

Leading Research Paper Tissue Engineering

A comparative analysis of scaffold material modifications for load-bearing applications in bone tissue engineering[☆]

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Abstract. To facilitate optimal application of appropriate scaffold architectures for clinical trials, there is a need to compare different scaffold modifications under similar experimental conditions. In this study was assessed the effectiveness of poly-ε-caprolactone (PCL) scaffolds fabricated by fused deposition modelling (FDM), with varying material modifications, for the purposes of bone tissue engineering. The incorporation of hydroxyapatite (HA) in PCL scaffolds, as well as precalcification through immersion in a simulated body fluid (SBF) to produce a biomimetic apatite coating on the scaffolds, was assessed. A series of *in vitro* studies spanning 3 weeks as well as *in vivo* studies utilizing a subcutaneous nude mouse model were carried out. PCL and HA–PCL scaffolds demonstrated increasing tissue growth extending throughout the implants, as well as superior mechanical strength and mineralization, as evidenced by X-ray imaging after 14 weeks *in vivo*. No significant difference was found between PCL and HA–PCL scaffolds. Precalcification with SBF did not result in increased osteoconductivity and cell proliferation as previously reported. Conversely, tensile forces exerted by tissue sheets bridging adjacent struts of the PCL scaffold caused flaking of the apatite coating that resulted in impaired cell attachment, growth and mineralization. The results suggest that scaffolds fabricated by FDM may have load-bearing applications.

Key words: bone tissue engineering; fused deposition modelling; polymer scaffolds; hydroxyapatite; precalcification.

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There is a need for an alternative to autogenous bone as graft material for the reconstruction of cranio-facial and orbital defects, or as filler material in orthopaedic surgery and fracture fixation. Bone frac-

tures and trauma-related injuries account for more than 1.3 million surgical procedures per year in the United States⁸, and reconstructive surgery requiring bone grafts is carried out every day in hospitals

worldwide. Bone tissue engineering promises such an alternative, and many groups have developed strategies based on seeding of cells onto 3-dimensional biodegradable polymeric scaffolds. Fabrication techniques that have been described for these scaffolds are myriad and include fused deposition modelling (FDM)⁶, solvent casting/salt leaching¹³, emulsion freeze-drying²², phase separation¹⁰, 3D printing¹⁷ and gas foaming/salt leaching¹⁵, to name just a few. Two major structural classes of biodegradable polymer scaffold made using these techniques are the 'foams' or those with a structure like a porous sponge²⁴, and scaffolds fabricated by fused deposition modelling with a reproducible internal architecture much like that of a honeycomb⁶. Foams have been described to have a structure much like that of trabecular bone⁵, and were the earliest polymer structures explored for use in bone and cartilage tissue engineering. Many studies have demonstrated limitations with this architecture in bone tissue engineering, such as limited tissue growth confined to the surface of the foam^{2,7}, as well as poor structural properties, in particular low strength and stiffness^{4,23}.

Scaffolds made of poly-ε-caprolactone (PCL) and fabricated by fused deposition modelling have also been described²⁶, with a highly reproducible internal structure based on layer-by-layer deposition of thermoplastic material (Fig. 1A). Such scaffolds were designed to retain their mechanical properties for 5–6 months and then gradually degrade over a period of 2 years²⁶. With a fully interconnected pore network, these scaffolds were shown to support proliferation of cells throughout the scaffold^{6,4}, as well as retain their mechanical properties after implantation in rabbits²⁰, pigs¹⁸ and mice¹⁹. This tissue engineering strategy may have a load-bearing application, such as in the reconstruction of long-bone defects or of large critical sized defects in the calvarium or orbit.

There is a need for studies to compare the effect of various material modifications under similar experimental conditions before tissue engineering moves to clinical trials. This is essential to ensure that the optimal scaffold architecture for a specific application is used in patients. In this study, the effect of incorporation of hydroxyapatite (HA) (Fig. 1B and C), as well as precalcification in simulated body fluid (SBF) (Fig. 1D and E), was compared with untreated PCL FDM scaffolds for the purposes of bone tissue engineering. The growth of osteoblasts on these scaffolds

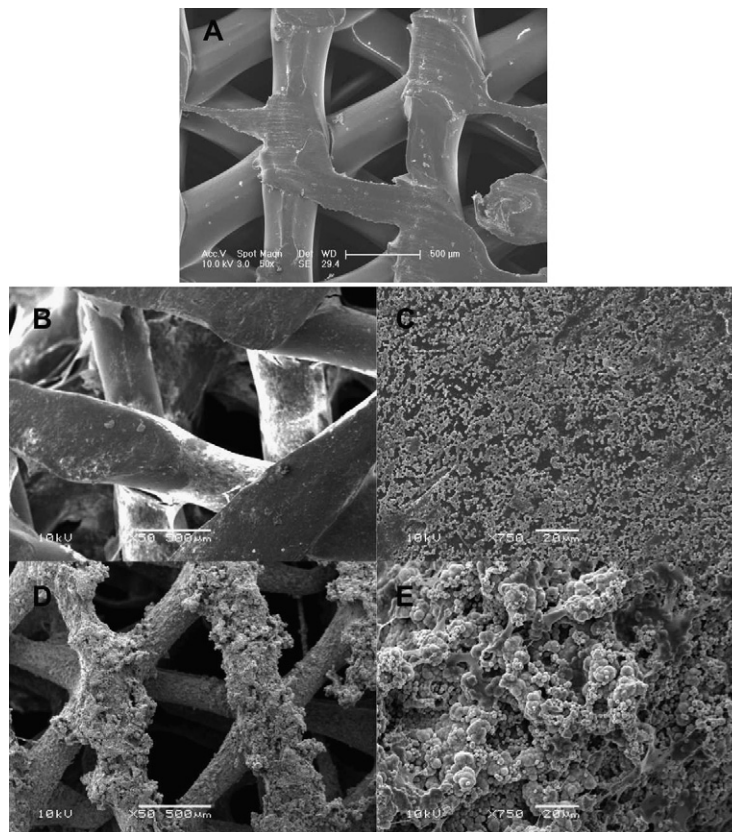


Fig. 1. Scanning electron micrographs showing: (A) PCL scaffold with a lay-down pattern of 0°/60°/120° fabricated by FDM; (B) HA-PCL scaffolds have a fine apatite coating, obvious at high magnification (C). (D) Precalcification of PCL scaffolds with simulated body fluid produces a coarse, prominent apatite layer, obvious at high magnification (E). Original magnification: (A, B and D) $\times 50$ and (C and E) $\times 750$.

was evaluated *in vitro* and in a nude mouse model, as well as comparing the degradation characteristics *in vivo*. A subcutaneous nude mouse model was ideal for this experiment, due to the different envisioned applications of these scaffolds in the clinic.

Previous studies have shown that blends of HA and polymers as composite scaffolds enhance osteoconductivity, as well as improving mechanical properties, cell survival and proliferation^{12,9}. Improved cell survival has been postulated to be related to the maintenance of a neutral pH, due to basic resorption by-products of HA buffering acidic resorption by-products of aliphatic polyesters⁴. Surface modification of polymer scaffolds by the formation of a bone-like apatite layer through immersion in SBF has also been reported^{14,25}, and MG63 cells were shown to attach to Bioglass-filled polylactide foams after immersion in SBF². This form of surface modification is used with the same aim as composite scaffolds incorporating HA: to enhance osteoconductivity and improve the mechanical properties of the scaffold. To the authors' knowledge,

this is the first report of a direct comparison between different material modifications in a similar experimental setting for the purposes of bone tissue engineering.

Materials and methods

Fabrication of scaffolds by fused deposition modelling

PCL scaffolds were fabricated by FDM as previously described by HUTMACHER et al.⁶ For HA-PCL scaffolds, PCL powder (catalog no. 44, 074-4, Aldrich Chemical Co., Milwaukee, WI, USA), Mn of approximately 80,000 Da (gel permeation chromatography) and melt index of 1.0 g/10 min (125 °C/44 psi ASTM D1238-73), and HA (microemulsion-derived CaPO₄ powder) were dried separately for 24 h in a vacuum oven at 120 and 40 °C, respectively. Composite pellets of PCL-HA were then formed by casting a solvent mixture of 20 g PCL-HA blend with 15% HA by weight. The composite material was cut into 5 mm \times 5 mm pellets and stored in a desiccator until used. The PCL-HA pellets were melt extruded to form monofilaments

using a one-shot extruder (Alex James & Associates, Greenville, SC, USA). HA-PCL scaffolds were then fabricated by FDM as described. PCL and HA-PCL scaffolds (PCL/HA = 85%/15%) with a lay-down pattern of 0/60/120 and a porosity of 65% were used. All scaffolds had a dimension of 10 mm × 10 mm × 8 mm. Prior to seeding they were sterilized by centrifugation in 70% ethanol. Ethanol was removed by washing 3 times for 1 h each in changes of phosphate-buffered saline (PBS). Subsequently, the scaffolds were transferred into 24-well plates, and dried in an incubator at 37 °C for 24 h.

Biomimetic coating using a SBF

To produce the biomimetic coatings the materials were submitted to a previously developed procedure¹⁶. The PCL scaffolds were 'impregnated' with a sodium silicate gel. This treatment was aimed at generating nucleating sites for the formation of the apatite layers. After the treatment the samples were immersed in 50 ml of SBF (pH 7.4, 37 °C) with an ion concentration nearly equal to that of human blood plasma (SBF 1×). After 7 days, the ion concentration of the SBF solution was raised to 1.5×, to induce growth of apatite nuclei. SBF solutions were changed every 2 days. The coating period was 30 days in duration. After coating, samples were rinsed in de-ionized water and dried at room temperature.

Cell isolation, seeding and culture

Human calvarial osteoblasts were isolated via explant culture from a piece of calvarium obtained from an infant undergoing reconstructive surgery. Non-osseous tissue was mechanically removed and the specimen was manually broken up into fragments measuring 5 mm × 5 mm. These fragments were extensively washed in PBS and seeded into T-75 culture flasks. The cells were cultivated in M199 medium (GIBCO, Rockville, MD, USA) supplemented with 10% foetal calf serum and 1% penicillin-streptomycin (GIBCO). Osteogenic supplements (ascorbate diphosphate, β-glycerophosphate) were added. Medium was changed every 4 days.

Cell growth and differentiation *in vitro* in combination with scaffolds

After 2 weeks of explant culture in T-75 flasks, cells were detached with 0.25% trypsin-EDTA, centrifuged, washed with PBS, and seeded on polymer scaffolds. Three different groups of scaffolds were defined: (1) PCL FDM scaffolds, (2) HA-

PCL FDM scaffolds and (3) PCL FDM scaffolds treated with SBF. Cells were seeded at a density of 1×10^5 cells on scaffolds with dimensions of 10 mm × 10 mm × 8 mm. The scaffolds were cultivated in M199 medium as described, and medium was changed every 3–4 days. To facilitate osteogenic differentiation, cells were stimulated with 10 μM dexamethasone on days 3 and 10 of culture. Cell-scaffold constructs were maintained in 24-well plates. At various time points, supernatant was collected before fresh medium was added, and stored at -80 °C. The supernatant was subsequently analysed for expression of alkaline phosphatase (ALP) and osteocalcin. The cell-scaffold constructs were serially observed under a phase-contrast light microscope.

Confocal laser microscopy was also used to assess cell morphology, proliferation and attachment *in vitro*. Samples were first fixed in 3.7% formaldehyde at room temperature for 30 min. After rinsing twice with PBS for 5 min each, RNase A (200 μg/mL) was added and the samples incubated at room temperature for 30 min. Phalloidin (A12379 Alexa Fluor 488 phalloidin; Molecular Probes, Eugene, OR, USA) was then added at 1:200 dilution at room temperature and the samples were kept in darkness for 45 min. Samples were then counterstained with a 5 μg/mL concentration of propidium iodide (1 mg/mL; Molecular Probes) solution, dried and mounted for viewing under a confocal laser microscope (IX70, Olympus). Cell viability and distribution within the scaffolds were assessed with the fluorescent dye fluorescein diacetate (Molecular Probes) and propidium iodide as the counterstain. Depth projection images were constructed from up to 50 horizontal image sections (20 μm each) through the stained cell-scaffold constructs.

Metabolic assays (*in vitro*)

To assess phenotype of osteoblasts seeded on polymer scaffolds *in vitro*, ALP and osteocalcin assays were performed. Supernatant from different time points on days 1–20 of incubation was used. This was stored at -80 °C until assayed. For assessment of ALP ($n = 3$), the production of *p*-nitrophenol in the presence of ALP was measured by a bone-specific immunoassay (Metra BAP EIA kit; Quidel, San Diego, CA, USA). The concentration of the major N-terminal fragment of osteocalcin ($n = 1$) was assayed by an enzyme-linked immunoassay kit (Metra Osteocalcin; Quidel).

Animal surgery

Cell-scaffold constructs were implanted subcutaneously in the back of Balb C nude mice ($n = 3$) after 21 days in culture. The 3 groups of constructs (1–3) described above were implanted together in the back of a single mouse. Animals tolerated surgery well. The mice were killed after 6 and 14 weeks, and the specimen retrieved *en-bloc* for histology, mechanical testing and analysis of degradation of scaffolds. Housing and feeding of the animals was according to standard animal care protocols. The study was approved by the Animal Welfare Committee, National University of Singapore, and licensed by the National Institute of Health's guide for care and use of laboratory animals.

Mechanical testing and image analysis

Samples retrieved after 14 weeks *in vivo* were compared to matched unseeded scaffolds of similar dimension and fabrication for compression testing. Six different points on each construct were tested on an Instron 4502 uniaxial testing system with a 10-N load cell (Canton, MA, USA) using an indenter with a diameter of 0.625 mm. The specimens were firmly secured onto a vise to ensure immobilization and a flat testing surface. The compression test was divided into 2 steps. In the first loading phase, a load of up to 10 N was applied at 0.1 mm/min. Results of this phase were analysed and the stiffness (loading between 8 and 10 N) of each reconstructed area was calculated. The second phase included a holding phase at 10 N for 60 s, for deformation rate measurements (creep). Afterwards the indenter was unloaded at 2.5 mm/min. The explanted *en-bloc* specimen consisting of all 4 scaffolds implanted in the mice was imaged with an X-ray scanning analytical microscope (XSAM) (XGT-2000; Horiba, Tokyo, Japan).

Statistical analysis

Results of metabolic assays reported are means of at least 3 different samples of supernatant. Differences in mean values of ALP activity at different time points were analysed by unpaired, 2-tailed *t*-tests; *P* values less than 0.05 were considered significant.

Results

Cell growth and differentiation *in vitro*

Calvarial osteoblasts were successfully isolated and seeded onto the 3 different

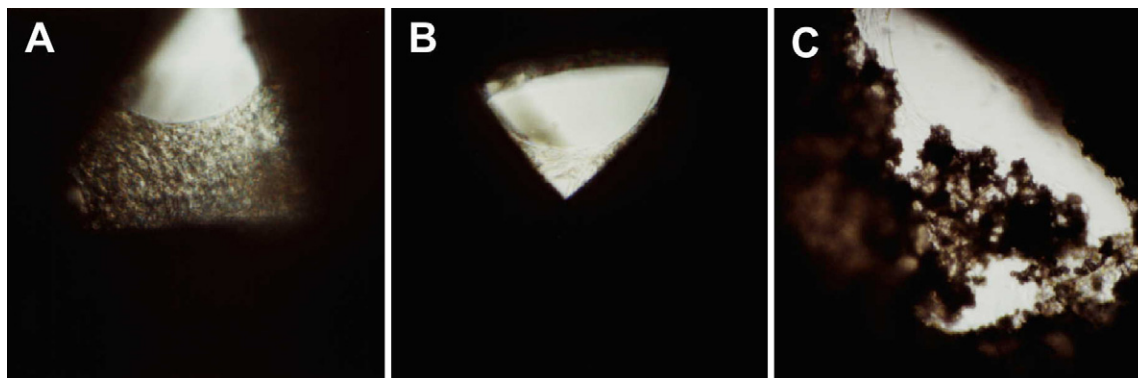


Fig. 2. Phase-contrast light microscopy of scaffolds 11 days after seeding. (A) PCL and (B) HA-PCL scaffolds show a tissue sheet bridging adjacent struts. (C) PCL scaffolds treated with SBF showing flaking of the induced apatite layer and subsequent detachment of cells from the scaffold bars. Original magnification: (A–C) $\times 100$.

groups of polymer scaffolds, after initial expansion in 2-dimensional culture for 2 weeks. When seeded onto all 3 types of scaffold, cells attached, spread and proliferated, adopting a stellate morphology typical of attached cells, with numerous filopodia and cell-to-cell contacts visible. The PCL FDM and HA-PCL FDM scaffolds demonstrated a constant rate of cell proliferation, with cells initially attaching to the scaffold bars and later forming sheets of cells bridging adjacent scaffold struts (Fig. 2A and B). Cells penetrated deep into the centre of the PCL scaffolds, where tissue sheets were even observed. Confocal laser microscopy confirmed the uniform distribution of cells throughout the PCL (Fig. 3A) and HA-PCL (Fig. 3B) scaffolds. Grossly, there was no difference in cell morphology or proliferation between the PCL and HA-PCL cell-scaffold constructs.

The PCL scaffolds treated with SBF did not support cell growth as well as the PCL or HA-PCL scaffolds. In the initial phase, cells attached to the bars of the coated PCL

scaffolds. It was observed subsequently that, with formation of tissue sheets bridging adjacent struts, sheets of cells detached from the bars of the scaffolds (Fig. 2C). Grossly, flaking of the apatite layer was seen on the bars of the scaffolds. This was probably a result of the apatite layer being too thick, due to an overly prolonged period of SBF immersion, such that the structural integrity of the coating on the PCL fibres was affected. Further studies to determine the optimum time for SBF immersion, so as to achieve a stable, continuous adherent apatite layer are required. Confocal laser microscopy showed that while there were still cells attached to the scaffold, these did not form a confluent layer, and were at a lower density than in the other 3 scaffold groups (Fig. 3C).

Analysis of ALP and osteocalcin expression by osteoblasts revealed a trend consistent with that expected of calvarial osteoblasts in culture for the PCL and HA-PCL scaffolds. Previously, it was shown that during culture of rat calvarial osteo-

blasts, expression of osteocalcin synthesis occurred subsequent to initiation of ALP activity and together with the formation of mineralized nodules^{1,4}. ALP has been described as an early marker in osteogenesis, while osteocalcin is a late marker that is produced synchronously with production of extracellular matrix^{1,4}. ALP production peaked immediately after seeding in all scaffolds (Fig. 4A), as measured on day 1, falling to a trough and subsequently reaching a peak again between days 17 and 20 after seeding. During the course of the *in vitro* study, ALP production was consistently lower for the SBF-treated PCL scaffold than the PCL and HA-PCL scaffolds, the difference increasing substantially from day 10 after seeding and reaching statistical significance on days 17 and 20 ($P < 0.05$). The lack of ALP expression for the apatite-coated PCL is in concordance with that observed by light and confocal laser microscopy, and was probably due to an increasing number of non-viable cells as the apatite layer sloughed off the bars of the scaffold.

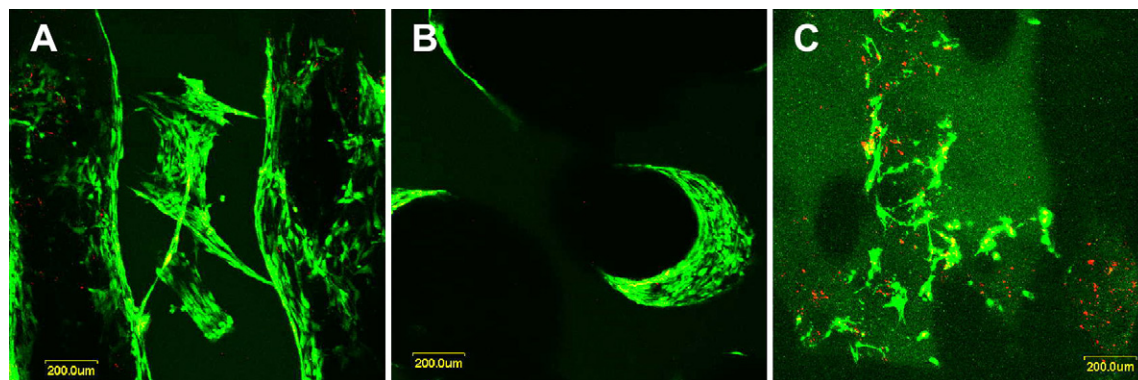


Fig. 3. Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images shows 3-dimensional distribution of cells within the scaffolds. (A) PCL and (B) HA-PCL scaffolds show even distribution of cells along the bars of the scaffolds, extending into the centre of the construct. (C) Few cells are seen on the PCL scaffolds treated with SBF, and these do not form a confluent network with cell-to-cell contact, unlike cells on the other 3 scaffolds. Original magnification: (A–C) $\times 50$.

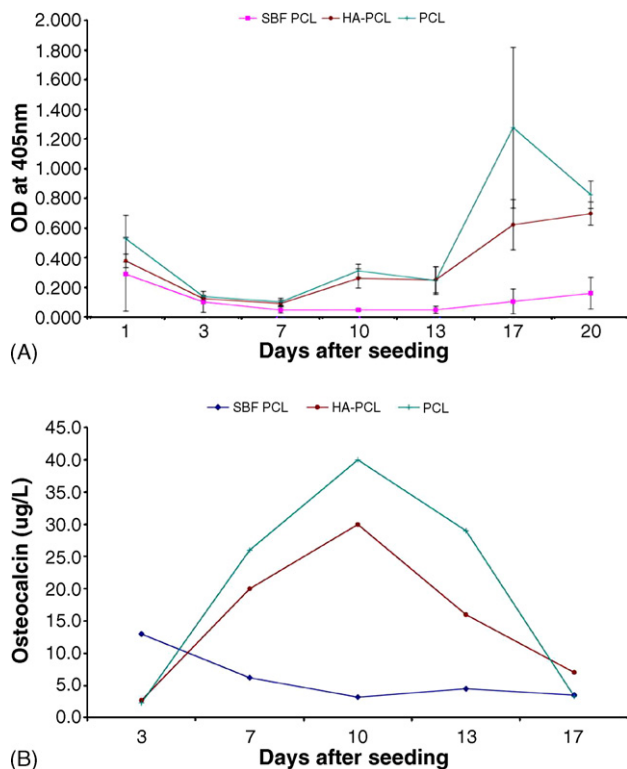


Fig. 4. Metabolic assays were performed to assess expression of osteogenic markers during a 21-day period *in vitro*. (A) ALP expression peaked immediately after seeding in all scaffolds, then slowly fell and peaked again between days 17 and 20 for the PCL and HA-PCL scaffolds. From day 10 onwards, ALP expression for the SBF-treated PCL scaffolds was consistently lower, reaching statistical significance on days 17 and 20 ($P < 0.05$). (B) Osteocalcin (OC) expression peaked at day 10 for the PCL and HA-PCL scaffolds.

Similarly, for osteocalcin expression (Fig. 4B), while a peak was observed for the PCL and HA-PCL scaffolds 10 days after seeding, no peak was visible for the SBF-treated PCL scaffolds. Formation of extracellular matrix was observed throughout the PCL and HA-PCL scaffold architecture, increasing gradually and filling the whole scaffold by 3 weeks (data not shown), as previously demonstrated^{4,21}. Production of extracellular matrix accompanied osteocalcin expression in these scaffolds. For the SBF-treated PCL scaffolds, the lack of osteocalcin expression can be explained by an inability of cells to attach properly to the scaffold bars, and subsequently being unable to secrete and produce extracellular matrix.

Comparative analysis of different scaffolds in a nude mouse model

The different scaffolds loaded with cells after a 3-week period of *in vitro* culture were implanted simultaneously into the back of Balb C nude mice (Fig. 5A).

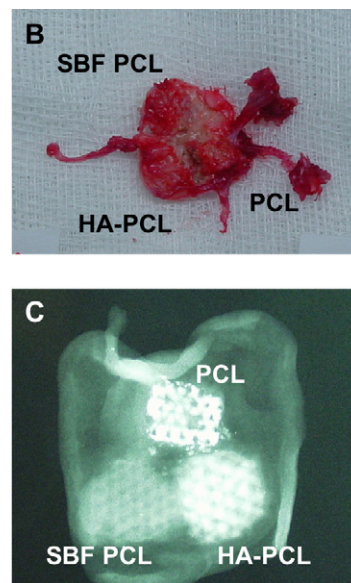
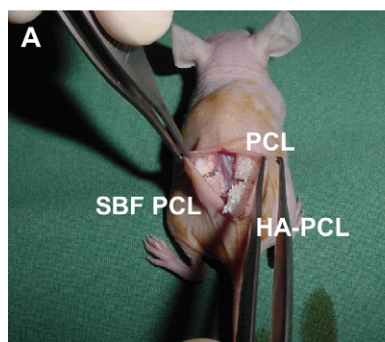


Fig. 5. Cell-seeded scaffolds were implanted subcutaneously in the back of Balb C nude mice after 21 days in culture (A). Explantation of the implant 14 weeks later showed evidence of vasculogenesis (B). In this specimen, 4 vessels can be seen emanating from the PCL construct, and 1 from the HA-PCL construct. (C) An X-ray taken of the explanted specimen showed evidence of mineralization (bright white areas) in PCL and HA-PCL scaffolds. This was less pronounced in the SBF PCL scaffold.

The implants were well integrated into the surrounding tissue and there was no evidence of encapsulation or excessive fibrosis. Gross examination of the explanted specimen 14 weeks after implantation revealed evidence of vascular ingrowth, particularly pronounced for the PCL and HA-PCL scaffolds. In the representative specimen shown (Fig. 5B), 3 blood vessels can be seen emanating from the PCL scaffold, and 1 from the HA-PCL scaffold. Gross evidence of vasculogenesis was absent for the SBF-treated PCL and poly(DL-lactic-co-glycolic acid) (PLGA) foams. Increased vasculogenesis in the PCL and HA-PCL scaffolds would be expected, considering increased cell proliferation and expression of osteogenic markers, as shown in the *in vitro* study. An X-ray micrograph of the explanted specimen (Fig. 5C) showed that while mineralization (bright white areas) was prominent in the PCL and HA-PCL scaffolds, it was less pronounced in the SBF-treated PCL scaffold and PLGA foams. This is to be expected on the basis of the findings of the *in vitro* study described above.

Mechanical testing was performed to assess the structural integrity of unseeded control scaffolds and specimens explanted after 14 weeks in a nude mouse model. The mean stiffness (Fig. 6A) of the PCL, HA-PCL and apatite-coated PCL was

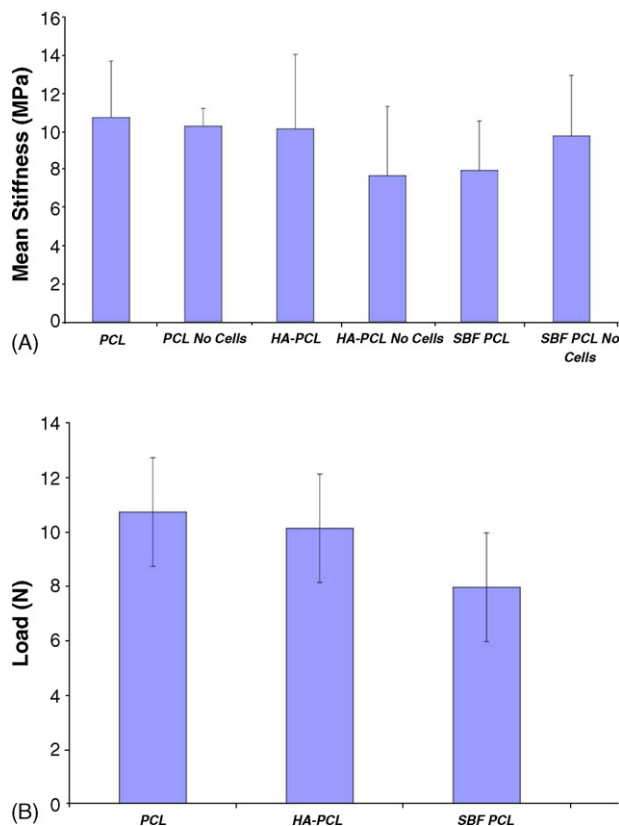


Fig. 6. Mechanical testing was performed on explanted scaffolds after 14 weeks *in vivo* with matched control unseeded scaffolds. (A) Mean stiffness is similar for PCL scaffolds. Stiffness increased after *in vivo* culture in the PCL and HA-PCL scaffolds, but decreased for the SBF PCL scaffolds. (B) Maximum load that could be supported before indentation of the scaffolds was greatest in the PCL and least in the SBF PCL scaffolds.

similar, as would be expected in scaffolds with the same architecture and composition, but the stiffness of the apatite-coated PCL scaffold after 14 weeks implantation was less than that of a control scaffold. Conversely, the stiffness of the PCL and HA-PCL specimens increased after the period of *in vivo* implantation; this was expected to occur due to ingrowth of tissue and formation of extracellular matrix. The decreased stiffness of the SBF-treated PCL scaffolds after implantation could be explained by poor attachment of cells to the unstable apatite layer *in vivo*, and therefore decreased mechanical strength. The maximum load that could be supported before indentation of the scaffold (Fig. 6B) was in the order of PCL, HA-PCL, SBF-treated PCL; again, this was as expected.

Discussion

For tissue-engineering strategies to progress to clinic trials, adequate studies must be performed to compare different scaffold modifications and cells under similar experimental conditions. Here, it was

sought to compare different modifications on a scaffold designed for load-bearing applications in bone tissue engineering. Scaffolds fabricated by FDM have adequate mechanical strength, and allow ingrowth of tissue throughout the implant, as demonstrated. The fabrication process allows customized shaped implants to be generated from computed tomography (CT) data⁶.

The effect was investigated of incorporation of HA into the scaffold material, and precalcification with SBF to produce a biomimetic apatite coating in PCL scaffolds made by FDM. While previous studies reported beneficial effects of HA in composite scaffolds^{9,12}, a significant difference was not found between PCL and HA-PCL scaffolds in the present experiments. It is postulated that, because of the fully interconnected network and large porous spaces of the FDM architecture, at baseline osteoconductivity is already relatively high, and therefore, HA incorporation did not result in a significant increase. In addition, the enhanced cell proliferation and mechanical properties attributed to HA

incorporation were reported from experiments conducted on 'foams', with decreased cell penetration and poorer mechanical properties compared to the FDM scaffolds.

While precalcification with SBF promises similar benefits to incorporation of HA into polymer scaffolds, it was found that, conversely, this was detrimental in the present experiments. Studies have reported attachment of MG63 cells to Bioglass-filled polylactide foams², as well as enhanced growth of human osteoblast-like cells on polymer-glass composites¹¹ and glass fibres³ over 14 days in culture. While these findings are disputed, the 3-dimensional porous architecture of the PCL FDM scaffolds dictates different mechanical constraints on cells attaching and growing on the bars of the scaffold. Cells seeded on scaffolds such as those used in this study will naturally form a tissue sheet bridging adjacent bars, as was shown here and previously^{4,6,20,21}. The tensile force exerted by this free tissue sheet on the scaffold bars is greater than would be expected for cells grown on foams or fibres, which attach individually to the supporting material. This would explain the poor attachment of cells to the SBF-treated PCL scaffold *in vitro*, and the observed flaking of the apatite layer. While precalcification with SBF might be a viable form of surface treatment for scaffolds of a 'foam' architecture or for 2-dimensional surfaces, the method as currently described may not be suitable for 3-dimensional scaffolds made by FDM. A thinner apatite layer, achieved through shorter immersion times in SBF and lower SBF concentrations, may yield more promising results.

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