Development and Characterisation of Completely Degradable Composite Tissue Engineering Scaffolds

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Chapter 6. 3D Cell Culture in solvent cast and phase-separated scaffolds: proliferation, differentiation and morphological characterisation.

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, 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 vivo* studies in animals and eventually in humans are part of the protocols necessary to approve implants for medical applications. Prior to *in vivo* studies, however, *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 offer the advantage of studying the scaffolds under very specific conditions, which can be easily reproduced and compared, as opposed to the highly complex environment present in living organisms. *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]. Thus, although 2D cell cultures are a useful first step in assessing the suitability of materials for tissue engineering, a 3D configuration is necessary in order to observe cell behaviour in relatively realistic conditions. 3D cell cultures are complex to perform due to the difficulty in cell seeding and maintenance. 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].

As described in the Introduction, Chapter 1, the study of scaffolds for bone tissue engineering must involve cell culture studies using osteoblasts or cells from the osteoblastic cell lineage: cells that produce bone. 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. The expression of this enzyme is needed before matrix mineralisation. 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[5;7;8]. In the laboratory, when assessing biomaterials, osteogenesis is often demonstrated by the expression of ALP, OP, OC, collagens or mineralisation nodules[9]. 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[10].

Common cell systems employed in these studies include: primary cultures of osteoblastic cells derived from several sources (including mesenchymal stem cells), established clonal cell lines isolated from bone tumours (often osteosarcomas), non-transformed cell-lines and bone marrow cultures. Cells from stable osteosarcoma cell lines such as the MG63 and the SOAS-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 [11-13]. 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.) [14-17] or human sources [18-20]. Human bone mesenchymal stem cells (MSCs) can differentiate along multiple lineages such as osteoblasts, chondrocytes, adipocytes and haematopoiesis-supportive stroma. One of the challenges in their expansion in vitro however, is the maintenance of their phenotype. Furthermore, their expansion is limited because the cells tend to senesce and lose their multidifferentiation potential with time in culture [18;21-23].

Various studies have been performed seeding osteoblasts or osteoblast-like cells onto degradable polymeric scaffolds [19;24;25] 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 [25-28]. 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. The presence of bioactive substances such as bone morphogenetic protein, demineralised bone matrix, calcium phosphate ceramic or ascorbic acid in the material have been shown to enhance osteoblastic differentiation and bone matrix production [14;16]. Dynamic culture conditions in a bioreactor under perfusion or low pressure have also been shown to tailor differentiation [4;29;30].

The objective of this study was to determine the biological properties of the scaffolds developed during this thesis by assessing the effect of scaffold architecture and composition on cell behaviour. The 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. A preliminary study

using primary human mesenchymal stem cells (MSCs) was also performed, as a complement to the first study.

Materials and Methods

The cell culture study was performed on scaffolds with four different compositions and processing techniques (Table 6.1): 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). (Details on the solvent casting and phase separation processes have been discussed in detail in Chapters 2 and 3 respectively.) The scaffolds measured 10mm in diameter and 2 mm in height.

Name of composition	**0%C**	**50%C**	**0%D**	**50%D**
Fabrication method	Solvent casting	Solvent casting	Phase Separation	Phase Separation
Solvent used	Chloroform	Chloroform	Dioxane	Dioxane
Glass wt%	0%	50%	0%	50%
Porosity	94%	95%	89%	90%
Pore size (μm)	[80-210]*	[80-210]*	[80-200]	[80-200]
Stiffness	300 kPa	190 kPa	4.72 MPa	7.10 MPa

Table 6.1: Fabrication methods, compositions and properties of the scaffolds used in the cell culture studies.

* The pore sizes of the solvent cast scaffolds cannot be measured directly, this range corresponds to the size of the NaCl particles used as the porogen agent, but, due to the extremely high porosity of the structures, real pore sizes are substantially larger (see Chapter 2).

MG63 cell culture

Cell Seeding

200,000 MG63 osteoblast-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 200μl of cells at 1,000 cells/μl, at two points on the surface of the scaffolds. This seeding method will be called two-point seeding from now on. 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. The scaffolds were transferred into fresh multiwells once a week in order to limit the amount of cells which left the scaffolds and attached to the bottom of the multiwell.

Cell Proliferation

Lactate Dehydrogenase readings

MG63 cell proliferation was monitored by means of a (Lactate Dehydrogenase) LDH Cytotoxicity Detection Kit (Roche). This kit measures the LDH activity released from the cytosol of damaged cells into the supernatant. For the assay, 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.

After the freezing cycles, the scaffolds were ground in order to release the cells from within the porous structure. The supernatant was then collected and centrifuged to remove debris which could hamper the spectrophotometric readings. The supernatants were diluted at 1:4 with medium before testing. 100μl of the diluted supernatant was added to 100μl of the assay Reaction Mixture (iodotetrazolium chloride and sodium lactate + a catalyst) and the mixture was incubated for 30 minutes at room temperature. Each composition was tested in triplicate. Fresh medium and medium which had been incubated in the presence of scaffolds without cells were used as controls.

The absorbance of the incubated mixture was measured at 450nm with a reference wavelength of 600nm on a PowerWaveX Bio-Tek Instruments Spectrophotometer. A calibration curve was computed prior to the assay, with 1,500,000, 1,000,000, 500,000, 200,000, 100,000, and 50,000 cells plated on multiwells. The calibration curve is thus not directly applicable to the scaffolds but can be used as an indication of the number of cells present and their proliferation trend. Cell proliferation was measured at days 1, 7, 14 and 21 of culture.

Total Protein Concentration

The total protein concentration was measured with a BCA Protein Assay kit (PIERCE). This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the selective colorimetric detection of the cuprous cation using a reagent containing bicinchoninic acid (BCA). The reaction product of the assay is purple-coloured and can be read at 562nm. It is formed by the chelation of two molecules of BCA with one cuprous ion.

Scaffolds were rinsed twice in phosphate buffered saline (PBS), and soaked in 500 μl of Mammalian Protein Extraction Reagent (MPER), in order to detach the cells from the scaffolds. The test was performed by adding 25μl of the MPER supernatant to 200μl of the kit's Working Reagent. The working reagent was composed of 50 parts of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part of Reagent B (4% cupric sulphate). The mixture was incubated for 30 minutes at 37°C and read at 562 nm. The calibration curve proposed by the assay kit was used to calibrate the absorbance corresponding to bovine serum albumin concentrations ranging between 0–2,000 μg/ml. The total protein concentration was measured at days 7, 14 and 21 of culture.

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.

Alkaline phosphatase

The ALP activity was measured with a Phosphatases, Alkaline Acid, Prostatic Acid assay kit (SIGMA Diagnostics). This kit depends on the hydrolysis of p-nitrophenoyl phosphate by the alkaline phosphatase enzyme, yielding p-nitrophenol and inorganic phosphate. When made alkaline, p-nitrophenol is converted to a yellow complex which can be read at 400-420 nm. The intensity of the colour formed is proportional to the phosphatase activity.

ALP was measured at days 7, 14 and 21 of culture in triplicate. Scaffolds were rinsed twice in 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, and lysate them. This is necessary in order to make ALP accessible to the membrane since it is attached to the cell membrane. The assay was performed by mixing 100μl of the MPER supernatant with 50 μl of substrate solution (p-nitrophenyl phosphate, disodium) and 50 μl of alkaline buffer solution (2-Amino-2-methyl-1-propanol). The MPER supernatant was centrifuged prior to the mixture in order to eliminate debris which could hamper spectrophotometric readings. The mixture was then incubated at 37°C. The reaction was stopped after 30 minutes with 100μl of 0.01N NaOH solution and read at 405 nm on a PowerWaveX Bio-Tek Instruments Spectrophotometer.

Osteocalcin

The osteocalcin concentration was measured by means of a Metra® Osteocalcin kit (Quidel corporation). This assay is a competitive immunoassay. It uses OC coated multiwell strips, a mouse anti-osteocalcin antibody, an anti-mouse IgG- alkaline phosphatase conjugate and pNPP substrate to quantify osteocalcin concentration. The cell culture supernatant (which contains the OC released by the cells) is added to the coated multiwells and incubated with the antibody. After incubation, the wells are thoroughly washed and the conjugate is added. The conjugate will attach to the antibodies which have not latched on to the OC on multiwells. Next, the pNPP substrate is added which reacts with the ALP conjugate creating the colouring which can be read on a spectrophotometer.

OC was measured on day 21 of culture in triplicate. 400 μl of supernatant from the scaffold culture wells were centrifuged to avoid debris. The supernatant was then analysed as described above. The results were normalised with the number of cells as given by the LDH readings.

MSC cell culture

MSCs were isolated from adult human bone marrow aspirates via density gradient centrifugation. The cells were expanded in non-differentiating MSC growth medium consisting of a-Minimal Essential Medium (α-MEM), with 20% foetal bovine serum (FBS), 1% penicillin-streptomicin (PEN-STREP) and 2mM glutamine, and were passaged every 7-10 days. The MSCs were seeded between the tenth and fifteenth passages which showed no significant difference between them.

The MSCs were dynamically seeded into the scaffolds using a Harvard Instruments syringe pump connected to a closed seeding chamber. The syringe pump is designed to operate at a fixed flow rate (L/min), and can perform alternate flow. Alternate flow means the step motor of the syringe pumpt can supply a positive or negative movement (infuse or withdraw) by manual selection (Figure 6.1 and Figure 6.2).

Figure 6.1: Photograph of the syringe pump used for the dynamic cell seeding with MSC cells.

The seeding conditions were optimised prior to the study. The syringe pump was loaded with 5ml of cells and medium and infused and withdrawn once at 0.4 ml/min, 4 scaffolds could be seeded simultaneously.

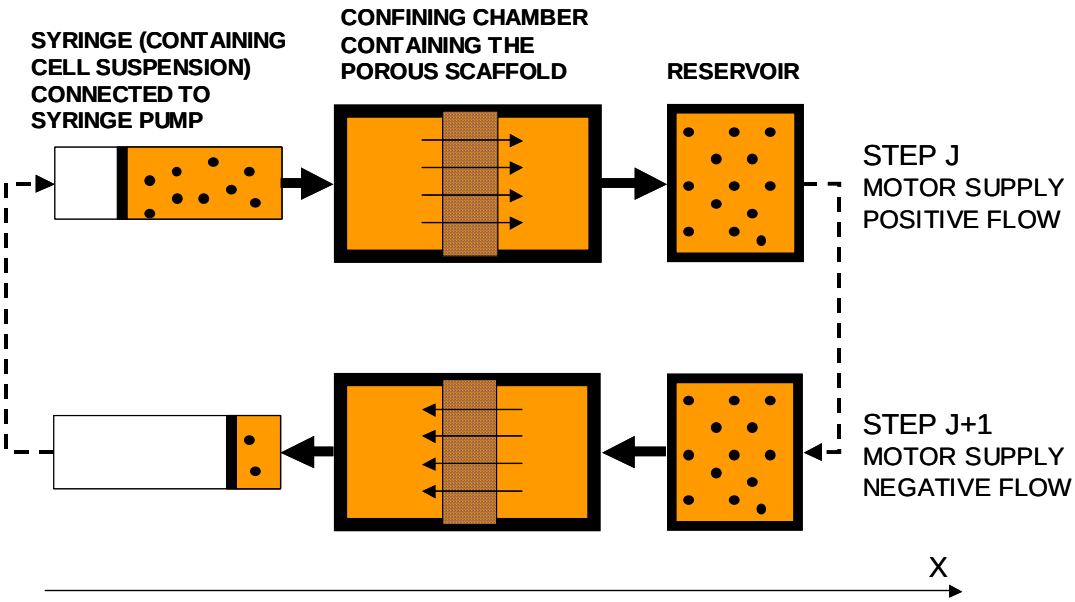

Figure 6.2: Schematic diagram illustrating the infusion and withdrawal cycles performed during the dynamic seeding of MSCs on the scaffolds**.**

The cell seeding density was 200,000 cells/per scaffold. The seeded scaffolds were maintained in multiwells in static culture for 21 days, in cell culture medium at 37°C in a controlled atmosphere of 5% CO_2. The cell culture medium was changed every 3 or 4 days. A total of 19 scaffolds were seeded per composition, thus 3,800,000 MSCs were needed per composition making a total of 15,200,000 MSCs used for the entire cell culture.

MSCs cultured in 2D were used as controls and standards and to verify their proliferative and differentiation potential. They were cultured with and without osteogenic medium. The osteogenic medium was supplemented with dexamethasone, ascorbic acid, and β-glycerophosphate.

Cell seeding efficiency

Cell seeding efficiency was assessed by counting the number of cells remaining in the syringe after the infusion and withdrawal with a haematocytometer. It was also measured by measuring cell viability with the AlamarBlue assay 24 hours after seeding.

Cell proliferation

Cell viability and proliferation were monitored by the AlamarBlue assay (Biosource TM) which had the advantage of being a non-destructive testing assay. The AlamarBlue assay can be used to measure cell viability and proliferation by measuring the reducing environment of the proliferative cell and the innate metabolic activity of the cell respectively. AlamarBlue works similarly to the tetrazolium salts. The internal environment of the proliferating cell is more reduced than that of non-proliferating cells due to the presence of certain compounds and cytochromes. As AlamarBlue is reduced (accepts electrons) by these compounds it changes colour from the initial indigo blue to a fluorescent pink state. AlamarBlue is then monitored spectrophotometrically at 570nm and 600nm due to the overlap of the maxima of its oxidised and reduced state. The readings are then expressed as a percent reduction of AlamarBlue as a function of the time of incubation.

The scaffolds seeded with cells were placed in culture medium with 5% of AlamarBlue solution and replaced in the incubators for 3h at 37ºC. 100μl of the cell culture medium was then read spectrophotometrically. Cell viability and proliferation was measured at days 1,7,14 and 21.

Sterilisation Protocol

For both the MG63 and the MSC studies, the scaffolds were sterilised prior to cell culture with gamma-radiation at 8Grad. The surface characterisation studies (Chapter 5) were performed with ethylene oxide sterilisation. Given the growing concern on the effects of sterilisation on biomedical polymers, and specifically on the traces of poisonous gas that may remain trapped within porous polymeric structures after sterilisation with Ethylene Oxide [31], gamma radiation was chosen as a better adapted sterilisation approach.

The MG63 cell seeding involved using a syringe to inject the cells, tweezers to hold the scaffold during the seeding, and individual cell culture plates on which the seeding was performed. The syringe and cell culture plate were changed for each seeding. The tweezers were sterilised overnight with UV radiation under the cell culture hood, and were soaked in ethanol between each seeding. All other operations were performed under normal cell culture sterile conditions.

The MSC cell seeding involved a large amount of instrumentation: the syringe pump, a seeding chamber containing the scaffolds, a 5ml syringe, tubing to connect the seeding chamber to the syringe, tweezers to place and remove the scaffolds before and after seeding, as well as the tools used to screw the seeding chamber tightly closed. Initial problems with contamination induced the enhancement of the initial sterilisation protocol. The final protocol that involved no contamination problems was the following. Scaffolds were gamma-radiation sterilised as described above, and soaked overnight in an antibiotic and antifungal solution together with the cell culture medium. The syringe pump was thoroughly cleaned before each seeding with laboratory detergent and water. A fresh syringe was used for each seeding. The tubing, screws, wrenches and tweezers were autoclaved before each batch of seeding (a single composition). The seeding chamber, which could not be autoclaved, was thoroughly cleaned with laboratory detergent, and ethanol, and sterilised overnight with UV-radiation under the cell culture hood.

Statistics

Samples were seeded, placed in the cell culture multiwell randomly and tested at each culture time in random order. Proliferation and differentiation test were performed in triplicate. Measurements are thus presented as an average of three samples. The statistical significance of the differences between the averages of the results for all parameters studied was calculated using ANOVA tables with a Fisher multiple comparison test. Statistical significance was established at $p < 0.05$. These calculations have been performed with MINTAB^{TM} Release 14 Minitab Inc. software.

Results whose difference is not statistically significant are indicated with a horizontal line on the graphs. When values are not alongside eachother, they are indicated with a symbol (*, +).

Cell-Scaffold Morphology

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

Stereomicroscopy

Scaffolds were harvested at days 1, 7, 14, and 21 of culture, rinsed in PBS and stained with ethylene bromide and acridine orange to perform a live/dead stain. They were then viewed under a MZ16F Leica Stereomicroscope. Stereomicroscopy is a fast and simple method that allows the characterisation of cells in their media. If performed quickly, it can be used to illustrate the cells that are dead and alive. It does not offer however, information on 3D distribution, and cells tend to perish during the observations.

Histological Sections

Histological sections of scaffolds were performed after 21 days of culture. The scaffolds were dehydrated in successive baths of 70%, 96% and 100% ethanol. They were then soaked in two baths of Xilol and included in paraffin at 57°C. After inclusion in paraffin, the scaffolds were sliced and mounted on silanised object slides. They were then stained with methylene blue and Borax. Histological sections give very illustrative information on the distribution of cells and tissue within the construct. In the case of porous scaffolds, it can illustrate cell penetration into the scaffold and cell distribution at virtually any depth. Sample preparation is long and must be adapted to each material and cell type. Some information can be lost during sample preparation.

Confocal microscopy

Confocal microscopy is a powerful tool which allows viewing the cells in 3D within the construct. Cells can also be stained in order to see different cells parts and thus observe in detail how the cells attach to their substrate. Some disadvantages associated to this technique are the limited depth penetration and the lengthy sample preparation.

Samples of scaffolds after 21 days of culture were viewed with a Leica SPII confocal microscope. The scaffolds were stained with phalloidin and Hoechst as explained below. Phalloidin stains the actin filaments of the cells and Hoechst stains the nuclei. Several solutions were prepared:

- PBS-Gly: 0.15g Glycine + 100ml PBS 10mM

- Fixation solution: 760 μl of Paraformaldehyde + 240 μl of saccarose 1M + 1ml of distilled water + 2ml of PB 0.2 M.
- Permeabilisation solution: 25μl of Tritori + 50 ml of PBS-Gly
- Blocking solution: PBS-Gly BSA 1%
- Phalloidin and Hoescht staining solution: PBSGly + 1:1000 parts of phalloidin + 1:500 parts of Hoechst.

The scaffolds were rinsed first in PBSGly and then soaked in the fixation solution for 10-20 minutes. They were then rinsed again in PBSGly, soaked in the permeabilisation solution for 10 minutes, rinsed again in PBSGly and soaked in the blocking solution for 20 minutes. After these steps, the scaffolds were incubated in the phalloidin and Hoechst solution for 1 hour at 37°C in the dark, rinsed again in PBS-Gly and tapped dry. The samples are then mounted onto an object slide, soaked with Mowiol and covered with a cover slip sealed with a hot glue gun.

Imaging was performed on the confocal microscope using reflection in order to view the scaffold material, and various fluorescent modes in order to see the actin filaments and cell nuclei. The reflection images were superimposed onto the fluorescence images in order to have all images in one.

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 prepared by fixating in glutaraldehyde, postfixating in osmium tetraoxide, and dehydrating in ethanol. The samples were then critically point dried and gold-sputtered prior to visualisation.

Results

MG63 cell culture

The static cell seeding of the MG63 on the various types of scaffolds was performed in a single day. The samples collected for the measurements were always chosen randomly in order to avoid any effect of the seeding order or sequence.

Cell proliferation

The LDH readings indicate that the MG63 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 (Figure 6.3). For the phase-separated scaffolds, proliferation levels were similar with and without glass throughout the assay (Figure 6.4). 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.

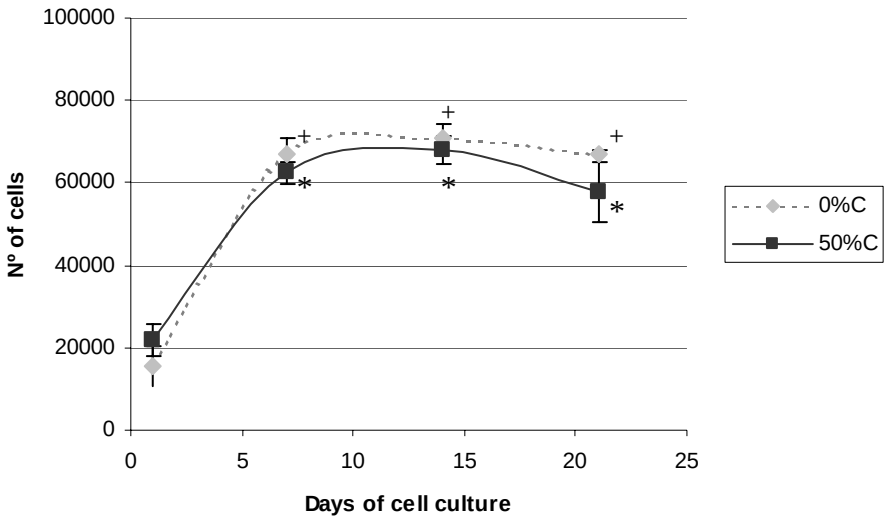

Figure 6.3: MG63 proliferation measured by LDH on the solvent cast scaffolds with and without glass particles (50%C and 0%C respectively) during the 21 days of cell culture. *, +: the differences between readings on day 7, 14 and 21 are not statistically significant.

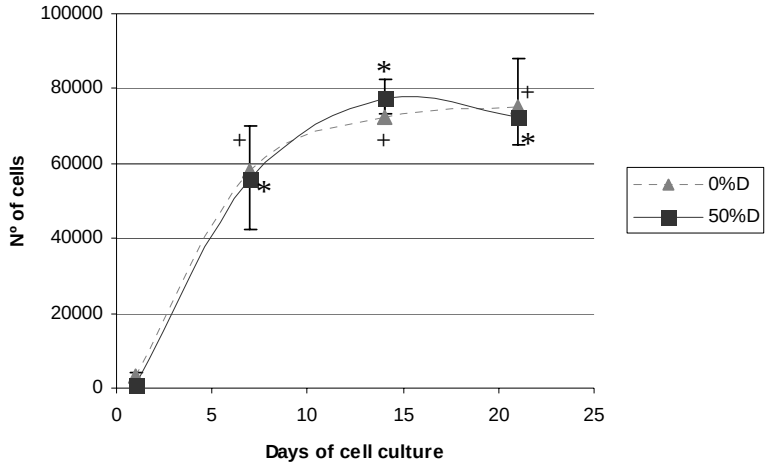

Figure 6.4: MG63 proliferation measured by LDH on the phase-separated scaffolds with and without glass particles (50%D and 0%D respectively) during the 21 days of cell culture. *, +: the differences between readings on day 7, 14 and 21 are not statistically significant.

The total protein content results on all compositions peaked on day 14 and then decreased on day 21 (Figure 6.5). 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.

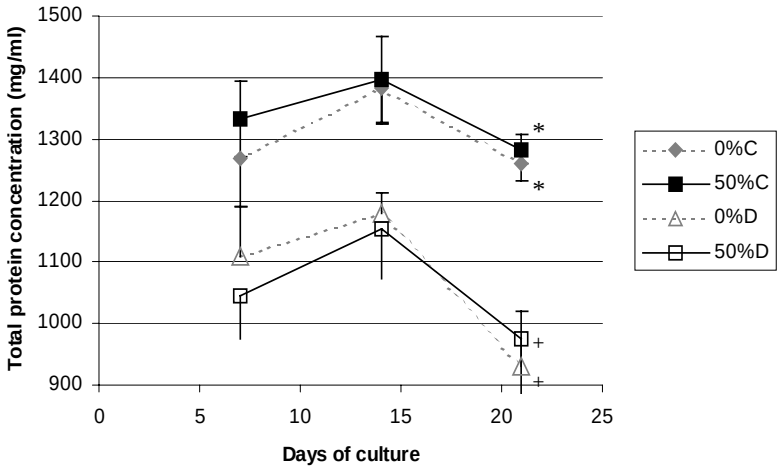

Figure 6.5: Total protein content of the MG63 cell cultures on the solvent cast (C),and phase-separated (D), scaffolds with and without glass particles (50%D and 0%D respectively) during the 21 days of cell culture. *,+: the differences between 0%C and 50%C, and 0%D and 50%D are not statistically significant.

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 6.6 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).

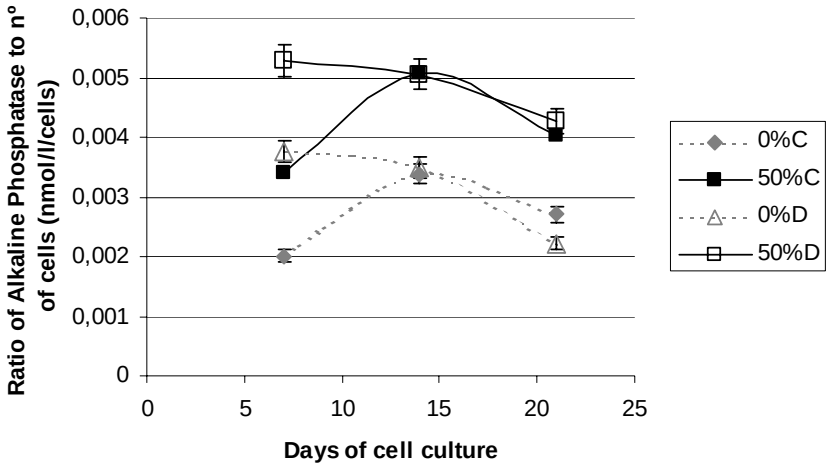

Figure 6.6: MG63 differentiation results illustrated as the ratio between Alkaline Phosphatase concentration and the number of cells measured by LDH. The samples include solvent cast (C),and phase-separated (D), scaffolds with and without glass particles (50%D and 0%D respectively) during the 21 days of cell culture.

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 (Figure 6.7), though the 0%D scaffolds tend to sustain higher OC concentration.

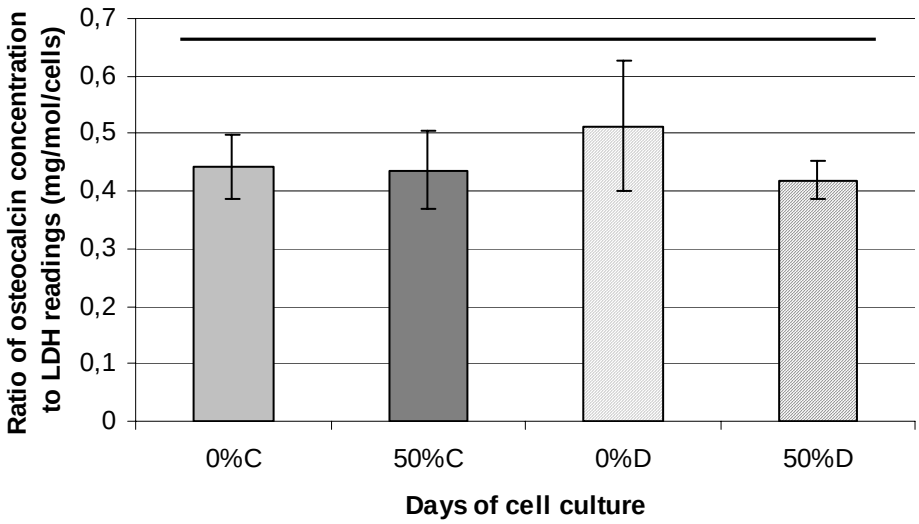

Figure 6.7: MG63 differentiation results illustrated as the ratio between osteocalcin concentration and the number of cells measured by LDH. The samples include solvent cast (C),and phase-separated (D), scaffolds with and without glass particles (50% and 0% respectively) during the 21 days of cell culture. The differences between the results are not statistically significant.

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 (Figure 6.8). Qualitatively, the phase-separated scaffolds seem to have a higher density of cell 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 (Figure 6.9).

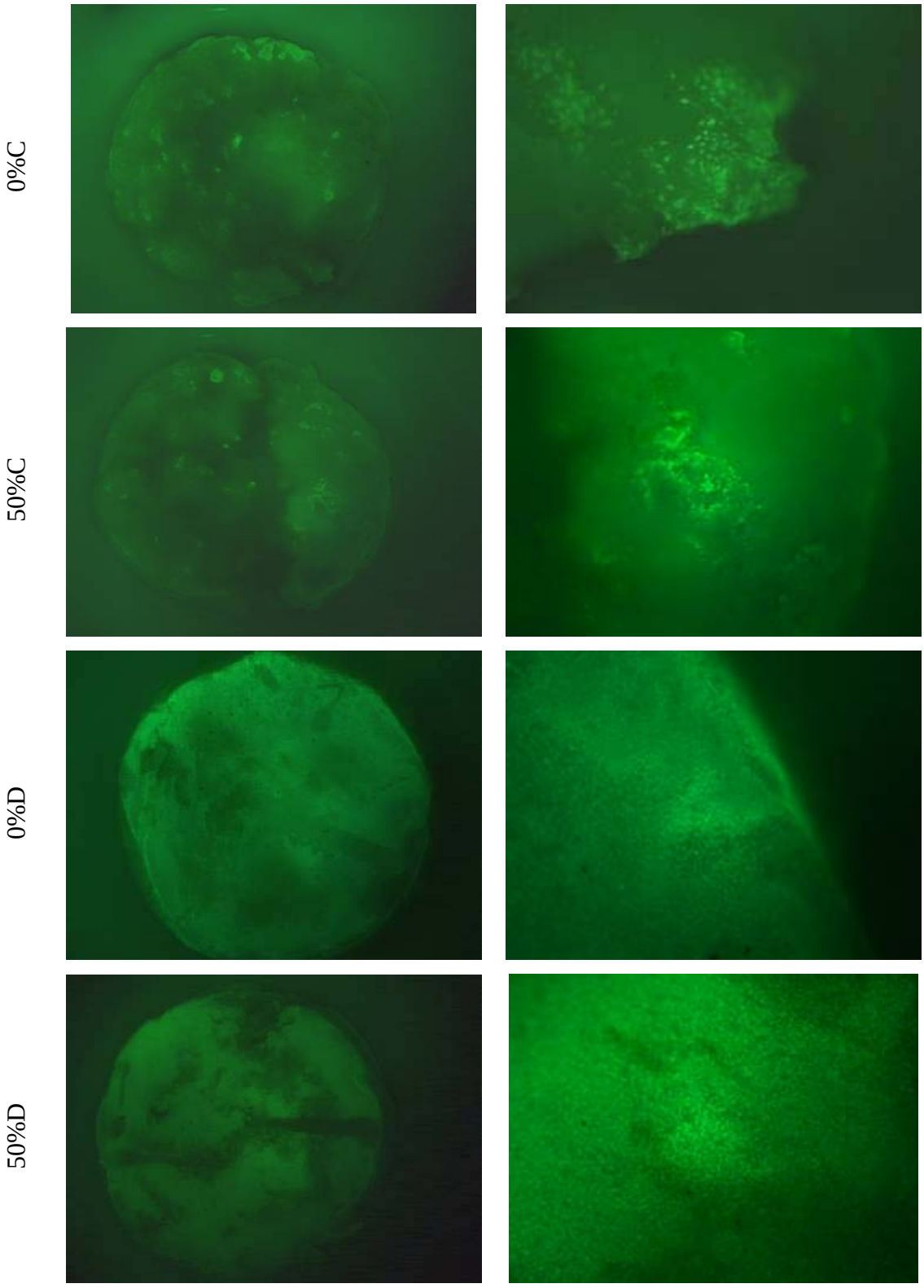

Figure 6.8: Stereomicroscope images of the live MG63 cells seeded on the scaffolds after 21 days of cell culture. The left column corresponds to views of the entire scaffolds (approximately 10mm in diameter) and the right column are close-up images.

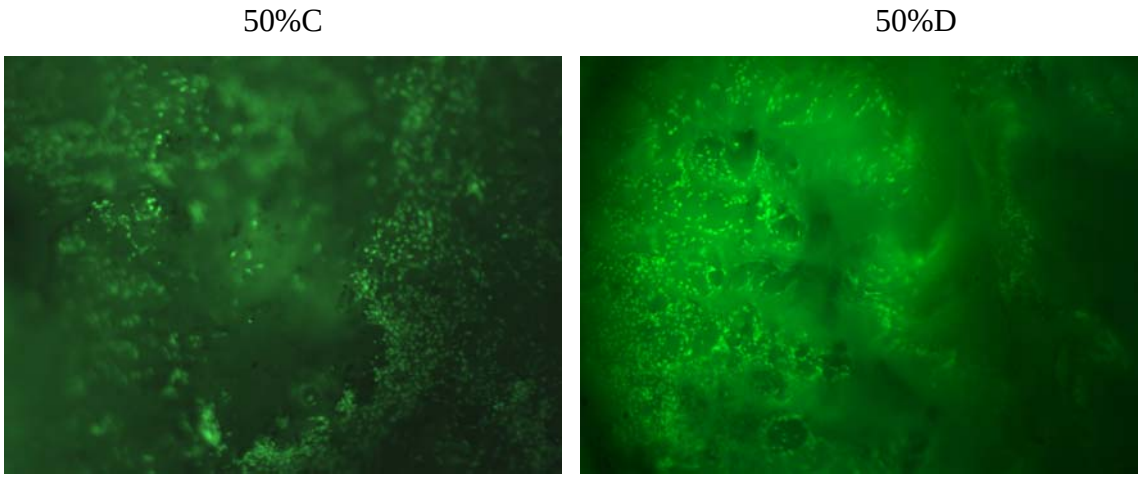

Figure 6.9: Stereomicroscope images of live MG63 cells seeded on the scaffolds after 14 days of culture. The images show how the cells adapt to the porosity and architecture of each scaffold.

The histological sections of the scaffolds were rather complex to produce due to the difference between the hardness of the PLA and that of the glass particles. This mechanical mismatch made the histological cuts uneven and they were often torn during the manipulation. Despite these limitations, the images obtained from the sections after 21 days of culture confirm the stereomicroscope images and give further information. The cells seem to thrive on the scaffolds, and are densely coated on the exterior of the phase-separated scaffolds (Figure 6.10). 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. Interestingly, at higher magnifications (Figure 6.11), cells can be seen to attach to the scaffold structure and specifically to glass particles.

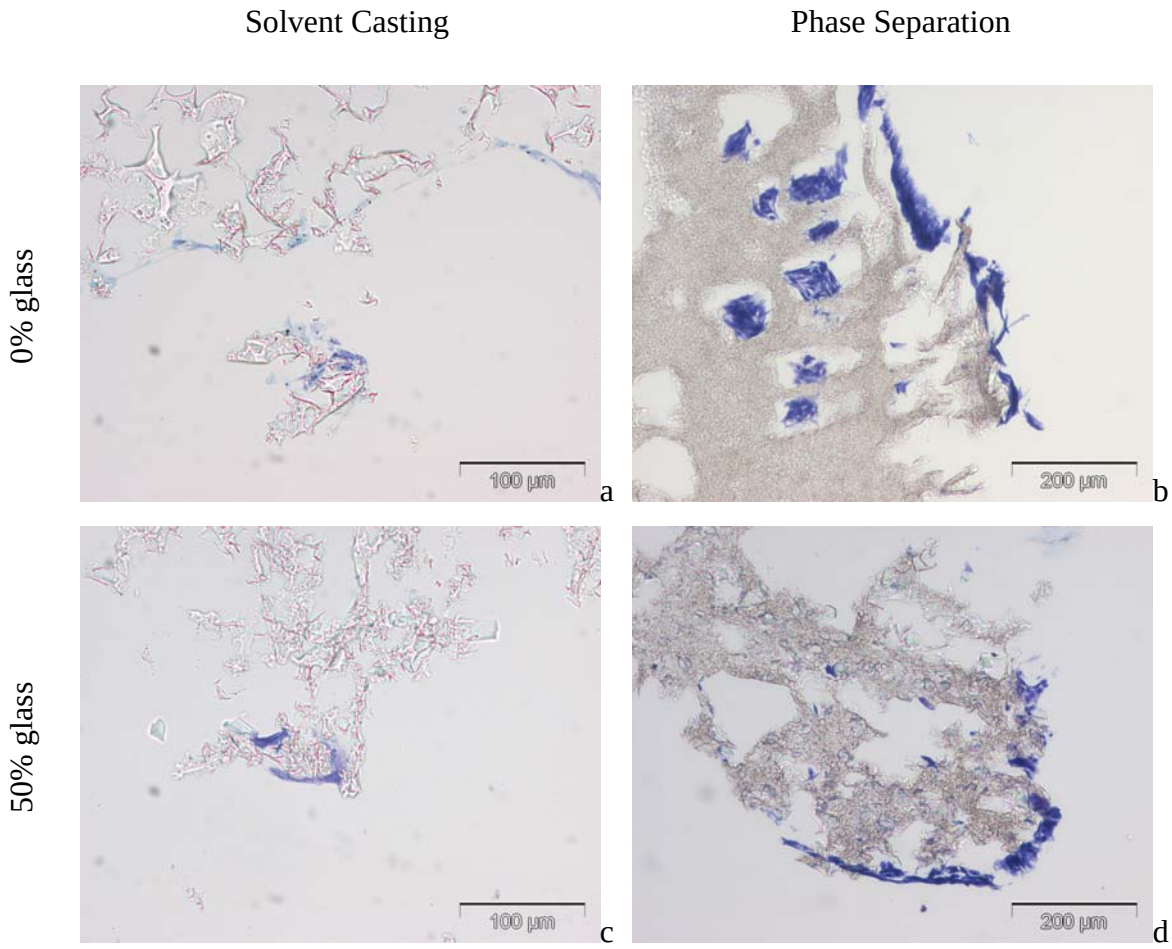

Figure 6.10: Histological sections of the scaffolds after 21 days of culture. The MG63 cells are stained with methylene blue. The scaffolds made by phase-separation (0%D and 50%D), images b and d, show a thick layer of cells on their surface.

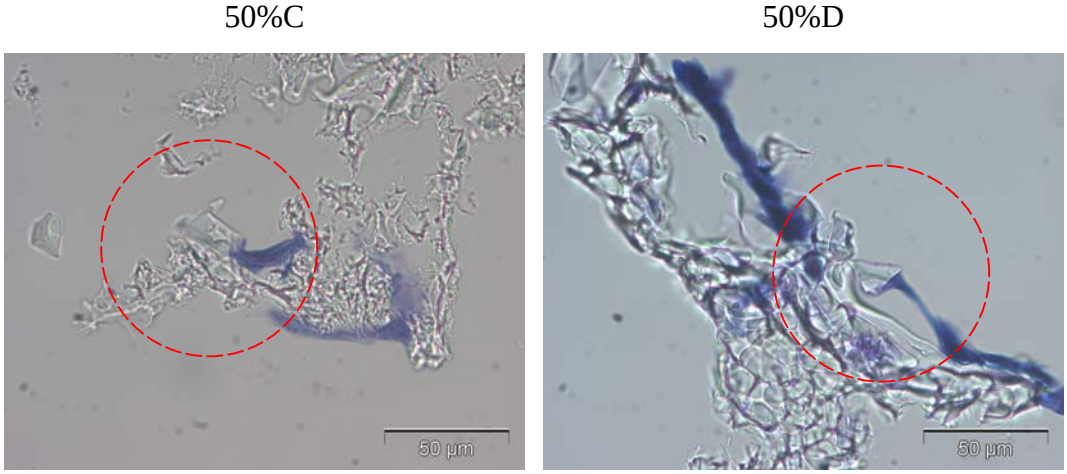

Figure 6.11: Histological sections of the scaffolds with glass particles after 21 days of cell culture. The images show the MG63 cells attaching directly on the glass particles (red dotted circles).

Confocal microscopy gives intricate images of 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 (Figure 6.12). Figure 6.13 shows images of the interior of the scaffolds. In order to view the interior, the scaffolds were cut with a scalpel and the fresh surface was viewed under the confocal microscope. Cells were more difficult to find within the phase-separated scaffold, whereas they were relatively abundant in the solvent cast ones.

50%C 50%D

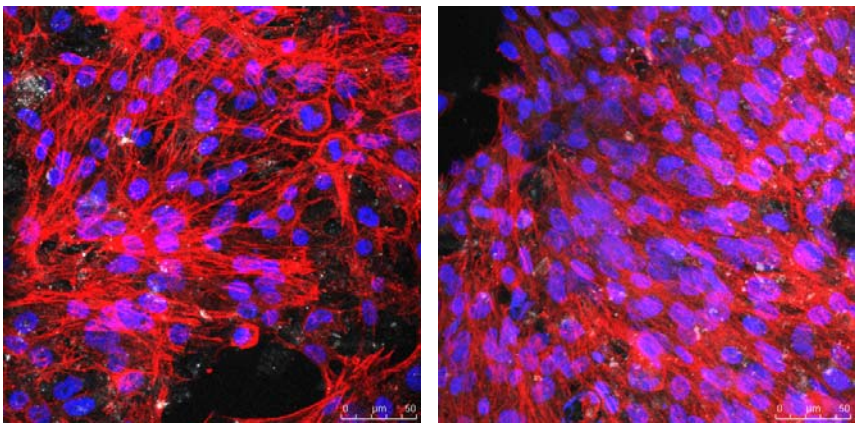

Figure 6.12: Confocal microscope images of the MG63 cells on the surface of the scaffolds made of PLA and glass after 21 days of culture. The cell nuclei are stained blue and the actin filaments appear red. (Scale bars correspond to 50μm)

0%C 0%D

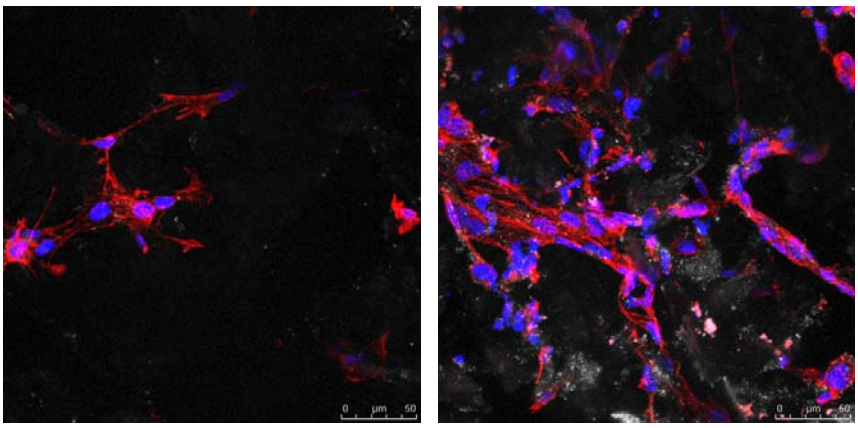

Figure 6.13: Confocal microscope images of the MG63 cells in the interior of the scaffolds made of PLA only after 21 days of culture. The cell nuclei are stained blue and the actin filaments appear red. (Scale bars correspond to 50μm)

SEM images complemented the previous qualitative analysis by stereomicroscope, confocal microscopy and histological sections. Figure 6.14 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 (Figure 6.15).

The SEM has proven a very powerful tool in the cell–scaffold characterisation. It allows the observation of the cells in great detail; how they adapt to the scaffold porosity and architecture (Figure 6.16) and whether they are outstretched or rounded (Figure 6.17).

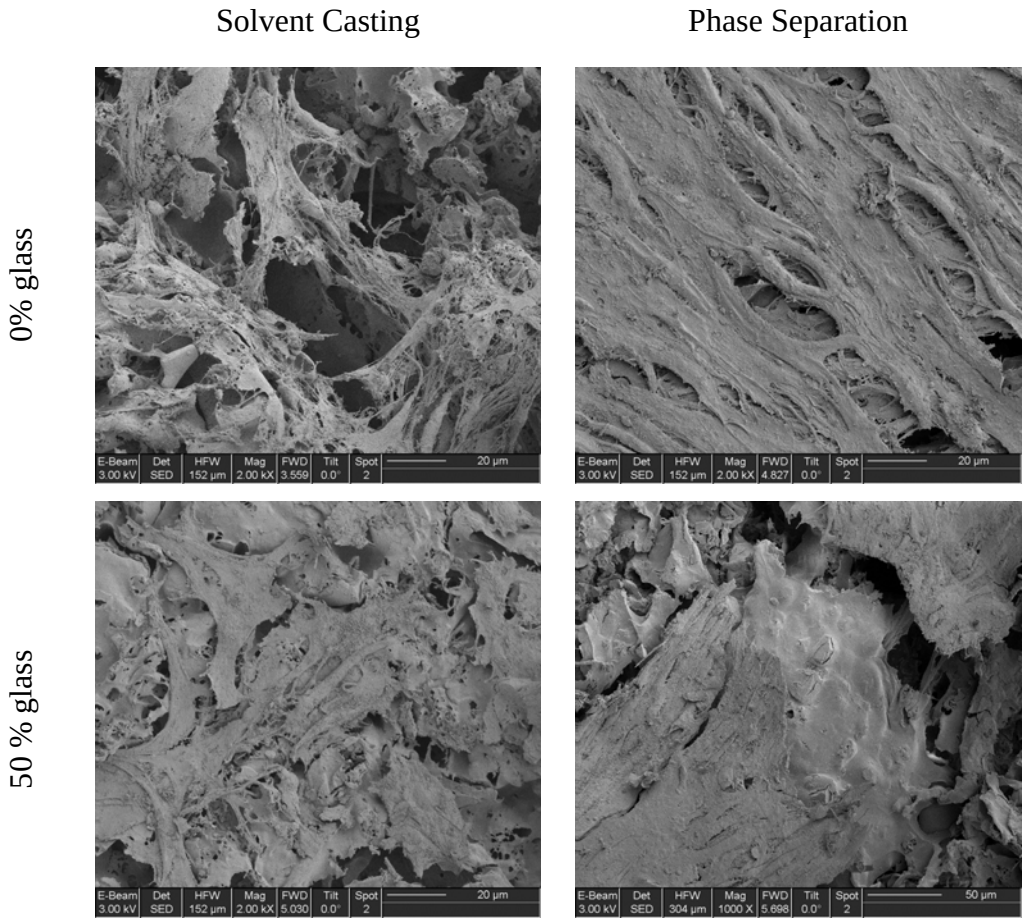

Figure 6.14: SEM images of the surface of the scaffolds after 21 days of MG63 cell culture.

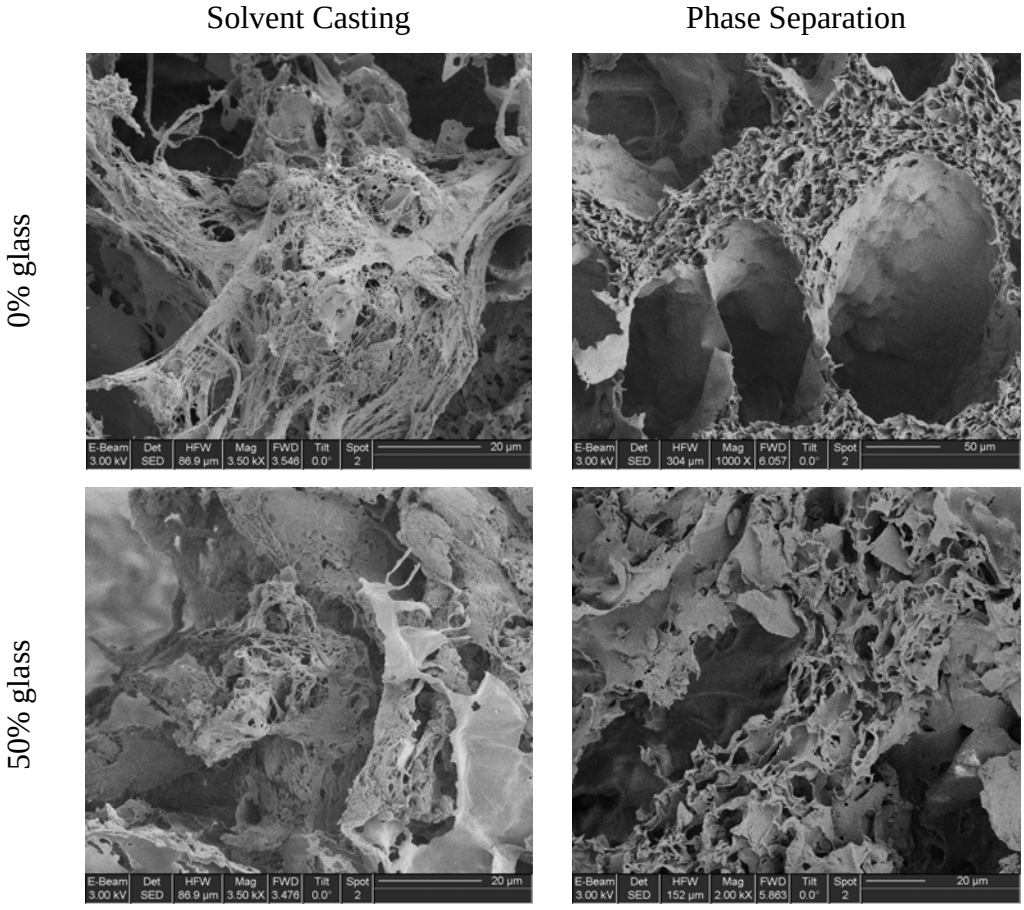

Figure 6.15: SEM images of the interior of the scaffolds after 21 days of MG63 cell culture. The solvent cast scaffolds, with and without glass, harbour cells which have created extracellular matrix. No cells appear on the image of the phase-separated scaffolds with 0% glass (O%D).

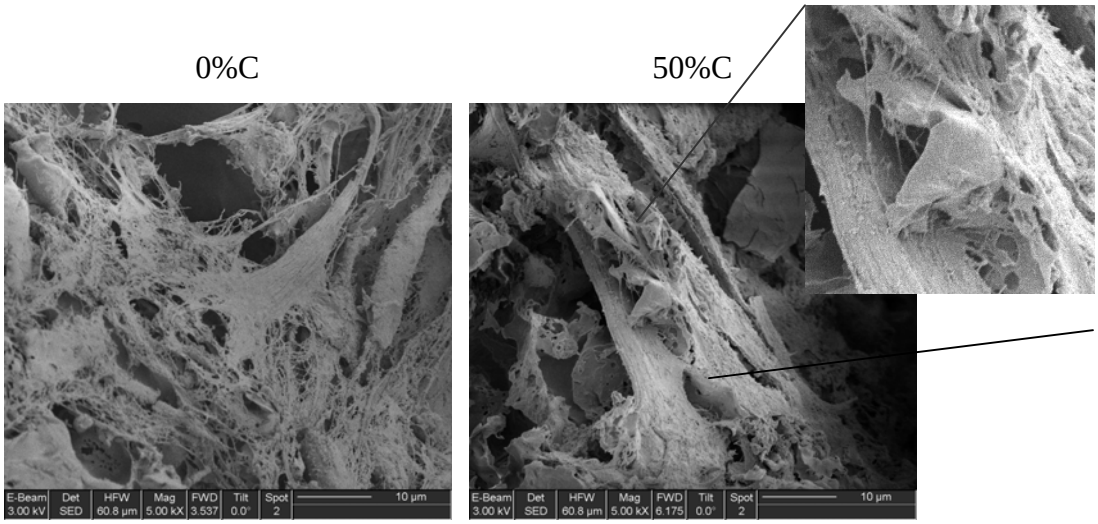

Figure 6.16: Focused Ion Beam images of the interior of the solvent cast scaffolds. These images show details of how the cells stretch and adapt to the porosity of the scaffolds. In the case of the scaffold with glass (50%C), the cell processes attach directly onto the glass particles (see close-up in upper right-hand corner).

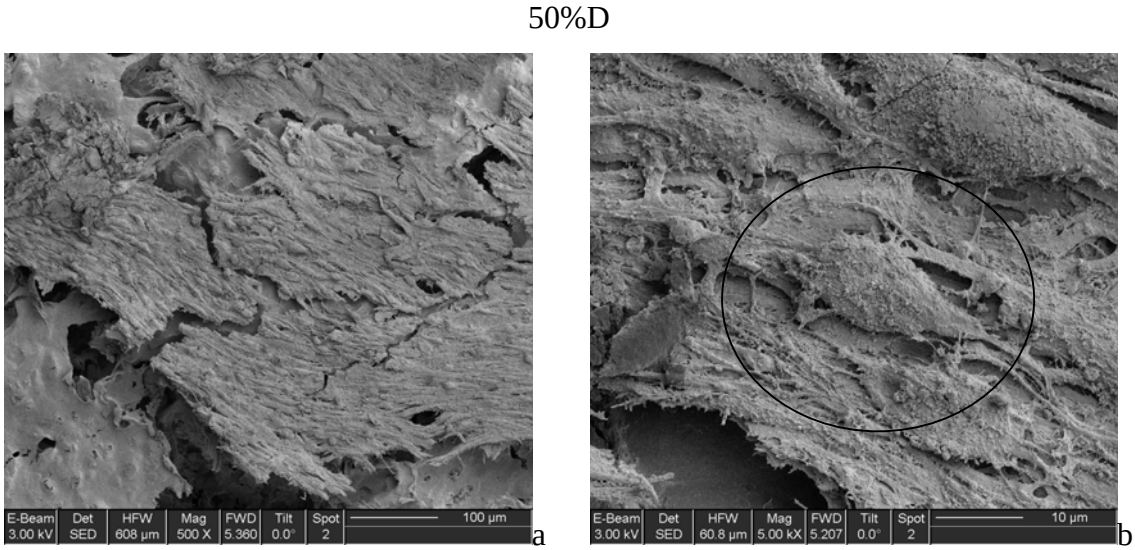

Figure 6.17: SEM image of the cells on the surface of the 50%D scaffold. The image at high magnification (b), shows great detail of the shape of the cell and its processes, the cell circled in black shows dorsal activity and creation of extracellular matrix.

MSC culture

The cell seeding conditions were optimised to a single infusion and withdrawal cycle (a single push and pull of the syringe) at 0.4 ml/min with the syringe pump in order to maximise the number of cells that remained within the scaffold. A single

infusion/withdrawal cycle was preferred to more cycles due to the simplicity of manipulation and to reduce the time spent seeding the scaffolds of each composition (Figure 6.1). In addition, the dynamic seeding process involved a higher risk of contamination due to the time, the number of instruments and the manipulation involved. Some contamination problems which occurred during the preliminary tests were solved by enhancing the sterilisation protocol and optimising the cell seeding time.

MSC seeding efficiency

Cell seeding efficiency was measured both by counting the cells left over in the perfusion medium after the dynamic seeding, and by evaluating cell viability with AlamarBlue after 24 hours of culture. Immediately after seeding, compositions 50%C and 0%D contained approximately 150,000 cells each, whereas compositions 0%C and 50%D had around 100,000 (Figure 6.18). After one day of cell culture, the 50%C composition continues having the highest number of cells (highest AlamarBlue reduction), but composition 0%D has approximately the same amount of cells as the remaining composition (Figure 6.19). Thus, cell seeding efficiency seems highest on the 50%C scaffolds.

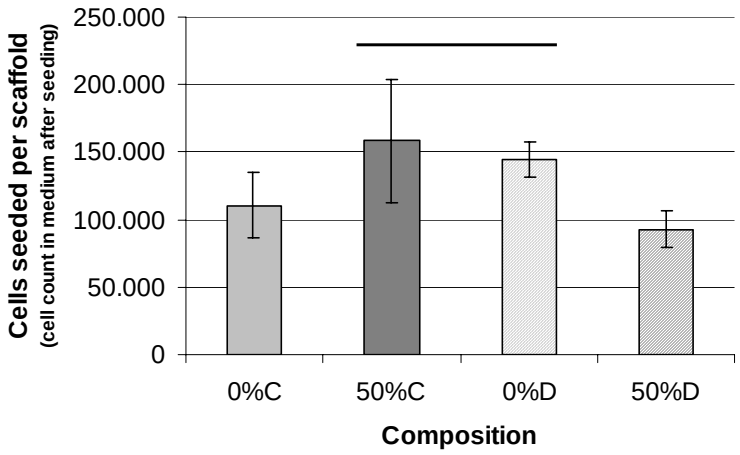

Figure 6.18: Cell seeding efficiency of the MSC on the scaffolds measured by counting the cells remaining in the medium after the infusion and withdrawal cycle.

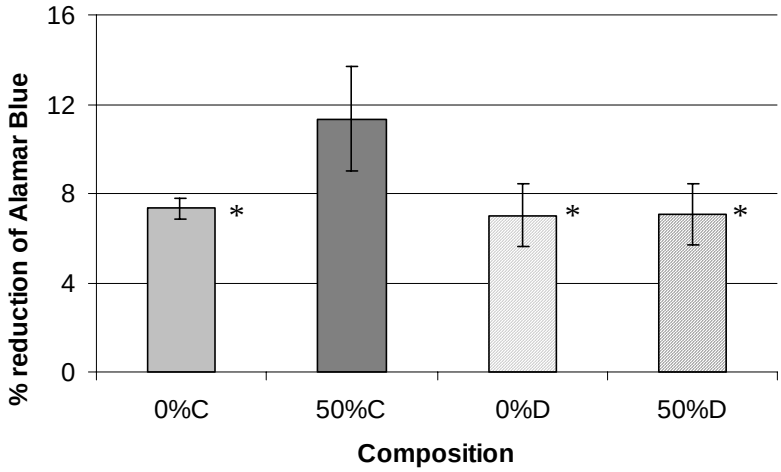

Figure 6.19: Cell seeding efficiency of the MSC on the scaffolds measured by the AlamarBlue reduction 24 hours after seeding. *: differences between compositions 0%C; 0%D, and 50%D were not statistically significant.

MSC viability and proliferation

The AlamarBlue assay allowed cell proliferation to be followed on the same cells during the 21 days of cell culture. The large number of cells which remained seeded initially in the 50%C scaffolds, do not all remain viable at 7 days of culture, as can be seen in the dip in AlamarBlue reduction on Figure 6.20. After day 7 however, the MSCs seeded on the 50%C scaffolds proliferate until day 21. The 0%C scaffolds gave steady proliferation values during the entire cell culture study. The results of the proliferation on the phase-separated scaffolds can be seen in Figure 6.21. Despite a large standard deviation between the results, 50%D scaffolds support significantly lower cell proliferation on days 7 and 14. In general, the % reduction of AlamarBlue is lower on the phase-separated scaffolds than on the solvent cast ones.

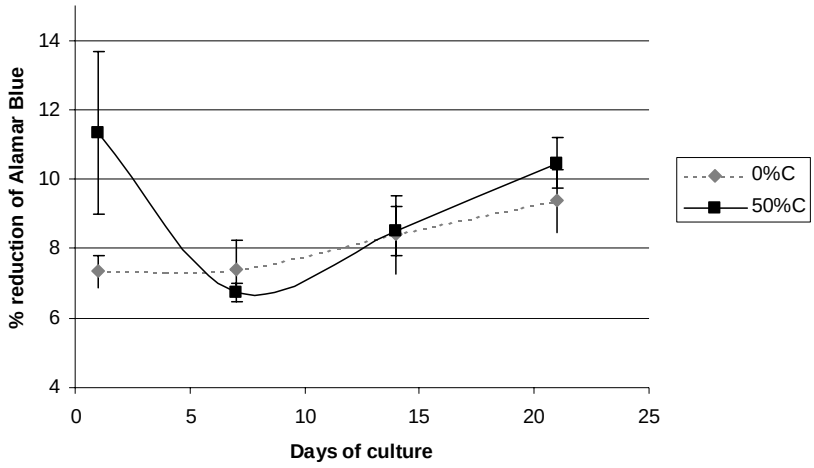

Figure 6.20: Proliferation of the MSC cells seeded on the solvent cast scaffolds during the 21 days of cell culture

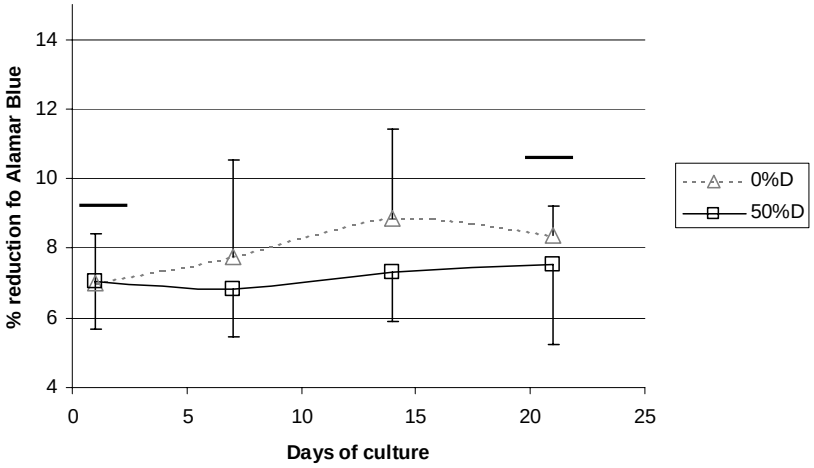

Figure 6.21: Proliferation of the MSC cells seeded on the phase-separated scaffolds during the 21 days of cell culture. (standard deviation bars are displayed in one sense to clarify the graph).

Cell-scaffold morphology

The SEM images of the scaffolds seeded with the MSC prove very useful to understand the distribution of the MSCs within the scaffolds (Figure 6.22). The cells on the solvent cast scaffolds are hardly visible on the surface of the scaffolds at low magnifications (Figure 6.22a), implying they are mostly proliferating inside the scaffold structure. Those on the phase-separated scaffolds on the other hand, tend to form a thick

layer on the surface of the scaffolds, but they have not completely occluded the surface porosity (Figure 6.22b). Larger magnifications, show the MSCs on both types of scaffolds stretching to bridge pores and adapting to the porosity.

Very high magnifications give beautiful displays of the cells in the scaffold environment. Figure 6.23 shows the detail of what seems to be a cells sitting on their extracellular matrix which had evolved cell processes that attach onto the glass surfaces.

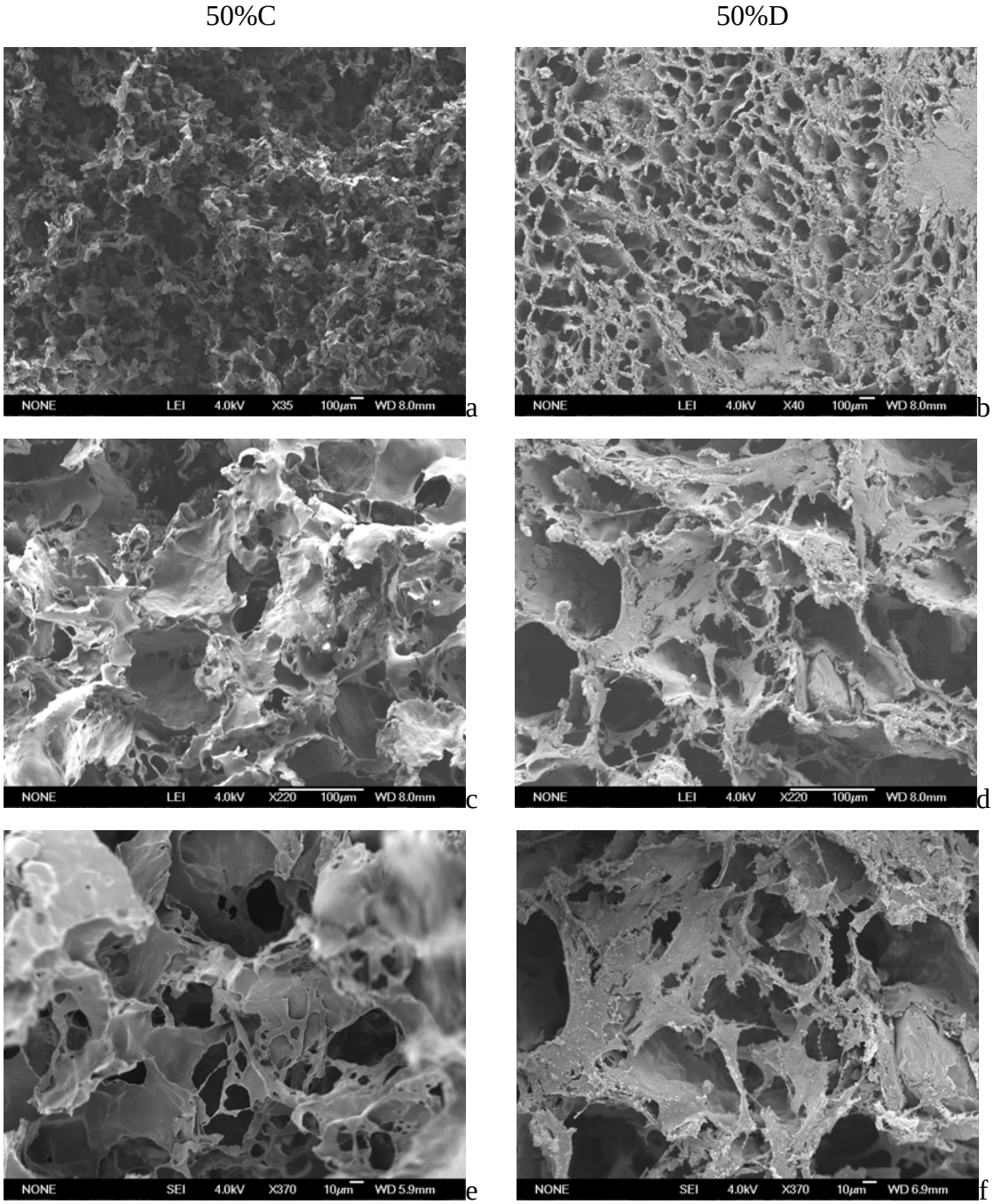

Figure 6.22: SEM images of the MSCs seeded on the scaffolds after 21 days of cell culture. The cells on the solvent cast scaffold samples (left column) seem very thick and sheet-like due to an excess of gold sputtering on the sample.

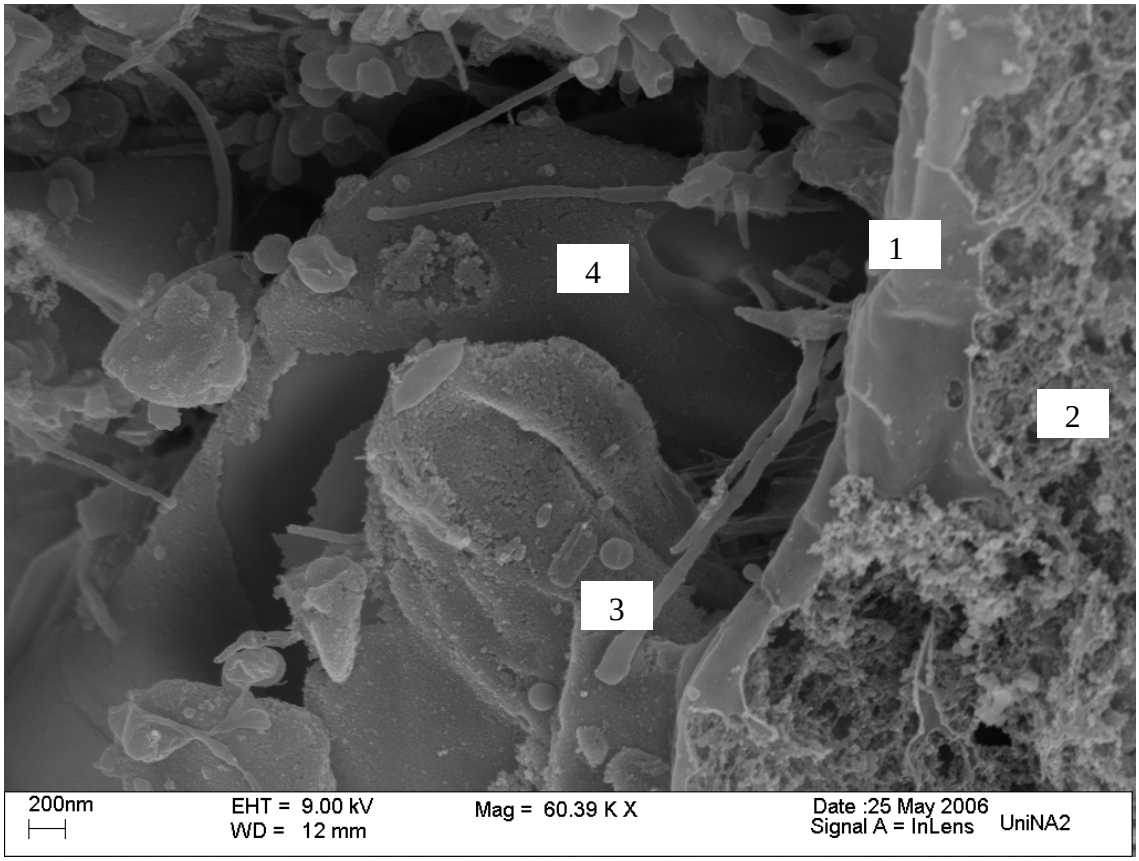

Figure 6.23: High magnification SEM image of the MSCs in the 50%D scaffold. This high resolution image allows the appreciation of what seems to be a cell (1) on its extracellular matrix (2), which has developed filopodia (3) that attach to the phosphate glass particle surface (4). The surface of the glass seems have formed a hydrated layer that is peeling off.

Discussion

The cell culture study described in this chapter has characterised the properties of the scaffolds as supports for cell growth. Since the non-cytotoxic nature of the PLA and the calcium phosphate glass materials had been previously verified[32], the objective of the chapter was to assess the scaffolds' potential as templates for cell attachment, migration, proliferation and differentiation. The results prove the 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. Before discussing the cell culture results per se in detail, some issues concerning the practical aspects and limitations of the cell cultures will be considered.

The different cell sources used, MG63 and MSC cells, implied completely different approaches and complications related to the cell culture assays. 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. Indeed, they were readily available, and could be expanded into the numbers necessary to begin the assay in a short number of days. Thus, the assay was planned and performed on schedule without inconveniences. On the contrary, the MSCs, being primary cells were harvested from a human donor and were available as determined by an external protocol. Primary cells are more difficult to culture, their proliferation speed is lower than cell lines, they are more easily contaminated, and give higher variability. The MSCs took approximately 14 days to duplicate. Thus obtaining the 19 million cells necessary to begin the cell culture assay was a lengthy process. Besides, the number of cell passages that could be performed was limited before the cells lost their characteristic phenotype or attained confluence[21;22;33]. Due to these factors, scheduling the beginning of the MSCs culture tests proved very complex and had to be postponed several times.

Concerning the different cell seeding protocols, static cell seeding was far quicker and simpler that dynamic cell seeding. For static cell seeding, the instruments used to manipulate the scaffolds were sterilised in absolute ethanol before each seeding, and no problems with contamination were encountered. The two-point static seeding was a very fast process, thus the scaffolds from all 4 compositions were seeded on the same day.

The dynamic seeding was a far more complex process. The seeding was time-consuming and ideally needed two persons in order to reduce MSC dwelling time outside of the incubator to a minimum. Since the seeding cycle for 4 scaffolds took approximately 30 minutes to perform, the MSC's were only trypsinised (detached from their substratum) immediately before each seeding. Thus seeding a single scaffold composition (19 scaffolds) involved a full 8-9 hour day. Due to the low proliferation rate and limited initial population of the MSC's and the timetable availability of the cell culture facilities, the start of the MSC cell culture had to be spaced out in different stages. Despite its challenges, dynamic cell seeding allows a better distribution of cells within the scaffold structure. Indeed, the layer of cells growing on the phase-separated scaffold surface shown in Figure 6.22 is not as occlusive as those shown in Figure 6.14.

Thus dynamic seeding seems to enhance more uniform cell distribution throughout the scaffold structure. Furthermore, Hofmann et al.[34], found the dynamic seeding of MSCs induced a higher rate of live cell attachment than static seeding, in which more cells were seeded but many include dead cells that had been passively seeded onto the scaffolds.

In sum, the MG63 cell culture with static seeding is a far more straightforward approach, it is less time-consuming, and requires less scheduling and time flexibility than working with MSCs. Concerning cell sources and seeding methods, their choice must be carefully planned according to the goals and the scope of each cell culture assay. The use of the immortal cell lines can be considered a first step in the biological characterisation of the scaffolds, to be complemented and amplified with primary cell sources.

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, Figure 6.8, Figure 6.10 and Figure 6.14 all show 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.

The solvent cast scaffolds are more porous and much less stiff (Table 6.1) 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.[11] 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[35]. Davies et al.[9] postulate ideal pore sizes for cell invasion: pores measuring less than 200μm become occluded by cells, and pores larger than 500μm induce tissue in growth. The solvent cast scaffold pore-size cannot be calibrated exactly, but it can be assumed to be

larger that 500μm. Indeed, the high porosity and interconnectivity caused by adjacent NaCl particles, induce a very open interconnected porosity (see Chapter 2). In the case of the phase-separated scaffolds, the lower porosity, smaller real pore size (< 200μm) (Table 6.1) and higher stiffness may have prevented many of the cells from penetrating the structure. Finally, perhaps cells do attain the interior of the scaffold 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 both static and dynamic 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. 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. It also simplifies cell seeding conditions as discussed above, and could require only static pre-implantation culture if the scaffold is meant to be implanted with cells. These simplifications, evidently, reduce not only the effort involved, but the time and financial investment as well.

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 (Figure 6.3 and Figure 6.4). The total protein content at day 7 is also substantially lower for the phase-separated scaffolds (Figure 6.5). 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 (Figure 6.6). 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 Figure 6.6. 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. As Figure 6.7 presents, 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.[36] 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[37], 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[38].

In sum, 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[32;39-42]. 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[43]. The effect of the phase-separated scaffolds con 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. The coating of the glass particles by the PLA is not included as a factor, since the addition of water in the composition of the phase-separated scaffolds avoids the tight coating of the glass by the PLA (see Chapter 3).

The findings of the MG63 study should be complemented with the MSC study. Indeed, the MSC study confirms the scaffolds' ability to sustain cell growth, migration and proliferation. The SEM images also confirm the adaptation of the cells to 3D environment in the scaffolds; bridging the pores and attaching to the pore walls. The quantitative studies must be interpreted with caution however. It seems solvent cast scaffolds sustain higher proliferation than the phase-separated ones, as in the MG63 study, but these results may also be due to limitations in the applicability of the testing methods. Again, the study confirmed the scaffolds can sustain cells growth within their structure in static culture conditions, indicating their porosity could be adequate for tissue in-growth.

This cell culture study has numerous limitations, and should indeed be considered as a first step toward *in vitro* characterisation of the scaffolds. It has explored various cell sources, cell seeding, and testing protocols, and great insight has been gained in the capacity of the scaffolds to sustain cell growth. Further work should be performed in order to reinforce and confirm the results, insisting on aspects such as OC activity and cell invasion. Furthermore, there is the limitation in the applicability of traditional cell biology testing protocols to 3D materials. Most protocols are adapted to a 2D multiwell cell culture environment, and often assume the supernatant contains all the information needed to characterise the study. In the case of 3D scaffolds, the supernatant is absorbed and entrapped within the pores of the scaffold, and is practically impossible to extract completely. The effect of the scaffold entrapment is further affected by the nature of the scaffold: whether it is more like a sponge (solvent cast

scaffold) and thus can be squeezed easily, or whether it is rather stiff (phase-separated scaffolds).

Special care was taken during this study to perform the assays in the most repeatable possible manner. But differences between the scaffold stiffness have surely influenced the ease of supernatant extraction and sample grinding. Thus, the proliferation results on the phase separations scaffolds could also be due to its particular architecture. Even more so in the case of the AlamarBlue assay which did not destroy the scaffold itself. In addition, the choice of testing method has proven crucial. The AlamarBlue assay, for example, being non destructive, largely reduced the number of viable cells needed for each scaffold test.

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. In fact, the cell culture should perhaps last a week or two more, in order to ensure OC has had time to be expressed. Furthermore, the effects of cell seeding should be further explored and assessed.

Despite the limitation of the cell culture studies, and the complexity of adapting the biomaterials science area to the cell biology area, these studies have given precious information on the scaffolds' biological behaviour. The adequate planning and success of future cell culture studies will be greatly enhanced by the experience gained during this study.

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.
- Cells attach to pore walls and to glass particles, and develop processes that bridge pores in 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.
- Both static and dynamic cell seeding were performed. Although more complex to perform, dynamic cell seeding enhances uniform cell distribution and limits the creation of a thick layer of cells on the surface of the scaffolds.
- 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.

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