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Soluble phosphate glass fibres for repair of bone-ligament interface

M. BITAR, J. C. KNOWLES, M. P. LEWIS, V. SALIH*
Department of Biomaterials/Tissue Engineering, Eastman Dental Institute, University College London (UCL), 256 Gray's Inn Road, London WC1X 8LD, UK
E mail: v.salih@eastman.ucl.ac.uk

Phosphate-based fibres of the generic composition $(CaO)_{0.46}$–$(Na_2O)_n$–$(Fe_2O_3)_y$–$(P_2O_5)_{0.50}$ have been evaluated, *in vitro*, as three dimensional scaffolds for tissue engineering of the hard-soft tissue interface by assessing the fibre solubility and growth and functional gene expression of human cells. Primary human osteoblasts and fibroblasts were seeded onto scaffolds and maintained in culture for up to 21 days. Fluorescent immunolabeling revealed the spread cell morphology and significant proliferation pattern on these fibres, particularly on the 3 mol% Fe_2O_3-containing formulation. Real-time quantitative Polymerase Chain Reaction (rtQ-PCR) analysis of gene expression using TaqMan® Probes was preformed and it has been established that committed cell differentiation was maintained by both cell types, and was strongly related to the 3 mol% Fe_2O_3 glass composition. These novel, readily manufactured, soluble glass fibres offer a biocompatible and biochemically favourable alternative in the search for suitable degradable materials used in Tissue Engineering.

1. Introduction

Processes by which populations of cells form organised tissues comprise a complex series of coordinated steps in which important signals are supplied to the cells during key temporal and spatial events. During normal development and repair, tissue formation is a naturally occurring phenomenon and numerous advances in biomolecular sciences have elucidated some of the important underlying mechanisms associated with such tissue changes. The last decade has seen a large increase in research efforts with respect to the techniques of exposing various mammalian cell types to *in vitro* environments, with the aim of promoting normal tissue formation for potential use in the development of suitable tissue engineered constructs for replacing diseased or damaged tissues. More recently, tissue engineering approaches have focussed on the requirement to provide environmental signals to cell populations *in vitro* in order to promote cell proliferation and appropriate differentiation.

The potential health benefits to society of engineered tissue constructs are manifested in the ideal that unlimited quantities of functional tissues may be supplied to patients for use in applications where tissue availability is severely restricted, such as cell/organ donation and tissue morbidity. One specific area where this is an important feature is the supply of tissue for implantation to patients that require the restoration of function e.g. blood vessels and tissues including cartilage, liver and pancreas. Moreover, tissue engineering not only helps to replace tissue but also augment repair processes *in vivo*. In this case, tissue regeneration occurs directly within the patient and is enhanced and/or modified by 'engineered' intervention. Examples of this include numerous material developments for bone and ligament regeneration.

In orthopaedics, the regeneration of the cruciate ligament is a significant clinical problem [1]. Quite often, this injury occurs in younger patients as a consequence of sporting activities and/or trauma and, once ruptured, restoration of this tissue is difficult. Current therapies make use of synthetic materials, autografts and also allografts. However, all of these methodologies, although somewhat successful, are far from ideal and have inherent problems, such as long term fatigue failure, graft rejection, donor site morbidity and the requirement for revision surgery. Cell transplantation as a concept for tissue engineering involves (i) the isolation and *in vitro* expansion of the patient's cells relevant to the defective tissue; (ii) the subsequent seeding of these cells onto a scaffold constituting biocompatible and, in some cases, biodegradable materials; (iii) induction of an *in vitro* "tissue" that can later be implanted into the defective area where it may continue to develop into a functional structure.

This study attempts to utilise novel soluble phosphate-based glasses as potential scaffold materials for the purpose of cell transplantation [2]. Thus, such glasses possess several advantages allowing them to form excellent substrates for tissue engineering. These

*Author to whom all correspondence should be addressed.

include suitable biocompatibility, biodegradability, excellent physical properties and their simple and rapid laboratory production, as previously shown by our department [3–8]. The overall aim of this paper was to develop methodology to assess the potential of attaching human fibroblasts and osteoblasts to a suitable scaffold material to form the basis of an engineered tissue construct for use in the repair of the ligament/bone interface. This would be achieved by biocompatibility assessment of iron-containing phosphate-based glass compositions to be used as substrata, for both human primary osteoblasts and fibroblasts, by means of cell proliferation and maintenance of phenotype specific to each cell type.

2. Materials and methods

2.1. Human cell culture

Craniofacial osteoblasts (HOB) were isolated from mandibular (alveolar bone) fragments using enzyme digestion and explant culture [9]. Oral fibroblasts (HOF) were obtained directly from punch biopsies of buccal oral mucosa. All tissues were obtained with approval from the local ethics committee and after informed patients' consent. On retrieval, biopsies were placed in tissue culture growth medium (GM) consisting of D-MEM, supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin prior to cell isolation. Passages 4–6 (HOB) and 12–14 (HOF) were used throughout the studies. Cultures were maintained in a humidified atmosphere, at 37 °C and 5% CO_2/95% air. Cell seeded fibre constructs were placed in 55 mm petri dishes (Bibby Sterilin, UK), in 5 ml GM, whereas cells grown in 24-well plates (Orange scientific, Belgium), were placed in 1.5 ml GM, and used as control where applicable. GM was changed every 2 days.

2.2. Production/characterisation of glass fibres

Using the precursor materials NaH_2PO_4, $CaCO_3$, P_2O_5 and Fe_2O_3 (BDH), three quaternary $[(CaO)_{0.46}–(Na_2O)_n–(Fe_2O_3)_y–(P_2O_5)_{0.50}]$ formulations were manufactured (Table I). In comparison, apreviously studied ternary $((CaO)_{0.48}–(Na_2O)_{0.02}–(P_2O_5)_{0.50})$ composition was also manufactured. Briefly, chemical components were weighed, mixed thoroughly, placed in a Pt/10% Rh crucible (Johnson Matthey, Hertfordshire, UK) and heated for 75 min, at 1200 °C, in a Carbolite RHF 1500 furnace (Carbolite, Derbyshire, UK). The glass melt was subsequently poured onto a steel plate to cool at room temperature. Glass batches were transferred into a Lenton furnace incorporated Pt/10% Rh crucible and left to melt and homogenize, at 1300 °C, for 60 min. At a temperature range of 1230–1270 °C, specific to each composition, an appropriate melt viscosity was reached and fibres were drawn, via a 1mm outlet in the crucible base, onto a stainless steel drum rotating at a frequency of 800 rpm.

TABLE I Ternary and quaternary phosphate based glass compositions

	Composition (mol%)			
Glass code	P_2O_5	CaO	Na_2O	F_2O_3
$(CaO)_{0.46}–(Na_2O)_{0.04}–(P_2O_5)_{0.5}$	50	46	4	0
$(CaO)_{0.46}–(Na2O)_3–(Fe_2O_3)_1–(P_2O_5)_{0.50}$	50	46	3	1
$(CaO)_{0.46}–(Na2O)_2–(Fe_2O_3)_2–(P_2O_5)_{0.50}$	50	46	2	2
$(CaO)_{0.46}–(Na2O)_1–(Fe_2O_3)_3–(P_2O_5)_{0.50}$	50	46	1	3

The fibre configuration was transferred onto 32 × 32 mm custom made stainless steel frames; fibre ends fixed using cyanoacrylate glue gel (Henkel UK Ltd.) and the 4 mm frame edges coated with cell repellent biocompatible RTV 118 silicone (Techsil Ltd., UK) to prevent non-fibre associated cell adhesion and cyanoacrylate-medium contact [10]. A single parallel configuration was produced resembling bone and ligament tissue architecture respectively. Scaffolds were sterilised in 70% ethanol and immersed in GM for 24 h prior to seeding.

Fibre bundles were embedded in an epoxy resin block (Struers, UK) which was consequently cut perpendicular to the fibre axes, polished and mounted on an inverted light microscope stage equipped with an image capture device. Digital images of sections, X20 magnification, were acquired and fibre diameters measured using Image-Pro Plus software (Media Cybernetics UK, Berkshire, UK). Solubility rates per glass formulation were determined by measuring weight loss over time in GM. Fibre bundles weighing 100 mg were placed in 6 well-plates (Orange Scientific), incubated in 5 ml GM to ensure consistency with *in vitro* culture conditions and fibres were dried and weighed for up to 10 days. The medium was replaced every 2 days.

2.3. Immunocytochemistry

To establish the survival and proliferation patterns on each glass composition, cells were seeded at 3.2 × 10^3 cell/cm^2, fixed at days 1, 7 and 14 with 100% ice cold methanol, nuclear DNA was stained with propidium iodide 1:1000 (Sigma-Aldrich, Dorset, UK) for 30 min and a minimum of 200 nuclei were directly counted, at ×20 magnification, in adjacent microscopic fields. Morphology of adherent cells was assessed by visualizing the intermediate filament protein, Vimentin. At 7, 14 and 21 day time points the cells were fixed as above, labeled with cy3 conjugated mouse monoclonal anti-vimentin antibody (1:200, Sigma-Aldrich) for 30 min. The nuclei binding DAPI stain (Sigma-Aldrich), at 1:1000 dilution, was included in the labeling process after which, digital microscope images were obtained via a CoHu CCD device. Leica DMIRB inverted fluorescence microscope(Leica Microsystems (UK) Ltd., Bucks, UK) was used to carry out cell counting and imaging for all specimens.

2.4. Real-time quantitative PCR

Seeding density was increased to 3.2 × 10^4 cell/cm^2 to take into consideration the assay sensitivity level and to

maximise the retrieval of cellular RNA**.** Gene expression was compared in both HOB and HOF cells seeded on glass fibres, in GM, against control cells seeded in 24-well plates (Orange). Additionally, and to investigate the osteogenic media effect on the HOB constructs, GM was supplemented with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate (Sigma-Aldrich).

Adherent cells were lysed at days 14 and 21 in culture, and total RNA extracted, using a RNeasy Mini Kit (Qiagen Ltd., UK), and converted into cDNA (high capacity cDNA archive kit, Applied Biosystems, UK). Conjugated to FAM (6-Carboxyfluorescein) reporter dye, TaqMan Probes (Applied Biosystems) were used to target corresponding bases in the cDNA strands. The presence of osteoblast-(Osteonectin and Cbfa-1) and fibroblast-associated (Collagen-I and P4HP3) nucleotide sequences, and their transcription rates, were assessed by quantifying the amplified FAM signal after 40 thermal cycles. This process took place using 25 μl reaction volume, in 96 reaction plates using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Relative quantification studies of Q-PCR, using the 7300 SDS software, were conducted against the expression of an rRNA encoding house keeping gene, 18S.

2.5. Statistical analysis

Statistical analysis was applied using a one-way ANOVA test using SPSS for windows (release 11, SPSS UK Ltd., UK) followed by Tukey post-hoc at 95% confidence interval; $p < 0.05$ was considered significant.

3. Results

3.1. Characterisation of glass fibres

Degradation rates, which were the *slowest* rates, demonstrated the best cell response. Thus, calculating the hourly percentage weight loss in solution demonstrated that the 3 mol% Fe_2O_3 glass to have the slowest degradation rate (Fig. 1(a)). Mean fibre diameters for the 1, 2 and 3 mol% Fe_2O_3 glass forms were 30.07, 30.36 and 29.37 μm, respectively. Thus, 30 μm was considered the average diameter of fibres used in scaffold fibre alignment. All glass compositions displayed significant weight loss by day 10 in GM (Fig. 1(b)).

3.2. Cell viability, proliferation and morphology

A clear proliferation pattern, for both cell types, was associated with composition as a significant increase in cell numbers occurred after 14 days in culture (Fig. 2). No growth in the seeded population was associated with the 2 mol% Fe_2O_3 glass fibres and the average cell number for HOB and HOF cells, at the most advanced time point, were shown to be significantly lower compared to those on the 3 mol% Fe_2O_3 glass. Cell survival ceased on ternary glass fibres and the 1 mol% Fe_2O_3 quaternary form at day 7 and these compositions, therefore, were excluded from further studies. Fluorescent microscopy evaluation of the cytoskeleton, at days 14 and 21 in culture, revealed the presence of confluent HOB and HOF cell populations where cells exhibited typical flat and well spread morphology aligned parallel to the fibre axes (Fig. 3).

3.3. Gene expression

No significant differences were noticed in osteocalcin gene expression between HOB cells cultured on both fibre compositions, at days 14 and 21 in culture, and the control cells (data not shown). Osteonectin transcription, however, was up-regulated on 3 mol% Fe_2O_3 containing fibres as a function of time in culture (Fig. 4(a)). At day 14, equal Cbfa1 mRNA levels were expressed among cells seeded on the two glass compositions and the control surface whilst, at day 21, Cbfa expression, although reduced compared with control cells, was lower in cells grown on the 2 mol% Fe_2O_3 composition than those on the 3 mol% Fe_2O_3 fibres. Osteonectin synthesis matched that of the control cells (Fig. 4(b)). Analysis of HOF cells cDNA at day 14 revealed similar cellular expression of Collagen-I on the 3 mol% Fe_2O_3 glass and the control surfaces whereas, in contrast, 2 mol% Fe_2O_3 fibres induced a reduced expression of Collagen-I gene when compared to the control (Data not shown). At day 21 in culture, regulation of Collagen-I was equally expressed by the seeded fibroblasts on all surfaces. Quantification of 5B5 gene regulation between control and scaffold seeded HOF cells yielded similar results at both time points (Data not shown). 5B5 and Collagen-I transcription, overtime in culture, remained constant on all surfaces.

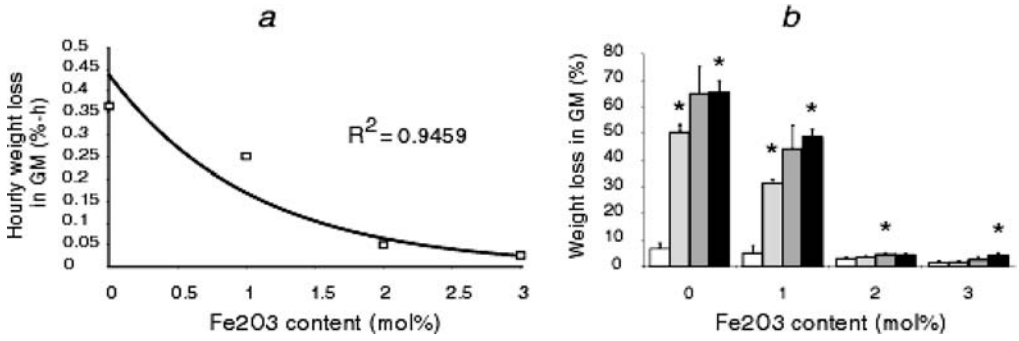

Figure 1 (a) Glass solubility rate in GM as a function of iron content; (b) weight loss values at days 1 (white bars), 4 (light grey bars), 7 (dark grey bars) and 10 (black bars) in GM. (∗) represents a significant increase in weight loss ($p < 0.05$), compared with weight values at earlier time points. Weight loss comparison was composition related against time in culture. ($n = 3$; error bars = ± s.d.)

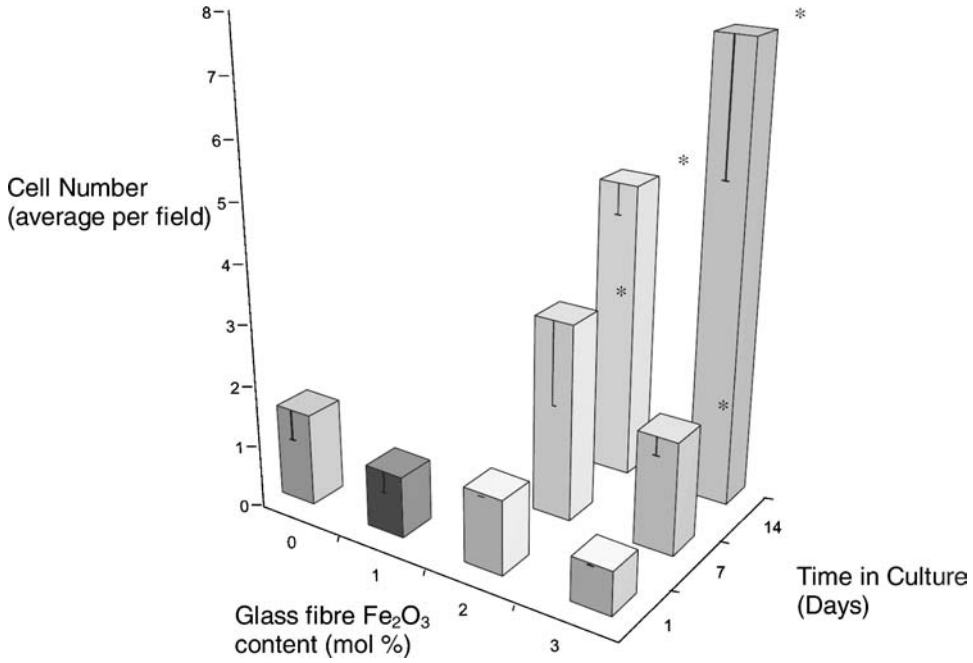

Figure 2 HOB proliferation patterns on fibres of 0, 1, 2 and 3 mol% iron content after 1, 7 and 14 days in culture. (*) indicates a significant increase in numerical value on one composition against time in culture, ($p < 0.05$) ($n = 3$, error bars $= \pm$ s.d.)

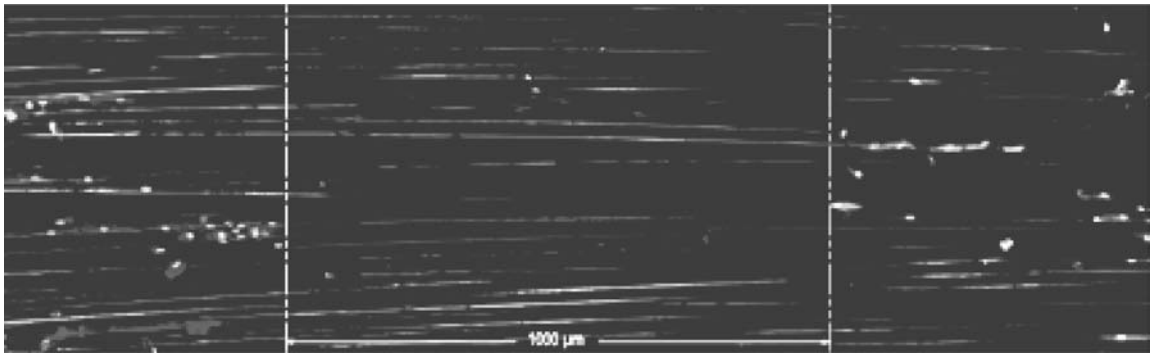

Figure 3 HOB cells (left side) and HOF cells (right side) seeded on $(CaO)_{0.46}$-$(Na_2O)_{0.01}$-$(Fe_2O_3)_{0.03}$-$(P_2O_5)_{0.50}$ after 3 days in culture. This composite light-fluorescent micrograph was composed from 3 adjacent microscope field images. Glass fibres were pseudo colored and the nuclei were labeled with DAPI.

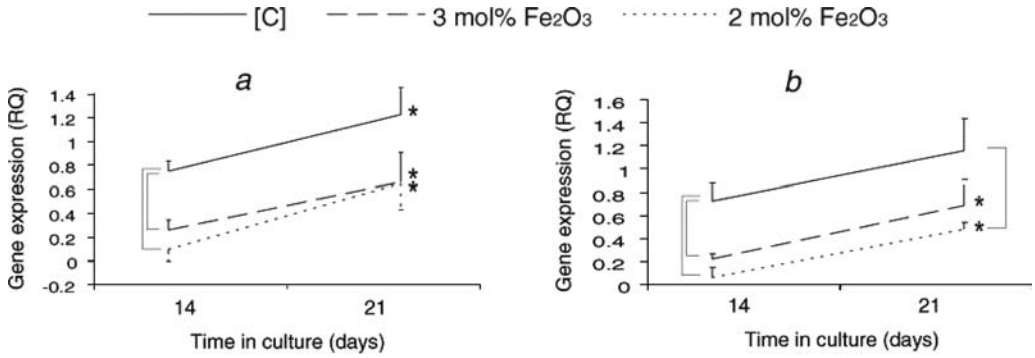

Figure 4 Relative quantification of gene expression of osteonectin (a) and Cbfa1 (b) in HOB cells seeded on 2 mol% Fe_2O_3 glass fibres, 3 mol% Fe_2O_3 glass fibres and tissue culture plastic (where C = control) after 14 and 21 days in culture. (∗) indicates significant gene up-regulation on one surface against time. Significant differences in gene expression for cells seeded on various surfaces, and at each time point, are linked using square brackets. ($p < 0.05$). ($n = 3$, error bars $\pm$ s.d.)

3.4. Gene regulation: Osteogenic medium effect

The inclusion in the GM of ascorbic acid and sodium β-glycerophosphate induced up-regulation of osteonectin and Cbfa-1 expression in control and constructs with HOB cells. At day 21, no significant differences in osteonectin expression were evident, among cells seeded on all substrates, whereas Cbfa1- mRNA levels were lower, compared to the control, in the 2 mol% Fe_2O_3 glass seeded cells (data not shown).

4. Discussion

The ability of any soluble substrates to provide a cell-tissue delivery system for repair, or to replace damaged

musculoskeletal tissues depends upon various factors. Adverse biological reactions evoked by the presence of a material must be negligible or limited and, most importantly, the material must support and maintain the processes of cell attachment, differentiated function and long term synthesis of ECM proteins [11]. In addition, normal tissue structure, coupled with elasticity is imperative if the material is to withstand the hydrostatic pressures and mechanical loads once implanted into the host [12]. The capability of producing three-dimensional templates of appropriate shape and form of the material is also important, such that *in vitro* cell invasion is permitted in a uniform manner throughout the entire structure and the efficient delivery of nutrients, growth and regulatory proteins is facilitated.

Phosphate containing glass forms have been designed and evaluated extensively by our group as bioactive materials combining structural and biodegradable properties [13]. These glasses have been shown to be bioactive by bonding to collagen in both hard and soft tissues [14]; to support the long term attachment and function of human craniofacial cells; and to elicit negligible inflammatory responses by the immune system [15, 16]. It is against this background, that quaternary iron-phosphate glass fibres have been assessed as scaffolds for engineering the hard-soft tissue interface [4, 17].

Constructs were produced so that fibre alignment was conducted in a uniform and consistent fashion, thus eliminating the experimental variability related to random arrangement of fibres and ensuring, at this stage, that composition variability was the main biocompatibility factor under investigation. As expected, glass dissolution rate was decisive in determining the nature of cell-composition interaction as the more soluble fibres, of the ternary and 1 mol% Fe_2O_3 formulations, failed to support the survival of cells beyond 7 days in culture, whereas substantial growth rate took place on the least soluble fibres i.e. 3 mol% Fe_2O_3. Cells displayed the well spread morphology critical for an *in vitro* functioning anchorage dependent population [18].

Osteocalcin transcription factor encoding gene, Cbfa1 expression and regulation is truly indicative of osteoblastic function as osteocalcin is synthesised, as a mineralization inhibitor, when remodeling of the bone matrix is activated [19, 20]. Production of the binding noncollagenous glycoprotein osteonectin, on the other hand, indicates active engagement in the heterogeneous nucleation during matrix mineralization [21, 22]. Both genes were activated and transcribed in the HOB cells attached to the glass fibres with mRNA levels, associated with the 3 mol% Fe_2O_3 composition, matching those of the control cells at days 14 and 21 in culture. The addition of osteogenic supplements to stimulate osteoblastic activity, promote mineralization and enhance possible scaffold-associated bone tissue development [23] has resulted in up-regulating Cbfa1 and ostenectin transcription. The presence of glass fibres, throughout the time in culture, resulted in no negative impact on the fibroblast phenotype as the transcription of COL 1, vital in maintaining soft connective tissue ECM [24, 25], has been maintained. Regulation of 5B5 gene encoding proline-4-hydroxylase (P-4-H), an enzyme implicated in precollagen molecule assembly, has also been expressed at a level parallel to that of the control cells as activated fibroblast characteristics were preserved when in contact with the fibre surface.

It is important when attempting to generate a complex structure, such as the bone-tendon/ligament insertion, to take the architecture of the tissue into consideration. In order to combine two histologicaly diverse entities so that an interface can be established, and evolve, the constituting cells must be seeded separately on one continuous incorporating scaffolding system whereby this continuity will later guarantee the establishment of a functional bond between the two elements. We have achieved successful co-culture concept implementation that was expressed by fluorescent microscopy as a central zone, void of cells, clearly separated two well defined opposing cell populations.

This implementation, however, must be further developed in future studies so that culture conditions specific for the initial development of each tissue can be individually provided. Evaluating the 3 mol% Fe_2O_3 incorporating phosphate glass fibres scaffolding system has outlined, and established, a foundation for a three-dimensional hard-soft tissue engineering device. This design must be developed by future studies so that the formation of a tangible volume of structural tissue can be induced through, for example, the inclusion of bone and tendon tissue growth factors. It is also imperative that the *in vivo* biomechanical processes are simulated in a bioreactor environment capable of delivering, through the application of cyclic loading and fluid dynamics, the appropriate mechanical stimulus to the evolving tissue. This environment should also be constructed so that the adequate and uniform diffusion of nutrients, waste and scaffold degradation by-products can be achieved by implementing a continuous flow culture design. *In vivo* models must be considered as *in vitro* experiments, albeit vital, provide limited insight into the establishment of a linear relationship between the *in vivo* integration of the engineered tissue and the scaffold degradation trend.

5. Conclusion

The 3 mol% iron containing quaternary glass fibres have demonstrated significant biocompatibility supporting a clear proliferation pattern, permitting a well spread morphology and maintaining the functional differentiation of the adherent HOB and HOF cells. Furthermore, these scaffolds can be used to accommodate the separate seeding of two cell populations in a co-culture arrangement. This can potentially lead to the *in vitro* simulation of the anatomical structure of a bone-ligament tissue interface.

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