused by adjacent NaCl particles, induce a very open interconnected porosity [35]. In the case of the phase-separated scaffolds, the lower porosity, probable lower pore interconnectivity, smaller real pore size (<200 μm) (Table 1) and higher stiffness may have prevented many of the cells from penetrating the structure. Finally, perhaps cells do attain the interior of the phase-separated scaffolds during seeding, but do not receive enough nutrients, when lodged in the scaffold, in order to survive.

It is important to underline the fact that static cell seeding of the solvent cast scaffolds induced cell penetration and survival in the interior of the scaffolds during 21 days. This result is fundamental. Indeed, the architecture of solvent cast scaffold allows for cells to invade and proliferate within the structure without external assistance. The permeability readings further confirmed that the scaffolds have very good interconnectivity.

The permeability values obtained for the solvent-cast scaffolds is comparable to that found by Chor and Li [36] who showed a permeability of 2×10^{-10} m^2 for polymer scaffolds (PVA, 150 μm pore size, Porosity 60%. Scaffolds' fabrication methods strongly affect their permeability values. For instance, Lee et al. [37] obtained a permeability of $2 \cdot 10^{-11}$ m^2 using rapid prototyping methods to elaborate Poly(Propylene Fumarate) scaffolds with 100% open pores (size 300 μm) and a rather small porosity of 19%.

Pore interconnectivity was quantitatively characterised at 99% by image analysis of 3D microtomographies [35]. This implies high chances of cells being able to perform similarly in in vivo conditions given that the cells will receive at least more nutrients through fluid flow in those conditions.

The quantitative results of cell proliferation also reveal differences between the phase-separated scaffolds and the solvent cast ones. LDH proliferation results for day 1 indicate that fewer cells are seeded on the phase-separated scaffolds than on the solvent cast ones (Fig. 4). The total protein content at day 7 is also substantially lower for the phase-separated scaffolds (Fig. 5). Proliferation results at 14 and 21 days, however, show the cells on the phase-separated scaffolds overtake those on the solvent cast scaffolds in number in the second half of the assay. Thus, fewer cells remain seeded on the phase-separated scaffolds, but those that do remain seem to proliferate at a higher rate than on the solvent cast scaffolds.

Both types of scaffolds suffered a decrease in proliferation at 21 days which can be associated to cell differentiation. In fact, ALP activity levels can be read since day 7 and day 14 on the phase-separated and solvent cast scaffolds respectively (Fig. 6). Indeed, the ALP attains it maximum on the phase-separated scaffolds at day 7, and then decreases. This behaviour coincides with the lower proliferation of the cells on the phase-separated scaffolds during the first weeks of culture. The ALP maximum is reached on day 14 for the solvent cast scaffolds and then decreases on day 21, coinciding as well with the slowing down in proliferation rate. Thus, it seems phase-separated scaffolds induce cell differentiation faster than solvent cast ones. The clearest effect on ALP activity, however, is the glass content. The differences between compositions with and without glass are marked on Fig. 6. Though each type of scaffold follows the previously described differentiation rate, the concentration of ALP is higher for the compositions with glass.

The ALP results are not corroborated by the OC readings however. There are no significant differences between the OC readings between the different scaffold types and compositions on day 21. The absence of differences could be due to the OC being released later in the differentiation

cycle (after day 21) or because all scaffolds have induced similar OC concentrations. Indeed, other studies using MG63 find no differences in the OC readings between their different materials. Price et al. [38] find equal OC readings on Bioglass®, polystyrene, titanium and cobalt-chrome substrates, and interpret that the OC had reached maximum levels or vitamin K was a limiting factor. Navarro [39], working with the same materials and cells as this study, finds no differences in the OC concentration between PLA and PLA and calcium phosphate glass materials after 11 days of culture, although the ALP readings had shown the glass favoured cell differentiation. Navarro proposes the OC, being a late marker, has not been released yet, or that differentiation should have been further stimulated with dexamethasone or ascorbic acid in the medium. Furthermore, Wang and Zhang [6] find similar OC levels on all their materials after 21 days of culture, whereas there were significant differences at 14 days.

Thus, the OC results may be incomplete, and should be measured on all the days of the study in order to have a complete characterisation. As such, they can only indicate that all the scaffolds have a similar OC level at day 21. There is, however, some discussion on the validity of OC readings with MG63 cells. Some authors argue that in the case of MG63 cells OC does not represent a valid reference parameter for cell phenotype, but rather a marker of cell functionality alone [40].

Overall, the ALP concentration results and the trends in proliferation seem to indicate that the presence of glass particles in the scaffolds enhances the differentiation of the MG63. These results are in accordance with the literature on calcium phosphate glasses [41–44]. In addition, the phase-separated scaffolds seem to increase and accelerate differentiation of MG63 cells as opposed to the solvent cast ones. Thus, the higher stiffness, the pore shape or the roughness of the phase-separated scaffolds somehow favour MG63 differentiation. Indeed higher roughness has been found to reduce MG63 proliferation and enhance differentiation in other studies [45]. The effect of the phase-separated scaffolds on cell differentiation could also be due to their growth in thick layers on the surface of the scaffolds, they may have attained confluence which could lead to cell differentiation.

This study includes some limitations. First of all the MG63 cells is a cell line from an osteosarcoma, thus they present the advantages of cell lines: less variability, high levels of proliferation and easy handling. The use of the immortal cell lines, however, should be considered a first step in the biological characterisation of the scaffolds, to be complemented and amplified with primary cell sources. Indeed primary cell sources, offer less reproducibility but are more realistic when it comes to characterising cell profileration and especially differentiation.

Concerning the cell seeding protocol, static cell seeding is a quick and simple seeding method. The instruments used to manipulate the scaffolds were sterilised in absolute ethanol before each seeding, and no toxicity problems were encountered.

Concluding from the cell number initial present and the number of cells residing in the scaffolds, the dynamic seeding methods yields a higher efficiency compared to the static seeding. The distribution of the cells is more even in the dynamically seeded scaffolds. Taking into account that for further culture of the cells for in vitro tissue engineering an even distribution is favourable for homogenous tissue development the perfusion seeding is more appropriate than the static seeding.

In order to establish proper differentiation results, more markers should be evaluated during the cell culture length. Based on this study, the ALP readings should be performed from day 1 of cell culture for example, and the OC should be evaluated throughout the study. Other markers as Collagen I and Ca^{2+} deposition can be determined, but the high porosity of solvent-cast samples made difficult to perform histologically staining due to the loose of material when making the thin cuts. Furthermore, the effects of fluid flow and fluid flow patterns on the long-term cultivation of cells in scaffolds, different velocities and seeding cycle values on cell seeding should be further explored and assessed.

5 Conclusions

- The solvent cast and phase-separated scaffolds both sustain osteoblastic cell growth, migration, proliferation and differentiation.
- The phase-separated scaffolds enhance the creation of a thick layer of cells and extracellular matrix on their surface that occludes the underlying pores. The colonisation of the interior of the scaffold is slower than for solvent cast ones.
- Solvent cast scaffolds are easily colonised by the cells that are distributed within the scaffold pore structure, and produce extracellular matrix inside the scaffolds.
- The presence of glass particles enhanced the differentiation of the MG63 cells.
- The phase-separated scaffolds sustained more and earlier differentiation of MG63 cells than the solvent cast ones.
- Cells proliferated within the scaffold structure in the absence of dynamic culture conditions.
- The results of this cell culture study seem to indicate that solvent cast scaffolds would be the best candidates for bone tissue engineering, due to their ability to sustain cell growth and in-growth in the absence of dynamic culture conditions. The higher differentiation

induction by the phase-separated scaffolds is outweighed by the ease of manipulation of the former.
- Dynamic seeding methods yields a higher efficiency compared to the static seeding.
- The developed perfusion system elevated seeding efficiency, indicating a good perfusion that is beneficial for further cell culture.

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