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Stratified scaffolds for osteochondral tissue engineering applications: Electrospun PDLLA nanofibre coated Bioglass[®]-derived foams

DM Yunos^{1,5}, Z Ahmad², V Salih^{3*} and AR Boccaccini^{1,4*}

Abstract

This work focuses on designing bilayered constructs by combining electrospun poly-DL-Lactide (PDLLA) fibers and Bioglass[®]-derived scaffolds for development of osteochondral tissue replacement materials. Electrospinning was carried out using a solution of 5 wt/v% PDLLA in dimethyl carbonate. The PDLLA layer thickness increased from 2 to 150 µm with varying electrospinning time. Bioactivity studies in simulated body fluid showed that HA mineralization decreased as the thickness of the PDLLA layer increased. A preliminary *in vitro* study using chondrocyte cells (ATDC5) showed that cells attached, proliferated and migrated into the fibrous network, confirming the potential applicability of the bilayered scaffolds in osteochondral defect regeneration.

Keywords

Bioactive glass, scaffolds, osteochondral tissue engineering, composites

Introduction

Articular cartilage injuries occur frequently as a result of trauma, osteoarthritis or in some cases due to tumors.^{1,2} Articular (hyaline) cartilage comprises cartilage rich extracellular matrix composed of a complex organization of type II collagen and other minor collagens in combination with hyaluronic acid and cartilage-specific proteoglycans (mainly aggrecan). The term 'osteochondral defect' is used to indicate complex damage of articular cartilage and the underlying and adjacent i.e. subchondral bone. Osteochondral defects require a unique repair response compared to that of chondral defects because cartilage is avascular, not innervated and has a low capacity for intrinsic repair.^{3–6}

Formation of fibrocartilage in the defect void⁷ has been often observed after long-term follow-up of repair procedures, made usually by conventional surgical methods such as abrasion arthroplasty, microfracture, and subchondral bone drilling.^{8–10} Several studies have shown that tissue engineering (TE) strategies have potential for regeneration of cartilage.^{3,6,11–14}

For osteochondral tissue engineering both bone and cartilage tissue engineering principles must be combined,

for example by using engineered osteochondral (bone–cartilage) composite scaffolds of predefined pore architecture and composition which could promote both bone and cartilage tissue formation. The bone aspect of the engineered osteochondral composite, made for example from a bioactive ceramic material, may further support anchoring of the graft within the defects since bone-to-bone interfaces bond stronger and faster than

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cartilage-to-cartilage interfaces.¹⁵ Indeed, osteochondral tissue engineering can be considered a typical case of the emerging field of 'interfacial tissue engineering' as discussed in the recent literature.^{16,17} The tissue engineering of interfaces refers to the approaches being proposed to regenerate specialized tissue areas that intimately connect two different tissues of different biochemical and mechanical characteristics. The interface area usually plays an important role in transferring mechanical load between the tissues, this being the case in the osteochondral tissue interface. Due to the complex biology and mechanics of this interface, the challenge for osteochondral tissue engineering includes developing scaffolds that integrate with both the surrounding cartilage, and the underlying bone tissue maintaining the mechanical properties and integrity of the interface.

Many strategies for developing tissue engineering scaffolds for osteochondral repair consider the design of bilayered scaffolds that should be able to regenerate both cartilage and subchondral bone.^{3,6,16} This scaffold approach involves the use of different combinations of materials, morphologies and specific properties in both regions of the scaffolds. Common approaches involve:⁶ (1) seeding autologous chondrocytes at the top of the scaffold creating a cell-scaffold construct for *in vivo* implantation,^{18–21} (2) two different cartilage and bone scaffolds assembled together either before or during implantation,^{22,23} and (3) an integrated bilayered composite structure that leads to a complete integration of bone and cartilage layers without needing a subsequent joining mechanism.^{24–26} Moreover, several strategies with single-layer materials have been put forward, as reviewed by Mano and Reis.³ It is accepted that bilayered structures are more challenging to design and fabricate but they are ultimately more suitable for regenerating osteochondral defects. Such bilayered scaffolds should be able to incorporate and support the growth of different types of cells in favorable local environments requiring adequate chemical signals and mechanical stimulation, leading to the growth of the two different tissues which are characterized by different biological requirements. Bilayered scaffolds should be, therefore, designed to mimic the native ECM for each tissue type independently (rather than attempting to fabricate a single construct which should incorporate the functional requirements of both cartilage and bone in a single structure).

Unlike traditional scaffolds, bilayered structures incorporate various materials, usually in a sandwich-like or stratified design, to form composite layered morphologies. In the present paper, results are presented, in which novel bilayered scaffolds based on Bioglass[®] derived foams (for the bone aspect) coated with a fibrous PDLA layer obtained by

electrospinning (for cartilage) have been developed and preliminary *in vitro* tests utilized to characterize the material. This work evolved from our previous study in which we presented the process of electrospinning of PDLA fibers on dense bioactive glass substrates²⁷ and shows for the first time the formation of tissue engineering scaffolds combining electrospun PDLA nanofibers and bioactive glass-ceramic highly porous scaffolds.

Materials and methods

Materials

Poly(D,L-Lactide) (PDLA) (Purasorb) was purchased from Purac Biochem, Gorichem, The Netherlands. PDLA (with a density of 1.26 g/cm³ and an inherent viscosity of 2.15 dL/g) was completely amorphous. Dimethyl carbonate (DMC) of >99% purity was used as solvent (Sigma-Aldrich, UK). 45S5 Bioglass[®] powder of mean particle size <5 µm was used to make 3D porous scaffolds using the foam replica technique developed earlier.²⁸ Briefly, polyurethane foam samples of the required dimensions were immersed in a 3% wt/v PDLA-DMC solution containing 66% wt/v Bioglass[®] powder for 3 min; the foams were manually retrieved from the suspension as quickly as possible and the extra slurry was removed vigorously by hand. The 'green bodies' were placed on tissue paper and dried under a fume hood at room temperature for at least 12 h prior to the subsequent heat treatment (sintering) process. Scaffolds of nominal dimensions 5 mm × 5 mm × 5 mm were sintered at 1000°C for 2 h and coated with PDLA by a dipping process similar to that described elsewhere.²⁹ Briefly, scaffolds were slowly immersed in a 5% wt/v PDLA-DMC solution and left in suspension for 2 h. Scaffolds were then taken out and placed on tissue paper to dry at room temperature for at least 12 h. Finally, the weight of the dried scaffolds was measured using an electronic analytical weighing balance and the dimensions were measured using a digital calliper (Mitutoyo, UK).

The composite scaffolds were then coated with PDLA nanofibers by electrospinning using a custom-made apparatus described previously.²⁷ The optimum conditions for PDLA electrospinning in terms of solution viscosity and applied voltage have been previously established.²⁷ A flowing solution of PDLA (5 wt/v%) in di-methyl carbonate (DMC) was used and electrospinning was carried out at an applied voltage of 8.5 kV for a fixed flow rate of 5 µL min⁻¹. In order to vary the thickness of the fibrous mesh, the deposition time was varied between 10 min and 2 h. The structure of the obtained bilayered scaffold design is schematically shown in Figure 1.

Figure 1. Schematic diagram of the proposed bilayered scaffold structure composed of a Bioglass®-based glass-ceramic foam covered with a thin mesh of PDLLA fibers obtained by electrospinning.

Materials characterization

Electrospun nanofibrous bilayered structures were sputter coated with gold (EMITECH K550) and the scaffold morphology before and after immersion in simulated body fluid (SBF) was observed by using a scanning electron microscope (JEOL JSM-5610LV). EDS analysis was carried out using the INCA software connected to the SEM operated at an accelerating voltage of 10 kV. X-ray diffraction (XRD) analyses were also performed to investigate the possible formation of crystalline hydroxyapatite (HA) on scaffold surfaces after SBF immersion.

Bioactivity studies in simulated body fluid (SBF)

SBF solution was prepared according to the standard procedure introduced by Kokubo et al.³⁰ In typical experiments, PDLLA fiber coated Bioglass® scaffolds were immersed in 30 mL SBF (pH 7.4) in clean centrifuge tubes. These were placed inside an incubator at a controlled temperature of 37°C. The SBF was replaced twice a week. Samples were extracted from the SBF solution after 7, 14, and 28 days. Once removed from the solution, samples were rinsed gently with deionised water and left to dry at room temperature in a desiccator prior to further characterization. SEM observations and EDS analyses were carried out on the surface of the Bioglass®-based scaffolds after different SBF immersion times to investigate the formation of hydroxyapatite layer on the surface of the scaffolds. In addition, XRD analyses were done to determine the crystallinity of the formed calcium phosphate layer.

In vitro cell culture assessment

Preparation of chondrocytes cells line (ATDC). In vitro cell culture studies were carried out on various samples

using ATDC5 chondrocyte cell line. Trypsin, phosphate buffered saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS) and low-glucose Dulbecco's modified eagle medium (DMEM) were purchased from PAA the Cell culture Company (UK). Cell culture flasks were procured from BD Biosciences (Oxford, UK). Penicillin and streptomycin were purchased from Invitrogen (Paisley, UK).

The cells were resurrected from their frozen state by thawing them and transferring them quickly to centrifuge tubes containing 10 mL of warmed DMEM (supplemented with 10% v/v fetal calf serum, 100 U/mL penicillin, and 100 µg/mL of streptomycin). The cell suspension was centrifuged at room temperature for 3 min at 1000 rpm. The cell pellet was carefully re-suspended using 5 mL of DMEM and 2 mL of the re-suspended cell suspension was added in the tissue culture flasks (gas permeable; surface area of 80 cm²), followed by adding 8 mL of fresh DMEM. The flasks were incubated in a humidified atmosphere, in 5% CO₂ and at 37°C. Spent medium was changed every 2 days and the cells were regularly observed using phase microscopy. Once the cells were approximately 80% confluent, they were passaged to maintain growth.

For cell passage, medium was removed from the flask and washed with 5 mL of PBS followed by adding 2–3 mL (1 mL/cm²) of trypsin/EDTA on to the washed cell monolayer and incubated at 37°C for 2 min. The cells were examined under inverted phase microscope to observe the detachment of the cells and 8 mL (3–4× the volume of trypsin) of fresh DMEM was added to the flask. The cell suspension was then centrifuged for 3 min at 1000 rpm. The cell pellet was resuspended using 5 mL of fresh DMEM. Then, 3 mL of the re-suspended cell suspension was further divided into three new flasks (1 mL each) and 9 mL of fresh DMEM medium was added. The cells were then further allowed to expand until required. The cells were not passaged for more than 4 times and were frozen in ampoules and stored in liquid nitrogen at –196°C for further use.

Sterilization of samples. Samples were sterilized using ultra-violet (UV) light. The samples were kept in 24-well microplates and placed under UV light for 60 min, having been turned once after 30 min. This form of sterilization was employed in order not to affect the chemical or, indeed, physical composition of the materials had more conventional methods been employed such as autoclaving, dry-heat sterilization or alcohol treatment. Sterilization was followed by passivating the samples by immersing the samples in 1 mL of cell culture medium (DMEM) and incubating (37°C, 5% CO₂) for 24 h followed by cell seeding.

Cell proliferation of ATDCs in porous (3D) scaffolds

- (i) Cell seeding on substrates. The semi-confluent ATDC cells were released from the flasks by trypsinization and concentrated by centrifugation (1000 rpm, 3 min). A cell count was performed using trypan blue dye and haemocytometer. A seeding density of 50,000 cells/cm³ was used for the scaffolds. Initial cell seeding densities were the same at the time of introducing the cells to each scaffold, as quantified by performing a cell count using a haemocytometer on trypsinized cells from stock cultures. Both scaffolds with nanofibrous layer and without coating were investigated in this part of the study. The samples were seeded with the ATDC cells and all experiments were performed in 24 well microplates. 3D scaffolds were seeded with 5 μ L (50,000 cells) of cell containing DMEM and left for 3 h. After this time, 1 mL of fresh DMEM medium was added on to the wells containing the scaffolds. The plates were incubated in a humidified environment (37°C, 5% CO₂) for a period of up to 14 days. The medium of the wells was changed every second day and samples were analyzed at various time points to measure the cell proliferation. Cell culture studies were carried out on triplicates samples per experiment. Standard tissue culture plastic was used as the control surface.
- (ii) Assessment of proliferation of ATDC. The proliferation of ATDCs in bilayered porous scaffolds was assessed with Alamar Blue at 1, 4, 7 and 10 days post seeding with the Alamar Blue dye reduction assay. In this assay, Resazurin, a non-fluorescent indicator dye, is converted to bright red-fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells thereby generating a quantitative measure of viability (and cytotoxicity, as damaged and nonviable cells have lower innate metabolic activity and thus generate a proportionally lower signal than healthy cells). Cell seeded scaffolds were placed in new multiplate wells. For comparison of biocompatibility, cells were also seeded on to Tissue culture plastic (TCP) control surfaces using the same cell density. Then, cell numbers were assayed by adding 100 μ L (10% v/v) of Alamar Blue to each well, left for 4 h and fluorescence intensity of culture supernatant measured using absorption at 560 nm and emission at 590 nm for each culture time point.
- (iii) Preparation of SEM samples. Specimens from Days 1, 7, and 14 were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for a minimum of 24 h at 4°C. Subsequent dehydration using a series of graded ethyl alcohols (50%, 70%, 90%, and

100%) was performed. Samples were then critical point dried by immersion in hexamethyldisilazane for 1 min and left in a fume cupboard for 2 h. The dried samples were then attached to aluminum stubs, gold/palladium coated and examined under SEM (JEOL 5610LV, JEOL, USA) at low and high magnifications using an accelerating voltage of 15 kV.

- (iv) Statistics. The in-vitro cellular proliferation tests were performed on triplicate samples for each material, and the data represented as mean $\pm$ SD. Statistical difference was analyzed using ANOVA test and a p value of $p < 0.05$ was considered significant.

Results

Microstructure analysis

Figure 2 shows SEM images of the PDLA fibrous deposition on Bioglass[®]-derived glass-ceramic scaffolds. From the microstructure of the deposited PDLA fibrous structure, it is observed that the fibrous mesh became denser when deposition time increased from 10 min to 2 h, as shown in Figure 2(A)–(C). From the SEM images, it can also be seen that the PDLA fibers were deposited preferentially on the struts of the Bioglass[®] scaffolds, as indicated by the circles in Figure 2(A)–(C). At higher SEM magnification, the increased density of the fibrous mesh on the struts with increasing deposition time becomes evident (see Figure 2(A1), (B1), and (C1)). Moreover, SEM examination of the Bioglass[®] glass-ceramic scaffolds revealed that pores are highly interconnected and possess pores of up to $\sim$ 500 microns in size (Figure 2(A)).

Figure 3(A)–(C) shows SEM images showing different orientations of the PDLA fibers on scaffolds. Figure 3(A) is a top view of the PDLA rather homogeneous layer, while Figure 3(B) suggests that the PDLA fiber orientation on the struts was random. On the other hand, fibers deposited between strut points are seen to be partially aligned (see Figure 3(C)). This pattern was formed due to the structure of the underlying porous scaffolds and it was not observed in fiber depositions on planar surfaces, described in the previous investigation.²⁷ Figure 4(A) shows a SEM image of the interface in a PDLA/45S5 Bioglass[®] scaffold deposited for 2 h, where it is possible to observe the two distinct porous layers. Figure 4(B) shows the PDLA fibrous layer at higher magnification. The estimation of the PDLA fibrous layer thickness was obtained from SEM observation of the interface of the bilayered scaffolds (see for example Figure 4(B)). The fibrous layer thickness was estimated to be $\sim$ 150 μ m. Figure 5 shows a composite

Figure 2. SEM images showing the PDLLA fibrous deposition on Bioglass[®] derived glass-ceramic scaffolds (uncoated) at different deposition times: (A) 10 min, (B) 30 min, (C) 2 h, and (A1) 10 min, (B1) 30 min, and (C1) 2 h at higher magnification.

SEM image revealing the typical complete transverse section of the bilayered, stratified scaffolds.

SBF bioactivity studies

The bioactivity assessment of scaffolds after immersion in SBF for 7, 14 and 28 days is described in this section. Figure 6(A)–(C) show SEM images of the PDLLA fibers on a Bioglass[®] scaffold (coated) deposited for 10 min after immersion in SBF for 7, 14, and 28 days.

There is possible formation of CaP or hydroxyapatite (HA) crystals on the PDLLA fibers after 7 days immersion in SBF, as indicated in Figure 6(A). The PDLLA fibers seem to become coated with more crystals when immersion time in SBF increased from 14 to 28 days, as can be seen in Figure 6(B) and (C), as expected. EDS spectra on PDLLA fibers deposited for 10 min after immersion in SBF for 14 days (Figure 6(B)) show the presence of peaks corresponding to Ca and P, indicating calcium phosphate formation (Figure 7). Thus it can

Figure 3. (A)–(C) SEM images showing the different fiber orientations at different positions of the PDLLA fibrous layer on the surface of Bioglass® scaffolds deposited for 2 h.

Figure 4. SEM images showing the microstructure of a PDLLA/45S5 Bioglass® bilayered scaffold (coated): (A) interface of the 45S5 Bioglass® scaffold coated with PDLLA fibers after 2 h coating, (B) layer of PDLLA fibere observed at higher magnification.

Figure 5. SEM composite image showing the cross-section of a typical PDLLA/Bioglass® stratified scaffold fabricated in this investigation. (The PDLLA fiber layer was deposited for 30 min.)

be concluded that PDLLA fibers deposited for 10 min may become mineralized after immersion in SBF for 7 days and longer periods. This result was expected based on the evidence presented previously,²⁷ indicating the mineralization of PDLLA fibers on sintered Bioglass® substrates upon immersion in SBF.

Figure 8(A)–(C) show SEM images of PDLLA fibers on Bioglass® scaffolds deposited for 30 min after immersion in SBF for 7, 14, and 28 days. It can be observed that on some PDLLA fibers no HA formation has occurred after 7, 14, and 28 days immersion in SBF. Thus it can be concluded that as the PDLLA deposition time increases from 10 to 30 minutes the thickness of the PDLLA fibrous layer increases (see section above) and therefore, hydroxyapatite formation on the PDLLA fibers located on the top layer was reduced possibly because fibers are not in direct contact with the bioactive glass surface. It is well known that PDLLA is not bioactive in terms of induction of HA deposition, requiring the combination with a bioactive inorganic material, e.g. Bioglass®, to exhibit mineralization (HA formation).²⁹ Figure 9(A)–(C) show SEM images of the

PDLLA fibers on a Bioglass® scaffold deposited for 2 h after immersion in SBF for 7, 14, and 28 days. From the SEM images, it is seen that there was no HA formation on the PDLLA fibers. EDS spectra of the PDLLA fibers deposited for 2 h and after immersion in SBF for 7, 14, and 28 days (Figure 9(A1)–(C1)) also confirmed that no HA has formed on the fibers, showing only the C and O peaks corresponding to PDLLA.

Thus it can be concluded that PDLLA fiber layers deposited on Bioglass® based scaffolds for 2 h should not mineralize after immersion in SBF for 7, 14, and 28 days. This is a very important result for the application of the present stratified composites (Figure 1) in osteochondral tissue engineering, since it is required that the cartilage side does not mineralize, e.g. where the PDLLA mesh should support the attachment, growth, and proliferation of chondrocytes only. In summary, SEM observation and EDS analysis have confirmed that formation of HA (indicating bioactivity) on PDLLA fibers when immersed in SBF was reduced as the PDLLA fiber deposition time increased from 10 min to 2 h, and the thickness of the fibrous PDLLA layer increased from ~2 to ~150 μm.

Cell culture assessment of bilayered osteochondral scaffolds

This section presents the results of a preliminary assessment of cell compatibility of PDLLA fiber coated bilayered scaffolds for potential osteochondral tissue engineering applications. The main aim of this section is to report, for the first time, the cell–biomaterial interactions on these bilayered scaffolds in terms of chondrocyte cell attachment, spreading, and proliferation. The ADTC5 cell line was chosen in this study due to its performance as a suitable cell line for development of tissue engineering strategies aimed at cartilage generation.³¹

3D Bioglass®/PDLLA scaffolds coated with PDLLA fibers (bilayered scaffolds) fabricated using the optimum conditions of electrospinning were used. Scaffolds without the fiber deposition were also investigated for comparison purposes. The cells were cultured for up to 10 days. Cell proliferation was quantitatively measured using the Alamar Blue assay. The data are illustrated in Figure 10. Visual inspection indicates that cells can infiltrate into the scaffolds. The cell proliferation data shows that scaffolds coated with PDLLA fibers exhibit a higher cell proliferation than the scaffolds without PDLLA fibers at day 4, 7, and 10. However, at day 7 and 10, the cell proliferation of the scaffolds deposited with PDLLA fibers (bilayered scaffolds) exhibits higher ability to foster cells than the control (tissue culture plastic, TCP). Although overall cell

Figure 6. SEM images of PDLLA fibers on Bioglass[®] based scaffolds (coated) deposited for 10 min after immersion in SBF for (A) 7, (B) 14, and (C) 28 days.

number increased in all scaffolds during the culture period, PDLLA fibers coated scaffolds (bilayered) induced significantly higher ($p < 0.01$) cell proliferation compared to scaffolds without PDLLA fibers. These results can be confirmed by the SEM images, as discussed next.

At days 1, 7, and 14, selected cell-seeded bilayered scaffolds were fixed and were observed by SEM. Figure 11(A) shows that at day 1, several chondrocyte cells attached and proliferated on the surface of the PDLLA fibers of the bilayered scaffolds. Rounded cells suggest dividing cells during the process of cytokinesis. At higher magnification, it can be observed that cells are clearly attached to the fibers and formed a 3D cell-matrix network, as shown in Figure 11(B). In particular at day 7, many cells are clearly attaching and spreading to and amongst the fibers and also communicating with one another using shared cytoplasmic filopodia (suggesting cell-cell interaction), as shown in Figure 12(A)), such that they could only be recognised at high magnification (see Figure 12(B)). In comparison

to day 1, the cells proliferated and spread into a larger area and formed a cell-matrix network. The cells also showed guided growth according to the PDLLA fiber orientations, giving rise to a network guided by the architecture of the fibrous scaffolds, as shown in Figure 12(B). At day 14, the cell spreading and proliferation increased as can be seen from the higher density of cells, as shown in Figure 13. The cells appeared to adhere to the fibers, and they seem to have started to migrate through the pores and grow within layers of fibers as indicated in Figure 13. These results confirm that the PDLLA fibers deposited for 2 h using the optimum electrospinning parameters are suitable as a substrate for chondrocyte cells. Cells were able to attach, proliferate and migrate within the fibrous PDLLA layer.

Discussion

In this investigation, we have assessed the potential application of a fibrous PDLLA matrix combined with Bioglass[®]-based foams as suitable stratified

Figure 7. EDS analysis on PDLLA fibers deposited on Bioglass[®] scaffolds (coated) electrospun for 10 min and immersed in SBF for 14 days. (a) SEM image, (b) spectrum. (The Au peaks are due to the gold coating used for SEM sample preparation.)

scaffold for osteochondral TE applications. By means of electrospinning, uniform PDLLA fiber meshes were produced with fiber diameters in the range between ~ 100 nm and ~ 0.2 μm to form a 3D fibrous architecture on 45S5 Bioglass[®] based composite scaffolds. Results showed that the PDLLA fibrous matrix deposited on Bioglass[®] foams increased chondrocyte cell attachment and proliferation. Increased surface roughness due to electrospun fibers should lead to better cell attachment and proliferation of chondrocyte cells.^{32,33} SEM images confirmed qualitatively that the topography (roughness) of the 3D layered composite scaffolds has been altered due to the deposition of PDLLA fibers by electrospinning (see Figure 2). The surface roughness increased more than 13 times in terms of the rms value for 2D Bioglass[®] sintered pellets coated with PDLLA fibers.²⁷ This result is in agreement with the study by Chen et al.³⁴ who modified the surface of poly(ϵ -caprolactone) membranes via electrospinning. Topography (roughness) of the porous bilayered scaffolds could not be measured by white light interferometry because the surface is not flat. To produce a uniform fibrous layer, we have optimized the fabrication process and determined the electrospinning parameters, such as polymer viscosity, voltage, flow rate, and electric field, as previously discussed.²⁷ It should be pointed out that if even one of these parameters was not optimal, irregular fibers could form, including a high number of bead-containing fibers, suggesting

Figure 8. SEM images of PDLLA fibers on Bioglass[®] (coated) scaffolds deposited for 30 min after immersion in SBF for (A) 7, (B) 14, and (C) 28 days.

Figure 9. SEM and EDS results on PDLLA fibers deposited on Bioglass® scaffolds (coated with 5 wt% PDLLA) deposited for 2 h after immersion in SBF for (A) 7, (B) 14, and (C) 28 days and EDS spectra after immersion in SBF for (A1) 7, (B1) 14, and (C1) 28 days.

Figure 10. Cell proliferation up to 10 days for Bioglass®/PDLLA composites with and without PDLLA fibers deposited on the scaffolds (Error bar = $\pm$ SD; $n = 6$) (with and without fibers, ** $p < 0.01$).

their coordinated optimization during the electrospinning process. The optimum electrospinning parameters were obtained using flow rate = $5 \mu\text{L}/\text{min}$, voltage = 15 kV, distance of application of electric field = 15 cm, deposition time = 2 h and employing solutions of 5 wt% PDLLA. Optimal fiber fabrication parameters are specific for a particular poly(α -hydroxy ester).³⁵ To the best of our knowledge, electrospinning of polymer fibers on bioactive glass scaffolds for tissue engineering has not been tried before. However, electrodeposition of fibers on a variety of patterned and porous substrates to develop special nano and microstructures has been reported.^{36–38} For example, electrospinning of poly(ϵ -caprolactone) has been carried out using metallic meshes used as collector to produce special topological nanofibrous structures.³⁶ Usually the deposition behavior in terms of fiber orientation and mesh architecture can be affected by the structure of the collector and well-arranged fiber patterns and

Figure 11. SEM images showing ATDC5-cell adhesion and proliferation on PDLLA mesh of bilayered scaffolds at day 1 at (A) low and (B) high magnification.

ordered fibrous (nano)structures can be produced.^{36,37} In our case, the scaffold struts provide the point of contact of the fibers and determine the formation of a particular pattern of the mesh, as shown in Figure 2(B) and (C). By varying the porosity structure of the scaffold used as substrate it is possible to affect the fibrous topography of the deposited mesh which indicates the versatility of the method for a variety of scaffold designs.³⁸

A scaffold intended to be used in TE needs to have structure resemblance to the ECM of the tissue to be regenerated and the incorporation of a fibrous layer aims at mimicking, to some extent, the natural matrix for osteochondral TE applications.^{3,39} The fabrication of fibrous layers by electrospinning for cartilage

Figure 12. SEM images showing the ATDC5-cell proliferation and spreading on PDLLA mesh of the bilayered scaffolds at day 7: (A) low and (B) high magnification.

TE applications has been extensively investigated,³ as described in the introduction. The present results show that the deposition of a PDLLA fibrous layer on the surface of 3D Bioglass[®] composite scaffolds by electrospinning could be controlled reaching 150 µm in thickness which seemed to be sufficient to prevent HA mineralization from occurring in SBF. This effect is significant for the application of the bilayered constructs for osteochondral TE applications, which should not show mineralization on the cartilage side.

In this regard, Figure 14 shows schematically the structure of the optimal stratified scaffold, representing the desired structure of the scaffolds formed by PDLLA fibers deposited for 2 h on the basic 3D PDLLA coated Bioglass[®] foam. The PDLLA fibrous layer should reach in this case ~150 µm in thickness. After immersion in SBF for 14 days, PDLLA fibers which are

in direct contact with the Bioglass[®]-derived foam substrate can become mineralized due to the well known bioactive effect of bioactive glass surfaces.²⁸ It is expected that the formation of HA on the Bioglass[®]-derived scaffold surface in contact with the first layer of PDLA fibers will increase the bonding strength at the PDLA fiber/Bioglass[®] scaffold interface (e.g. as schematically indicated in Figure 14(B)). Although the PDLA fiber/Bioglass[®] scaffold interface strength was not quantitatively measured in this study, qualitative manual assessment of the integrity of the layered structure confirmed that the scaffolds can be manipulated for applications. It is also worthwhile to notice that the extent of mineralization of PDLA fibers will decrease with increasing distance from the interface and it will be completely suppressed

in the outer PDLA fiber layers, which are most distant from the bioactive glass–ceramic surface. From the EDS analysis reported in the previous section, it was confirmed that there is no HA formation on the fibers in the top layer after immersion in SBF for up to 28 days. This mechanism is very important to ensure: (1) strong interface bonding of the PDLA layer to the bioactive glass substrate and (2) smooth transition at the interface given by the gradual change of HA layer formation dependent on the distance from the Bioglass[®] scaffold surface.

The risk of possible delamination between the polymeric and composite constituents, respectively, was avoided since the bilayered constructs showed an integrated continuous interface assessed by SEM images and SBF in vitro assessment. It is hypothesized that the residue solvent could enhance the adhesion of PDLA fibers to the PDLA coated Bioglass[®] scaffold substrate, forming a strong bond, according to previous results reported by Chen et al.³⁴ The quantification of the interfacial adhesion strength has not been covered in the work and needs dedicated further investigation in the future. Nevertheless the bilayered scaffolds developed here were qualitatively robust and of adequate structural integrity for their handling for material characterization and for cell culture studies. The microstructure, in addition to the chemical composition and polymer molecular structure, are the primary determinants of the degradation profile of a 3D tissue-engineering scaffold,³³ although macroscopic features such as the morphology and architecture of the scaffold (e.g. porosity) are also important factors. Apart from the materials aspects, the environment (biochemistry) in which the material is placed is vitally important. In relation to our scaffolds, ultra-fine electrospun fibers would be more susceptible to hydrolysis compared with larger fibers when used in vitro and in vivo. The results showed that PDLA fibers can maintain their structural stability in SBF and in cell culture

Figure 13. SEM image showing ADTC5-cells migrating through the pores and growing within layers of the PDLA fibrous network at day 14.

Figure 14. Schematic diagram showing the development of the interface of a bilayered scaffold: (A) as fabricated and (B) stratified scaffold after immersion in SBF for 14 days.

media for up to 14 days, as shown by the SEM images in Figures 9 and 13, respectively.

Moreover PDLLA fiber roughness is hypothesized to act as anchorage for cells, to enhance cellular adhesion and spreading. A wide variety of cells, including chondrocytes, are able to detect changes in the surface topography and approaches to topographical control of cells have been reviewed by Wilkinson et al.⁴⁰ Our results also show that chondrocyte cells proliferated better on 3D-scaffolds coated with PDLLA fibers compared to 3D-scaffolds without fibers, as indicated by the data in Figure 10. Thus, it is hypothesized that in the present composite scaffolds PDLLA fiber roughness is relevant in controlling cell behavior when the ion release from the materials (e.g. material chemistry) during degradation is fixed. In this case the larger surface area achieved by the fibrous modification provides more binding sites for the cells to attach and proliferate. The enhanced roughness and fibrous topology should also contribute to stronger cell–cell interaction. It was reported that fibrous features with high surface area to volume ratio are able to absorb serum and ECM proteins for better cell adhesion. Woo et al.⁴¹ have reported that fibronectin and vitronectin preferentially adsorbed to a nanofibrous scaffolds at the rate of 2–4 times higher than on solid walled scaffolds. Thus the present bilayered scaffolds exhibit both advantages by having the basic 3D bioactive porous basic 3D Bioglass[®] based scaffold for releasing the relevant ions for bone regeneration and the PDLLA fibrous layer leading to improved chondrocyte cell adhesion. The smaller pore size of the PDLLA fibrous layer compared to the larger pore size of the 3D Bioglass[®] based composite scaffolds (see Figure 4) could also improved chondrocytes cell attachment. Indeed, investigations have shown that cells cultured in a 3D scaffold with large pore size (30 times the cell diameter, which is of the range 10–15 μm) are more likely to behave as in a monolayer culture condition, which is known to promote dedifferentiation of chondrocytes.⁴² On the other hand, too small a pore size may impair homogenous cell distribution within the scaffolds as well as access to nutrients.⁴³ To maintain the initial shape of the scaffold surface and the number of attached chondrocytes, adequate mechanical strength and highly cellular adhesivity are requirements for scaffolds in cartilage tissue engineering. Based on the current initial data we may reasonable conclude that our novel bilayered construct based on PDLLA fibers on 3D Bioglass[®] porous composite substrates can be potentially a suitable biomaterial to create a 3D scaffold with high cellular adhesivity and the required support for chondrogenesis in osteochondral tissue engineering applications. In the present scaffolds, PDLLA fibers penetrate into the Bioglass[®] scaffold region (as depicted

in Figure 5). In addition the interfiber depth is greater than 10 μm in many regions which would allow sufficient space and room for cells to migrate deeper into areas beneath the surface. As in the present study the culture times were relatively medium-term, longer term future studies would indeed need to be carried out to confirm cell migration to deeper layers.

It should be also considered that chondrocytes exhibit a profound change in their phenotype after isolation from the ECM. To maintain the chondrocyte phenotype through the process of cartilage regeneration, scaffolds must have the potential to support chondrogenesis while maintaining the chondrocyte phenotype. These aspects were not yet covered and will be considered for future studies. Another limitation of this study is that the results were derived from an *in vitro* experimental model. Therefore, the biocompatibility of the current layered scaffolds in living joints is still unclear and remains an important task for future investigations.

Conclusions

Stratified PDLLA fiber coated Bioglass[®] composite scaffolds were designed, fabricated, and characterized. Electrospinning at an applied voltage of 15 kV, flow rate of 5 $\mu\text{L}/\text{min}$ using a solution of 5 wt/v% PDLLA in DMC led to the development of a fibrous PDLLA layer on PDLLA coated Bioglass[®] derived foam. PDLLA fibers integrated well onto the composite scaffolds as the electrospun deposition time increased from 10 min to 2 h. The PDLLA fibrous layer thickness was controlled by deposition time up to $\sim 150 \mu\text{m}$. Bioactivity studies in SBF for 7, 14, and 28 days immersion time showed formation of HA, the extent of HA formation decreased as the thickness of the PDLLA layer increased. A preliminary study using chondrocyte cells (ADTC5) showed that scaffolds have chondrocyte cell-supporting ability. Cells were seen to infiltrate and migrate effectively into the porous network structure of the PDLLA fibrous layer. The PDLLA fiber stability was seen to be maintained up to 14 days in culture solution, however the effect of the attachment of cells and extracellular matrix produced by cells at longer cultivation periods could lead to accelerated PDLLA fiber breakdown.⁴⁴

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