Novel bioresorbable and bioactive composites based on bioactive glass and polylactide foams for bone tissue engineering

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Bioresorbable and bioactive tissue engineering scaffolds based on bioactive glass (45S5 Bioglass®) particles and macroporous poly(DL-lactide) (PDLLA) foams were fabricated. A slurry dipping technique in conjunction with pretreatment in ethanol was used to achieve reproducible and well adhering bioactive glass coatings of uniform thickness on the internal and external surfaces of the foams. *In vitro* studies in simulated body fluid (SBF) demonstrated rapid hydroxyapatite (HA) formation on the surface of the composites, indicating their bioactivity. For comparison, composite foams containing Bioglass® particles as filler for the polymer matrix (in concentration of up to 40 wt %) were prepared by freeze-drying, enabling homogenous glass particle distribution in the polymer matrix. The formation of HA on the composite surfaces after immersion in phosphate buffer saline (PBS) was investigated to confirm the bioactivity of the composites. Human osteoblasts (HOBs) were seeded onto as-fabricated PDLLA foams and onto PDLLA foams coated with Bioglass® particles to determine early cell attachment and spreading. Cells were observed to attach and spread on all surfaces after the first 90 min in culture. The results of this study indicate that the fabricated composite materials have potential as scaffolds for guided bone regeneration.

1. Introduction

In recent years, composite materials have gained increasing importance in the biomedical field because, unlike with monolithic materials, flexible tailoring of their properties can be accomplished, hence the materials show better adaptation to the complex environment and demands of the human body. Recent complete review articles covering the multitude of medical and clinical applications of composite materials have been published by Thompson and Hench [1] and Ramakrishna *et al*. [2].

A great deal of research in the biomedical field concerns the investigation of alternatives to the current practice of replacing damaged or diseased tissues with permanent implants due to problems related to the long-term performance of such implants [3]. One promising alternative is ''tissue engineering'' which combines engineering principles with the life sciences to enable the regeneration of tissues by exploiting the body's inherent repair mechanisms [4]. Tissue engineering requires a suitable temporary scaffold or regenerative allograft which, for bone tissue engineering, must fulfil a set of complex criteria as shown in Table I [5].

A wide variety of bioresorbable materials have been investigated as scaffolds for tissue engineering applications, including natural [6, 7] and synthetic polymers [8–12]. Synthetic polymers have the advantage to enable precise engineering of macro- and microstructure and control of material composition so that optimal conditions for cell survival, proliferation, and subsequent tissue formation can be created [8].

Synthetic bioresorbable polymers, in particular polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers, are successful candidates as scaffold materials [12]. They are currently used in clinics, for example, as resorbable surgical sutures and meshes or as drug delivery systems. A major drawback of these materials, once implanted, however, is the release of acidic degradation products which may lead to inflam-

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TABLE I Criteria for an ideal scaffold for bone tissue engineering

An ideal tissue engineering scaffold
1. is made from a material that is biocompatible, i.e. not cytotoxic;
2. acts as template for tissue growth in three dimensions;
3. has an interconnected macro-porous network for vascularization, tissue ingrowth and nutrient delivery;
4. bonds to the host tissue without the formation of scar tissue;
5. influences the genes in the bone generating cells to enable efficient cell differentiation and proliferation;
6. resorbs at the same rate as the tissue is repaired, with degradation products that are non-toxic and that can be easily excreted by the body, for example via the respiratory or urinary systems;
7. is made from a processing technique that can produce irregular shapes to match that of the defect in the bone of the patient; and
8. exhibits mechanical properties sufficient to be able to regenerate bone in load bearing sites.

matory responses [8, 9, 12, 13]. Another limitation is their lack of bioactivity, which means, for the case of bone tissue engineering, that they do not allow bone apposition or bonding on the polymer surface [14].

Certain ceramic materials, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and selected compositions of silicate and phosphate glasses, and glass-ceramics, for example, the commercially available Bioglass®, react with physiological fluids and form tenacious bonds to bone tissue through cellular activity. These materials are therefore known as ''bioactive'' [15].

Biodegradability and bioactivity can be combined in the form of composite materials to obtain optimized tissue engineering scaffolds exhibiting tailored physical and mechanical properties and controllable resorption rates in the body [16, 17]. Bioresorbable and bioactive composites are being developed worldwide, most commonly using combinations of polylactide (PLA), polyglycolide (PGA) and other resorbable polymers, and HA, TCP or bioactive glasses and glass-ceramics in different scaffold architectures [16–25]. The most usual approaches involve HA, TCP, and bioactive glass particles or fibers used either as fillers or in the form of coatings in porous polymeric biodegradable substrates, as reviewed elsewhere [16].

In the present work bioactive and bioresorbable composite materials were fabricated using macroporous poly(DL-lactide) (PDLLA) foams coated with and impregnated by bioactive glass (Bioglass®) particles following a previously developed technique [26]. The *in vitro* response of the composites in contact with simulated body fluid (SBF) was assessed. For comparison, the *in vitro* behavior in phosphate buffer saline (PBS) of composites made using Bioglass® particles as filler in PDLLA foams was also investigated. Cell culture studies with human osteoblasts (HOB's) were carried out to assess cell attachment and proliferation on Bioglass®-coated scaffolds. The present work complements recent research on the use of Bioglass® as the bioactive phase in the development of new porous resorbable composite scaffolds for bone tissue engineering [26–28].

2. Experimental

2.1. Materials and processing

Poly(DL-lactide) (PDLLA) foams were fabricated by a thermally induced phase separation process, often termed freeze-drying, which has been described in detail elsewhere [11a, b]. Briefly, 2 g of PDLLA (Purasorb®; Purac biochem, Holland), with inherent viscosity of 1.52 dl/g, were dissolved in 40 ml of dimethylcarbonate (99%, Acros) under magnetic stirring overnight. The solution was then transferred into a 150 ml lyophilization flask and frozen for 2 h by quenching in liquid nitrogen. The flask was connected to a vacuum pump (10^{-2} Torr), and the solvent was sublimated at $-10\,^{\circ}C$ for the first 48 h, followed by an additional 48 h at $0\,^{\circ}C$. The residual solvent was removed at ambient temperature until the foam reached a constant weight. In a separate experiment, the freeze-drying process was conveniently modified for the incorporation of bioactive glass particles in different concentrations (5–40 wt %) into the PDLLA matrix, as described elsewhere [29].

The bioactive material used was a melt-derived bioactive glass powder (Bioglass® grade 45S5, US Biomaterials Co., Alachua, FL, USA). The powder had a mean particle size $< 5\,\mu m$. The composition of the bioactive glass was (in weight percentage): 45% SiO_2, 24.5% Na_2O, 24.5% CaO, and 6% P_2O_5, which is the original bioactive glass composition developed by Hench and coworkers [15].

A slurry-dipping technique, which has been described in detail elsewhere [26], was used to coat and infiltrate PDLLA foams with Bioglass® particles. The technique involved the preparation of a stable slurry with 42 wt % of Bioglass® particles in distilled and deionized water. The foams were pretreated in ethanol for 12 h following a procedure described by Mikos *et al*. [30], in order to decrease the hydrophobicity of the foam thus improving infiltration of the glass particles into the pores and enhancing coating homogeneity. Immersion time in the Bioglass® slurry was 5 min. After withdrawal from the slurry, the samples were slowly dried on glass plates at ambient temperature in humid atmosphere.

2.2. Characterization and *in vitro* studies

For material characterization with scanning electron microscopy (SEM), the samples were cut with a razor blade to enable analysis of longitudinal and transverse cross sections. The coating quality and degree of infiltration was assessed on gold-plated samples using an accelerating voltage of 20–25 kV.

In vitro studies in simulated body fluid (SBF) [31] were performed using Bioglass®-coated and uncoated PDLLA foams. The samples were immersed in 75 ml of SBF in clean conical flasks, which had been washed using hydrochloric acid and deionized water. The conical flasks were then placed in an orbital shaker (New Brunswick Scientific, C24 Incubator Shaker), which rotated at 175 rpm at a controled temperature of $37\,^{\circ}C$.

The PDLLA /Bioglass® foams and uncoated PDLLA foams were left in immersion in SBF for time periods of 7, 14, 21, and 28 days. SBF was changed every seven days as cation concentration decreased during the course of *in vitro* studies, as a result of the changes in the chemistry of the materials, as discussed below. After immersion in SBF, the samples were characterized using SEM. Raman spectroscopy was used to verify whether HA formation had occurred on the surfaces of selected samples. For comparison, Bioglass®-filled composite foams (non-coated) were immersed in phosphate buffer saline (PBS) for up to 16 weeks to assess their bioactivity. The crystallinity of HA formed on the surface of these composites was investigated by X-ray diffraction (XRD).

2.3. Human osteoblast cell culture

Sterilization of the Bioglass®-coated and as-received foams was carried out using UV light source in a tissue culture laminar flow hood for 20 min on each side.

Primary human osteoblasts were isolated as described in the literature [32, 33]. Trabecular bone from femoral heads obtained after hip replacement surgery was cut into fragments of approximately 3 mm × 3 mm. Fragments were washed several times in phosphate buffered saline (PBS) to remove blood cells and debris with a final wash in culture medium. Fragments were then placed into culture flasks in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% Foetal Bovine Serum (FBS) with 1% glutamine, 2% penicillin/streptomycin, and 200 μM L-ascorbic acid 2-phosphate. The fragments were incubated at 37 °C in a humidified incubator with 5% CO_2. After culture for 7–10 days, fragments were subjected to trypsin (0.02%) and collagenase (0.162 U/ml) digestion for 20 min at 37 °C on a roller mixer. The resulting HOB cell suspension was then centrifuged at 1000 rpm and enzyme digestion of the fragments repeated. The digestion was performed five times in total and cells pooled. HOBs were cultured as described above on material samples or Thermanox™ and Bioglass®) discs as positive controls, for 30, 60, and 90 min, and 4 h, at a density of 40 000 cells/cm^2. Cells were tested for the osteoblastic phenotype using an alkaline phosphatase assay and their ability to mineralize in culture (data not shown).

After culture for various time periods, the samples were washed twice in PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. The samples were then washed in PBS and permeabilized in 0.2% Triton for 5 min at −20 °C. This was followed by washing in PBS containing 1% bovine serum albumin (PBS/BSA). Samples were stained using Alexa Fluor 488 phalloidin (Molecular Probes) at a concentration of 40 U/ml (according to manufacturers recommendations) for 20 min at room temperature. Samples were then counter-stained with propidium iodide at a concentration of 5 μg/ml in PBS for 30 s at room temperature.

The samples were washed several times, placed onto glass slides and mounted under coverslips using PBS/glycerol mountant (1:1). Samples were then viewed using a BioRad MRC 600 confocal laser scanning microscope.

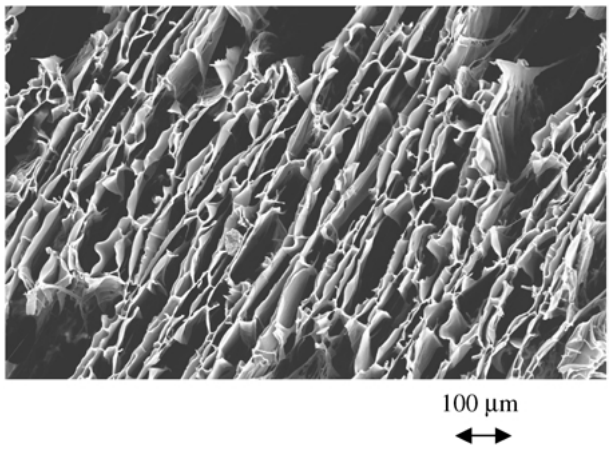

Figure 1 SEM micrograph showing a typical cross-section of PDLLA foams made by the freeze-drying process.

3. Results

3.1. Materials characterization

The typical morphology of PDLLA foams prepared by freeze-drying is shown in Fig. 1. The foam exhibits two distinct pores sizes, i.e. macropores of ≥ 100 μm average diameter and interconnected micropores with an average diameter of 20–30 μm. The tubular macropores are highly oriented as a result of the unidirectional cooling process. PDLLA foams coated by Bioglass® particles were fabricated using a slurry-dipping technique. Fig. 2(a,b) shows SEM micrographs of Bioglass®-coated PDLLA foams at different magnifications. No peel-off of Bioglass® particles or macrodelamination of the Bioglass® coating were observed. It could be observed that the material exhibited a thin, even film of Bioglass® that covered the surfaces of the foam. Using an immersion time of 5 min, Bioglass® particles deeply infiltrated the pores of the material and glass particles were deposited evenly along the internal surfaces of the porous structure. Due to the slow drying process used, the occurrence of microcracks was eliminated.

3.2. *In vitro* studies in SBF

The response of Bioglass®-coated foams in contact with SBF was analyzed qualitatively using SEM and Raman spectroscopy. Fig. 3(a) shows the development of HA crystals on the surface of the foam after 7 days in SBF. In Fig. 3(b) it is shown that complete covering of the surface with HA has occurred after 28 days in SBF. The formation of HA on the surface of the coated PDLLA foam samples with increasing days of immersion in SBF was confirmed by Raman spectroscopy. Fig. 4 presents the Raman spectra of Bioglass®-coated foams after having been soaked in SBF for 7 days. For comparison, the spectrum for a sample in the as-fabricated condition is also shown. The formation of the hydroxyapatite layer on the surface of the Bioglass®-coated PDLLA foam is indicated by the strong peak at 964 cm^{-1}, which corresponds to the symmetric stretching vibration of P-O in PO_4^{3-} tetrahedra belonging to hydroxyapatite crystals [34, 35]. For similar composite samples, the crystallinity of the HA structure formed on the surface of the foams has been also confirmed by XRD [26]. The 875 cm^{-1} peak in the Raman spectra (Fig. 4)

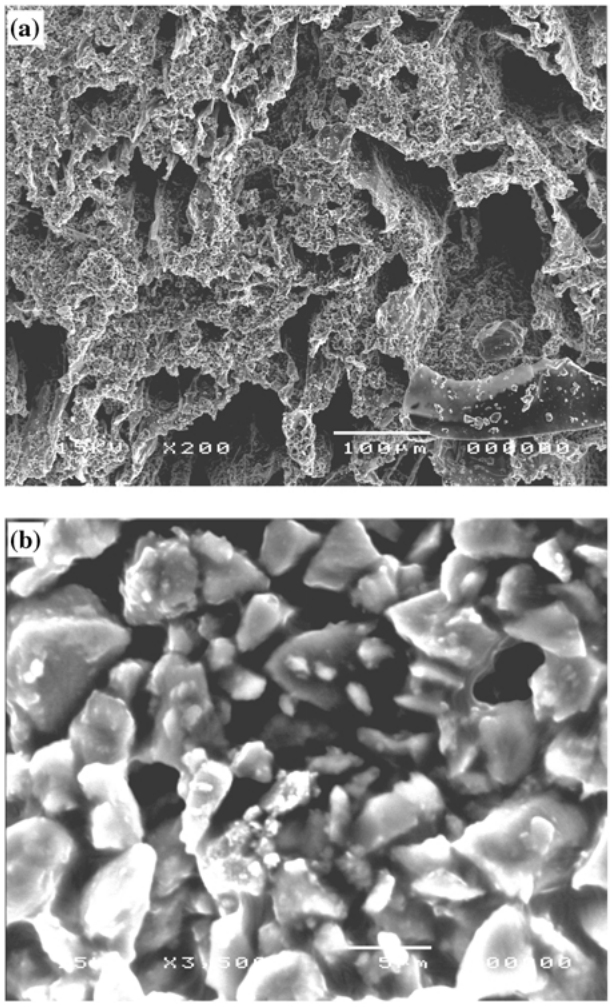

Figure 2 SEM micrographs showing the microstructure of Bioglass®-coated PDLLA foams produced by slurry-dipping. The efficient infiltration of Bioglass® particles into the pores (a) and the homogeneous coating microstructure (b) can be observed.

corresponds to C-COO stretching in PDLLA [36], therefore the ratio between the heights of the peaks at different immersion times may be used to quantify the relative amount of HA formed during immersion in SBF. The qualitative *in vitro* studies in SBF were successful in confirming the high ability for hydroxyapatite (HA) formation on the Bioglass®-coated foam surfaces, which is a measure of the considerable biaoctivity of the materials. It was also confirmed that the structure and morphology of the HA layer changed during immersion in SBF. Small HA crystals deposited after the first week of immersion, which developed into a continuous HA layer formed by coalescence of large crystals after the third week of immersion in SBF (see Fig. 3(a),(b)).

The results of the *in vitro* behavior of Bioglass®-filled foams after immersion in PBS are similar in that rapid formation of HA crystals on the samples' surfaces was found. Fig. 5 shows XRD diagrams of PDLLA/Bioglass® composite samples containing 40 wt % Bioglass®. Results for samples incubated for 7 and 28 days in PBS are shown. Well-defined HA peaks can be seen, which indicate the high bioactivity of the samples. A more detailed study of the *in vitro* behavior of samples containing different wt % of Bioglass® particles has been presented elsewhere [29].

3.3. Cell attachment study

Early attachment of HOBs was tested on the four material surfaces with the Thermanox™ and Bioglass® discs used as controls as shown in Fig. 6. It can be seen that for Thermanox™ discs attachment and spreading was observed at the earliest time point studied (Fig. 6(a)). This is due to the fact that the material is an ideal substrate for cell attachment [33]. After 90 min and 4 h,

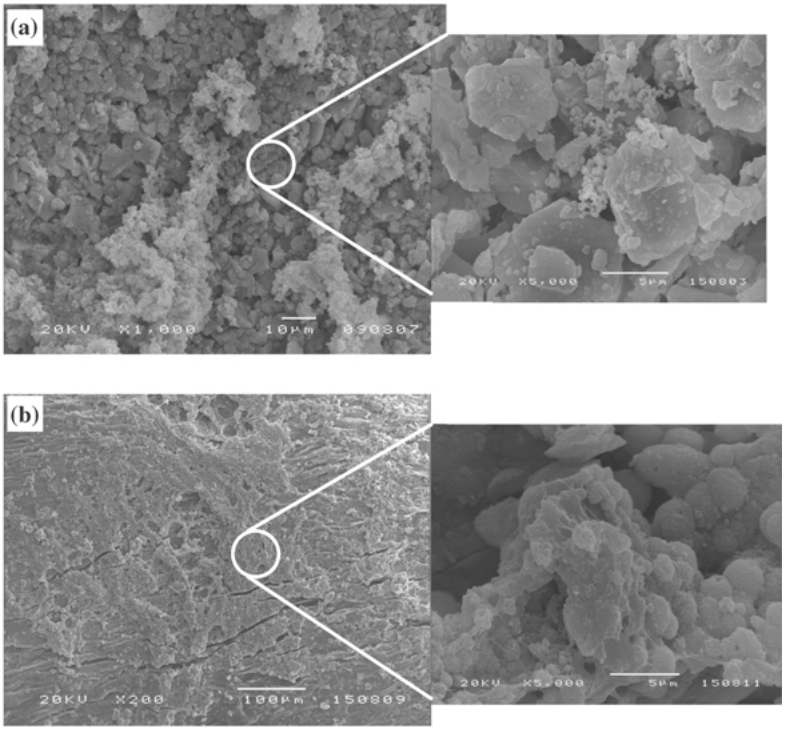

Figure 3 SEM micrographs showing surfaces of Bioglass®-coated PDLLA foams after degradation in contact with SBF for: (a) 7 days and (b) 28 days. The micrographs reveal formation of HA crystals and development of a surface HA layer.

TABLE II Summary of cell spreading of osteoblasts cultured on the material surfaces

Material/time	30 min	90 min	4 h
Thermanox™	Cell attachment, cells beginning to spread	More spreading with parallel stress fiber formation	More spreading and more stress fiber formation
Bioglass® disc	Attachment and early spreading	More spreading, cells appear spiky	More spreading with parallel stress fiber formation
PDLLA Foam	Attachment and early spreading	More spreading	Cells become polarized with parallel stress fibers
Bioglass®-coated PDLLA Foam	Attachment with little spreading	More cell spreading	More cell spreading

spreading increases further, and the formation of parallel stress fibers has occurred (Fig. 6(b),(c)). Cell attachment and spreading occurs more slowly on Bioglass® discs. After 30 min, some spreading is observed although to a lesser extent than with Thermanox™ discs (Fig. 6(d)). After 4 h in the cell culture similar developments of the cytoskeleton to those on Thermanox™ discs are detected. The formation of a spiky cytoskeleton is evident, which is a typical feature observed for cell attachment on materials with rough surfaces, as demonstrated by Kieswetter *et al*. [37] and by Anselme *et al*. [38]. The Bioglass® discs in this study have a 3 μm finish and are not as flat as the Thermanox™ control. Attachment and spreading on the uncoated PDLLA foams is shown with stress fiber formation observed at 4 h, along with a highly polarized morphology, which is likely to be due to the grooved topography of the material surface (Fig. 6(g)–(i)). Attachment and spreading of cells on the Bioglass®-coated PDLLA foams occurs at a slower rate than Thermanox™ and Bioglass® disc controls and cells appeared less polarized than compared to cells on the uncoated PDLLA foams (Fig. 6(j)–(l)). Table II summarizes the cell attachment and spreading results on the materials tested.

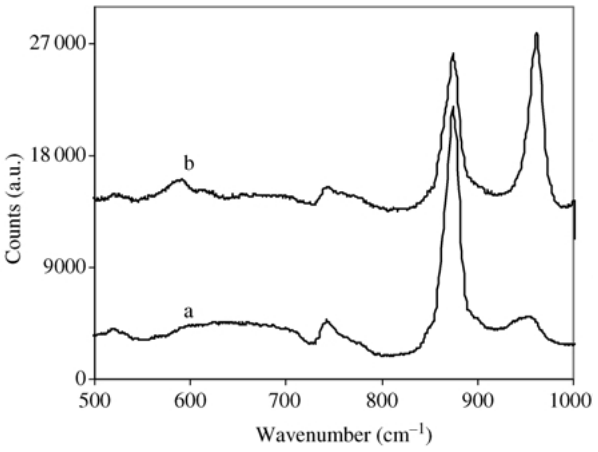

Figure 4 Raman spectra of Bioglass®-coated PDLLA foams (a) before and (b) after 7 days immersion in SBF, showing HA development on the surface of the SBF-treated sample.

4. Discussion

The development of PDLLA/Bioglass® composite materials for bone tissue engineering applications is interesting due to the fact that PDLLA degrades *in vitro* without generation of any crystalline remnants. Heideman *et al*. have proved recently the complete resorption of PDLLA implants from the extracellular space in animal studies, hence confirming their tissue tolerance [39]. The course of degradation is more questionable for slowly degradable, crystalline PLLA of high molecular weight. In some cases, degradation products are formed, including numerous stable and highly crystalline particles, which are responsible for a delayed inflammation and foreign body reactions at the site of implantation [40].

In the case of polymer/Bioglass® foams, the bioactive glass particles, applied as a coating of the pore walls, should act as a protective hydrolysis barrier affecting both the extent and rate of degradation of the polymer substrate, as suggested in recent investigations [27, 28]. The rapid exchange of protons in water for alkali in the glass should provide a pH buffering effect at the polymer surface, therefore acceleration of degradation will not

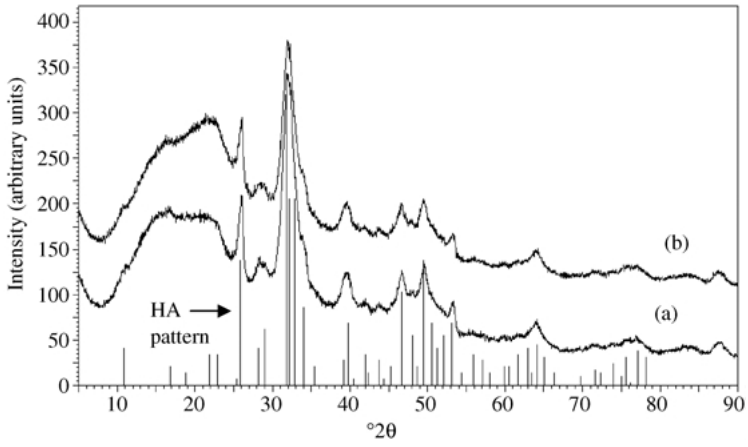

Figure 5 XRD pattern of Bioglass®-filled PDLLA foam composites (40 wt % Bioglass® content) after immersion in PBS for 7 (a) and 28 (b) days, showing development of HA crystals. Note that the intensity is given in arbitrary units, i.e. the relative height of the peaks does not correlate with the relative amount of HA present in the different samples.

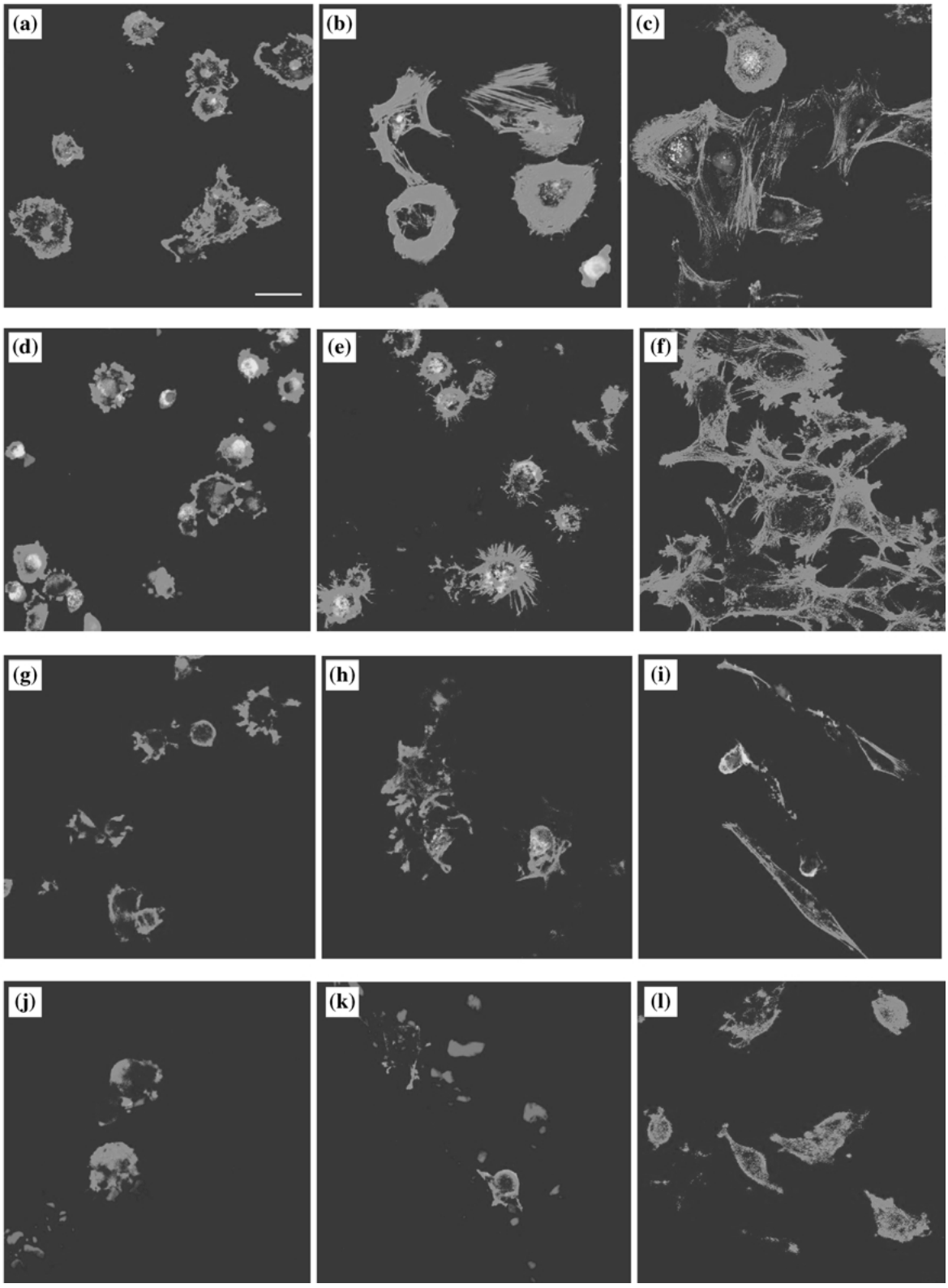

Figure 6 Confocal laser scanning micrographs of primary human osteoblasts cultured on Thermanox™ discs for 30 min (a), 90 min (b) and 4 h (c), Bioglass® discs for 30 min (d), 90 min (e) and 4 h (f), PDLLA foams for 30 min (g), 90 min (h) and 4 h (i) and Bioglass®-coated PDLLA foam for 30 min (j), 90 min (k) and 4 h (l). Cells were stained with FITC-conjugated phalloidin for the actin cytoskeleton and counterstained with propidium iodide.

occur due to small pH changes during bioactive glass dissolution.

The rapid formation of an HA layer on the Bioglass®-coated and filled PDLLA foams, both after immersion in SBF and PBS, indicates the high bioactivity of the materials. In particular for the Bioglass®-coated samples, it was found that the thickness of the HA layer increased with increasing time in solution and it was in all cases thicker than that formed on composites made using HA particles as bioactive phase [20]. After 21 days in SBF, for example, the HA layer had a thickness of $\sim 10\,\mu m$, as determined by SEM examination of sample cross sections, as shown elsewhere [26]. It is well known that Bioglass® has a higher index of bioactivity than HA, which makes this material more suitable for applications in bone reconstruction [15]. It

has been shown, for example, that there is much more bone formed in one week in the presence of Bioglass® than is formed when HA or other calcium phosphate ceramic particulates are placed in the same type of defect [41]. Recent investigations have shown also that there is genetic control of the cellular response of osteoblasts to bioactive glasses [3, 41]. Moreover, since Bioglass® is a class A bioactive material (as opposed to HA, which is class B) it has shown a strong bond also to soft tissues [15]. The application potential of the Bioglass® containing composites fabricated could therefore encompass both hard and soft tissue regeneration and repair.

Comparing the *in vitro* results of Bioglass®-coated and filled (40 wt % Bioglass® content) composites, one can conclude that both types of composites exhibit high bioactivity, both after immersion in SBF and PBS. A further investigation of the effect of Bioglass® weight content on bioactivity of similar PDLLA composite foams is being carried out.

Osteoblast adhesion is an essential parameter when investigating bone-biomaterial interactions, since the development of bone-implant interfaces depends on the direct interactions of bone matrix and osteoblasts with the biomaterial [33, 37, 38]. Cell attachment and spreading was observed on all the surfaces tested, although spreading and stress fiber formation was slowest on the PDLLA foams and Bioglass® coated foams. This may not impede the long term phenotype of the cells as *in vivo* osteoblasts are cubiodal rather than spread and flattened as seen on Thermanox™ or tissue culture plastic controls. Further investigation is necessary in order to make a clear statement on the cell behavior on the materials, particularly to assess the effect of the Bioglass® coating. Experiments are underway analysing cell infiltration into the porous network and expression of the long term phenotype, particularly whether the cells form a mineralized matrix earlier, on and within, the Bioglass® coated foams.

5. Conclusions

Bioactive and bioresorbable composites were developed based on three dimensional, macroporous poly(D,L)-lactic acid (PDLLA) foams, and Bioglass® particles. A cost-effective processing technique, slurry-dipping, led to stable and uniform glass coatings as well as adequate infiltration of Bioglass® particles into the porous network of the foams. Bioglass®-coated foams developed a surface HA layer after 7 days immersion in SBF. Bioglass®-filled PDLLA foams were also assessed for their *in vitro* behavior using PBS. HA formation, and therefore bioactive behavior, was detected in composites containing 40 wt % Bioglass® after 7 days in PBS. Initial osteoblast attachment shows progressive spreading on both uncoated PDLLA foams and Bioglass®-coated PDLLA foams. Further investigations are underway analyzing proliferation, osteoblast phenotype and cell infiltration into the porous network. The results available so far indicate that the fabricated PDLLA/Bioglass® foams, with their tailored, oriented porosity, high bioactivity, and favorable cell response are attractive scaffolds for uses in bone regeneration and repair.

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