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Three-dimensional matrices of calcium polyphosphates support bone growth *in vitro* and *in vivo*

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Novel macroporous calcium polyphosphate (CPP) scaffolds, with three-dimensional interconnected structure, were fabricated using a polyurethane sponge method. They were then employed in both *in vitro* and *in vivo* assays to examine their suitability as bone tissue engineering scaffolds. In the former, subcultured rat marrow cells were seeded on the scaffolds at 7.0×10^5 cells/sample and cultured for 2 wk. Cell-free controls were employed to monitor changes in the scaffold itself. In the *in vivo* assay, CPP rods were implanted in rat distal femur and recovered after 2 wk. Samples were examined by scanning electron microscopy following freeze-fracturing. Both *in vitro* and *in vivo* assays demonstrated the growth of bone within the scaffolds. *In vitro*, the bone/CPP interface was occupied by a morphologically distinguishable cement line, while *in vivo* non-mineralized fibrous tissue was seen at the interface together with bone ingrowth into the scaffold microporosity. The morphology of the individual surface grains of the CPP scaffolds employed *in vivo* changed to a more rounded form, while no change in geometry was observed in the *in vitro* cell-free group. These preliminary studies indicate that three-dimensional CPPs can be successfully used as scaffolds for bone tissue engineering.

1. Introduction

Calcium phosphates are generally considered materials of choice as bone substitutes. While calcium phosphate ceramics meet some of the needs for bone replacement they are limited by their inherent stiffness, brittleness and low fatigue properties relative to bone [1] and are generally not resorbed during bone remodeling [2]. Despite these problems, macroporous calcium phosphates have also been adopted as scaffolds for the restoration of bone stock through bone tissue engineering (TE) strategies [3, 4].

The underlying concept of TE is that cells can be isolated from a patient, the population expanded in cell culture and seeded on to a carrier and the TE construct is then grafted into the same patient to function as replacement tissue. Ideally, the scaffold should undergo degradation, either by osteoclasis or dissolution, within a predicted time, so that it is replaced by natural bone tissue. This has led to the adoption, by some, of biodegradable polymers as bone TE scaffolds [5] which may also serve as controlled delivery vehicles. Again these have, to date, proved less than ideal for bone applications because not only do they lack mechanical strength, which is thought to be an essential prerequisite for bony implantation, but it has also been shown that bone cells do not penetrate such three-dimensional matrices [6]. Indeed, we have demonstrated that scaffold macroporosity is a critical factor in cell migration and bone matrix elaboration *in vitro*. Thus, matrices with a nominal pore size of 200 μm [7], resulted in occlusion of pores by migrating cells, while similar scaffolds of 500 μm nominal pore size permitted three-dimensional tissue growth *in vitro* [8] which has led to the convincing demonstrating of new bone growth on the pore walls *in vivo* by Yoshikawa *et al.* [9].

Thus, the ideal scaffold for a bone TE construct has yet to be found. Recently, biodegradable calcium metaphosphates (CMP) and calcium polyphosphates (CPP) have been proposed as bone implant materials, both in fiber form [10] and as porous rods [11]. While the latter study demonstrated that sintered CPP rods were well tolerated by the surrounding tissue upon implantation in rabbit femora, these preparations only

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resulted in interconnected channels of approximately 100 μm.

To address the problems associated with current calcium phosphate TE scaffolds which are either of uncontrolled degradation or insufficient pore size, we report herein the employment of novel three-dimensional calcium polyphosphate scaffolds, possessing a macroporous structure with approximate pore size range of 0.3–1 mm which support bone growth both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Calcium polyphosphate scaffold preparation

Porous calcium metaphosphate (CMP) blocks were prepared by condensation of anhydrous $Ca(H_2PO_4)_2$ to form non-crystalline $Ca(PO_3)_2$. From the latter an homogenous melt was created by thermal treatment, quenched in distilled water, and the block was then milled to produce a CMP powder. Macroporous scaffolds were made using a polyurethane (PU) sponge method which we have reported previously [12]. The PU was burnt out and the resultant inorganic scaffold was sintered at 900 °C for 1 h to create a CPP. Different configurations were prepared for the *in vitro* and *in vivo* studies. Scaffolds used in the *in vitro* study were in block form, having approximate dimensions of 4 mm × 4 mm × 4 mm. Six scaffolds were used for each subculture (see below) and the whole procedure was repeated in triplicate. Cylindrical scaffolds having dimensions 2 mm × 4 mm were prepared for the *in vivo* study ($n = 8$).

2.2. Rat bone marrow cell culture

Rat bone marrow was harvested from young adult male Wistar rats (approximately 120 g) and cultured in α-minimal essential medium (α-MEM) containing penicillin G, gentamicin, fungizone, foetal bovine serum, and freshly prepared ascrobic acid, β-glycerophosphate, and dexamethasone ($n = 3$). We have reviewed this culture system in detail elsewhere [13]. On day 5, the cells were passaged, filtered and seeded on γ-irradiated CPP scaffolds ($n = 6$) at a density of 7.0×10^5 cells/sample. A cell-free scaffold was cultured in fully supplemented medium as a control. The subcultures were re-fed every second day until termination.

2.3. *In vivo* implantation procedure

Scaffolds ($n = 8$) were γ-irradiated and implanted aseptically in male Wistar rat (200–250 g) femora under 4% halothane, nitrous oxide and oxygen (2 : 1) anaesthesia. The implants were press-fitted into transfemoral drill holes made using a low-speed dental handpiece and 2 mm round burr, and the incision was closed using 4–0 polyglycolic acid sutures and skin staples. Each animal ($n = 4$) received one implant per femur and was allowed full activity postoperatively. Both the *in vitro* cultures and *in vivo* implants were fixed, after 2 wk, by immersion in Karnovsky's fixative for 24 h.

2.4. Scanning electron microscopy

In order to examine the interface and the inner pore volume, the implants were freeze-fractured in liquid nitrogen. The fractured specimens were dehydrated through a graded series of ethanol and critical point dried from carbon dioxide (Polaron CPD7501 Critical Point Dryer). A 3 mm layer of platinum was sputter coated on to the specimens (Polaron SC515) and was examined in a scanning electron microscope at an accelerating voltage of 15 kV (Hitachi 2500).

3. Results

Examination of the as-received material, Fig. 1, demonstrated the degree of interconnecting porosity and pore size. The pore sizes ranged from 0.3–1 mm. The latter was more easily seen by scanning electron microscopy. Fig. 2 shows the as-received material (a, b, and c) and similar fields of view of a sample cultured in fully supplemented medium for a period of 2 wk without cells (d, e, and f). It is clear from the lower power micrographs (a and d), that the surface structure was inhomogeneous and, at higher magnifications, the fused grain structures of the CPP material showed no significant changes during the 2 wk culture period at 37 °C.

Fig. 3 represents the appearance of the CPP scaffold after 2 wk culture in the presence of rat bone marrow derived cells. These cells populated the outer and inner surfaces of the three-dimensional scaffold and formed a continuous sheet following the surface contour of the scaffold as seen in Fig. 3a. Fig. 3b, c and d show higher magnification scanning electron micrographs of the matrix laid down within the scaffold. Fig. 3b

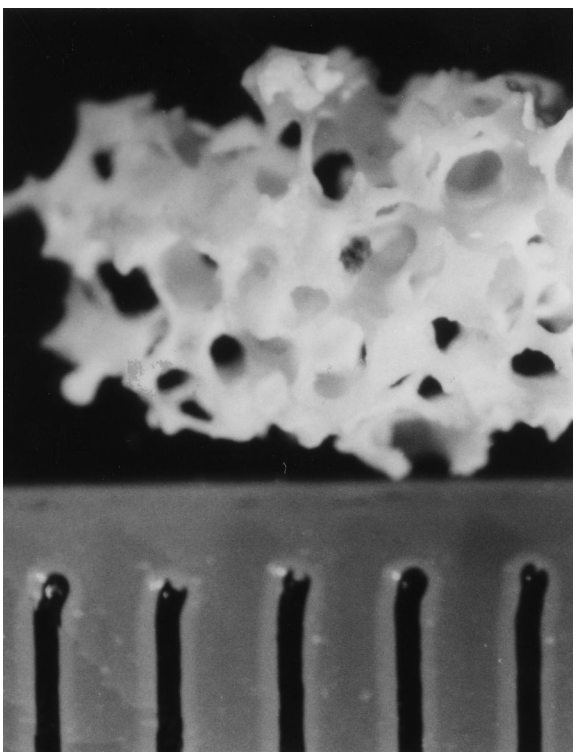


Figure 1 Light micrograph showing the CPP scaffold as-received. Field width = 4.5 mm.

demonstrates mineralized collagen fibers bridging between individual grains of the CPP scaffold. At this magnification the collagen would seem to be inserting into the microcrystalline surface layer of the CPP grain structure, which is more clearly visualized in Fig. 3c as a surface layer which is quite distinct from the underlying CPP surface. Cell processes also adhered to this surface layer, as shown in Fig. 3d, which enveloped the grain structure of the underlying surface.

Retrieval of the CPP scaffolds from rat femora after 2 wk demonstrated that bone had grown throughout the pore structure as evident in Fig. 4. Fig. 4a provides an overview of the site of implantation with respect to the neighboring cortical bone, although at this magnification, it is difficult to distinguish the implant from the reparative trabecular bone. Fig. 4b illustrates the distinction between bone, containing an osteocyte lacuna, and the neighboring implant with its characteristic grain structure. It should be noted with these freeze-fractured samples that the spaces appearing between the biological surface tissue and the CPP are artefacts of processing. Fig. 4c shows that a bone seam having grown along a surface of the CPP scaffold is anchored through the interdigitation of the bone matrix with surface microporosity of the scaffold. It is also evident that some of the surface grains of the scaffold, which have not been influenced by the freeze-fracturing technique, exhibited a rounded morphology. This morphological change was evident on both the lateral and apical facets of individual grains. The ability of the elaborated biological matrix to envelope

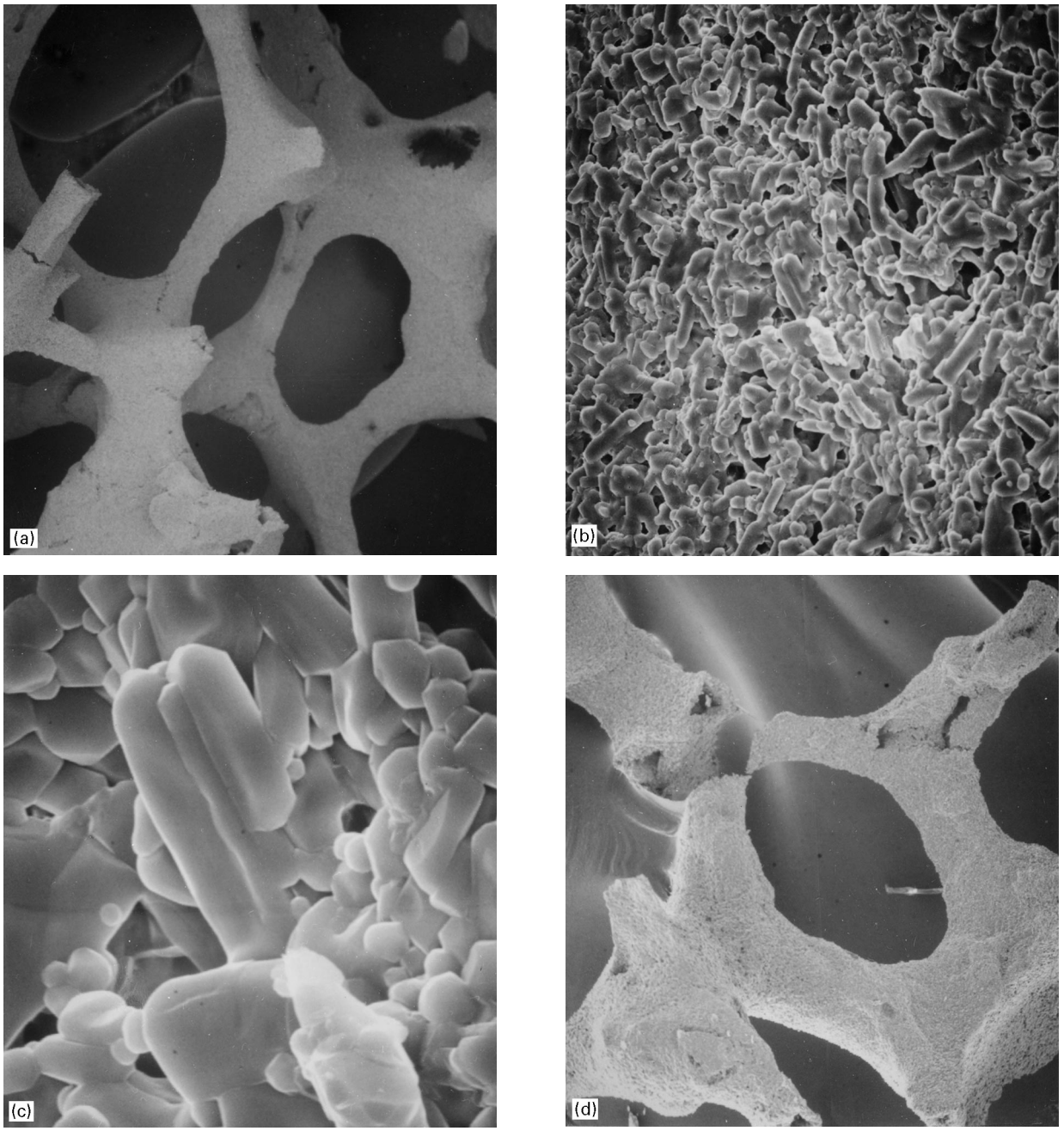


Figure 2 Scanning electron micrographs showing the surface structure of the CPP material (a–c) as-received, and (d–f) when cultured in fully supplemented medium without cells for 2 wk. The macroporosity of the CPP material is clearly seen in the lower magnification micrographs (a and d). Field width = 1.35 mm for (a) and field width = 1 mm for (d). Higher magnifications (b, c, e, and f) illustrate the individual grain structures in the as-received material and control material. Field width = 50 μm for (b) and (e) and field width = 10 μm for (c) and (f).

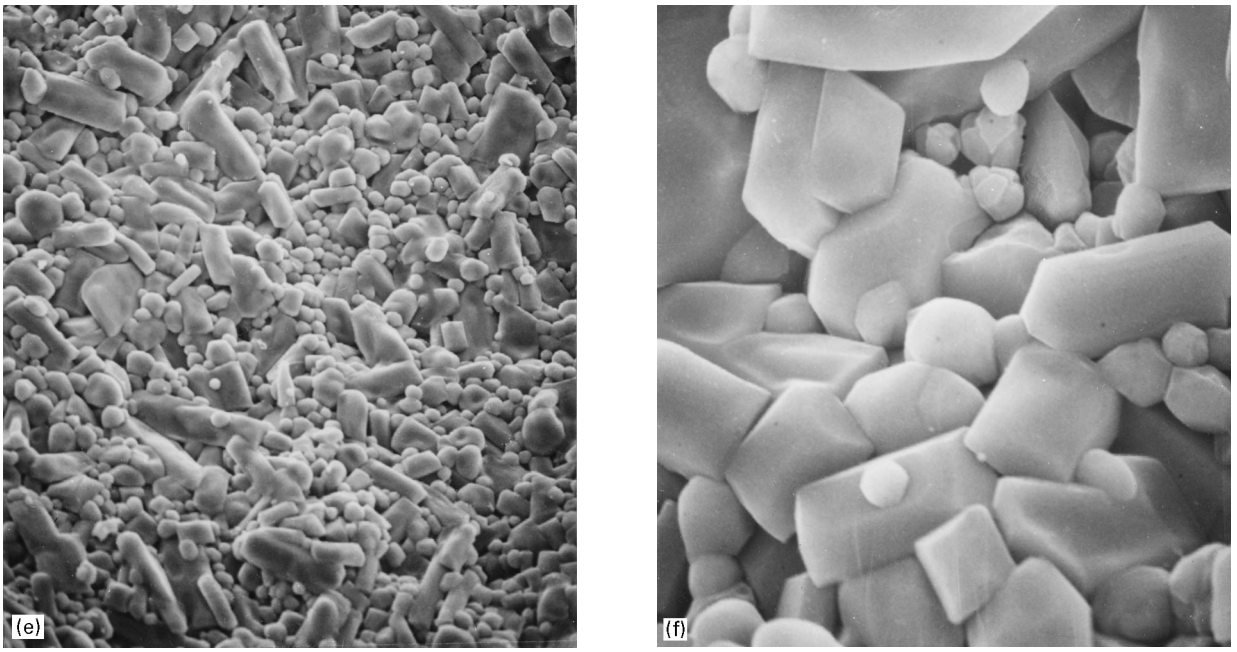


Figure 2 Continued.

the CPP scaffold is shown in Fig. 4D, where individual grains can be seen surrounded by a network of collagen fibres.

4. Discussion

To function as a bone TE construct, the scaffold has to support three-dimensional growth of osteogenic cells *in vitro* during the initial phase of the TE strategy which can then produce new bone tissue, on the pore walls. The success of the bone TE strategy is dependent on the macroporosity of the scaffold. Scaffolds with appropriate pore sizes are suited for the delivery of a large number of cells that may, upon grafting into the patient, accelerate bone remodeling at the implant site [14]. Our results clearly demonstrate that the CPP three-dimensional scaffold employed is able to support bone growth both *in vitro* and *in vivo*. During the 2 wk culture period, there was no significant change observed in the scaffold surface, although individual surface grains became rounded during the same period *in vivo*, indicating some initial surface degradation. Although it was not the purpose of this work to study the degradation behavior of CPPs, it is possible that this change in surface grain structure was the result of lowering of the extracellular pH during the initial phases of bone healing brought about by the interaction of cells, possessing membrane-bound

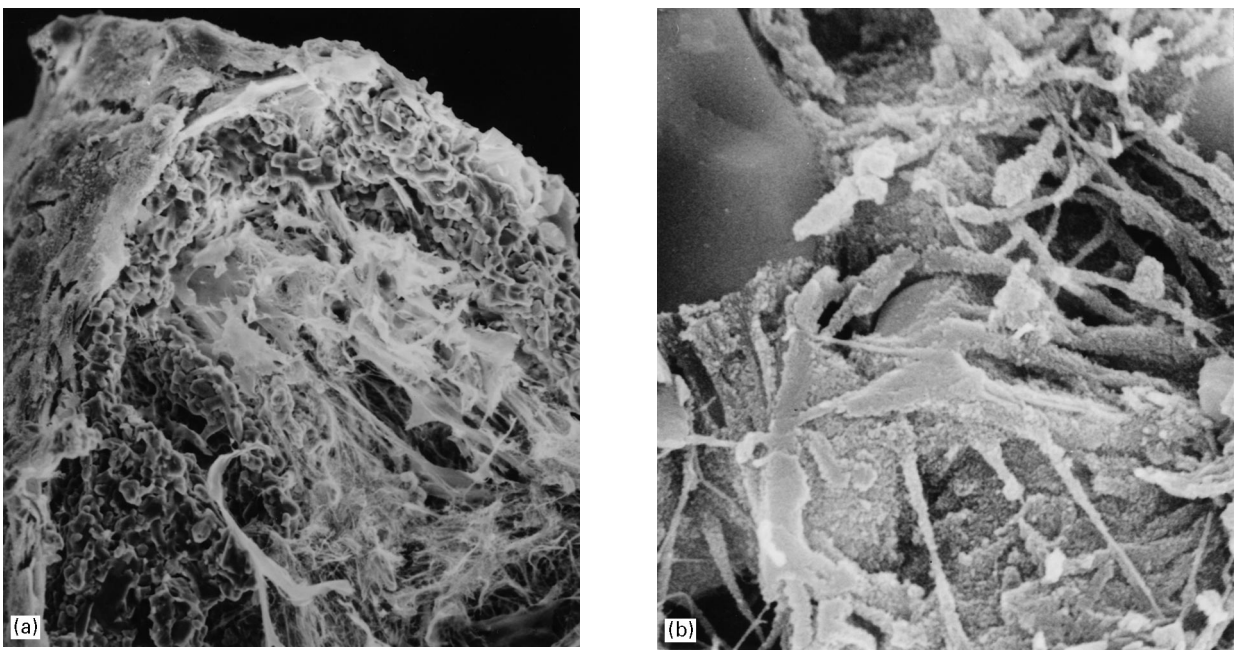
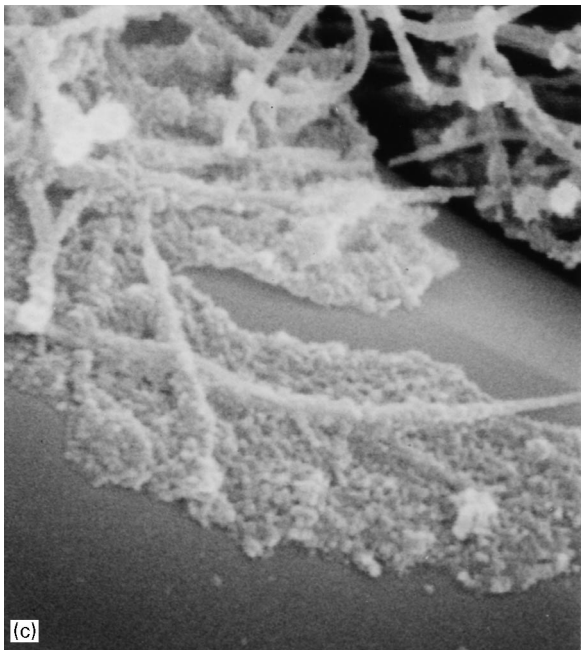


Figure 3 Scanning electron micrograhs showing the appearance of the CPP scaffold cultured in the presence of rat bone marrow cells for 2 wk. Field widths = 178, 6, 2, and 3.4 μm for (a)–(d), respectively.



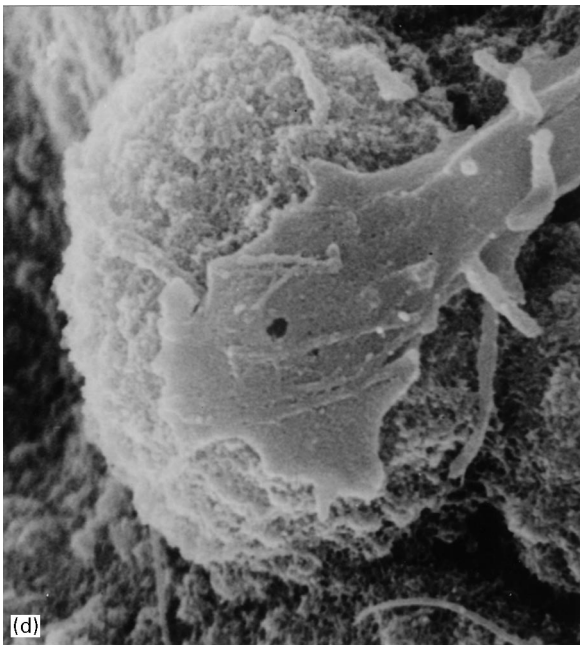


Figure 3 Continued.

proton pumps, with the material surface that we have observed in other calcium phosphate implants [15].

It is interesting to note that the tissue interface created *in vitro* and *in vivo* was quite different. This was expected because in the *in vitro* assay, the majority of the cells populating the scaffold surface, in our dexamethasone-containing culture medium, would be of osteogenic phenotype. We have already demonstrated the ability of such cells to form the cement lines represented in Fig. 3b, c and d [13]. However, implantation of these scaffolds would result in not only initial contact with blood, but also the occupation of the scaffold surface by the blood and perivascular connective tissue cells, prior to the infiltration of osteogenic cells. Thus, the retrieved *in vivo* interface shows the CPP surface occupied by fibrillar connective tissue, although bone tissue has evidently grown into the intergranular surface microporosity of the scaffold. These results illustrate the distinction between the biological interface created through classical biomaterials implantation, and that which is achieved in the *in vitro* stage of the TE strategy involving seeding the scaffold with a population of osteogenic cells.

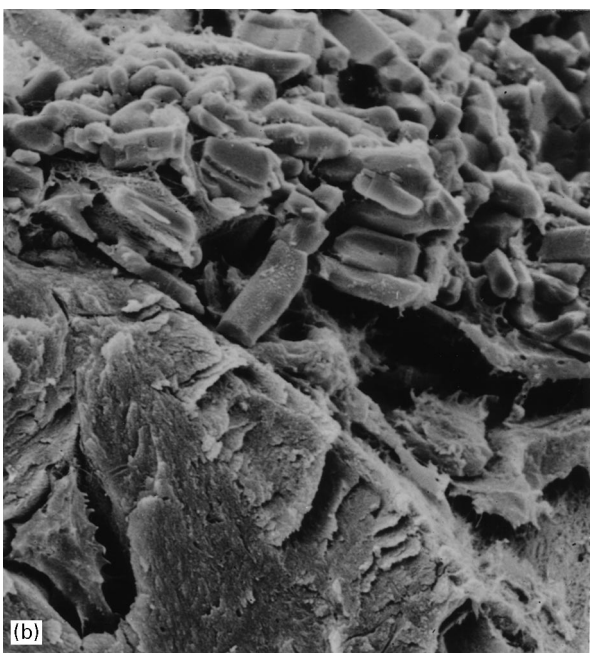
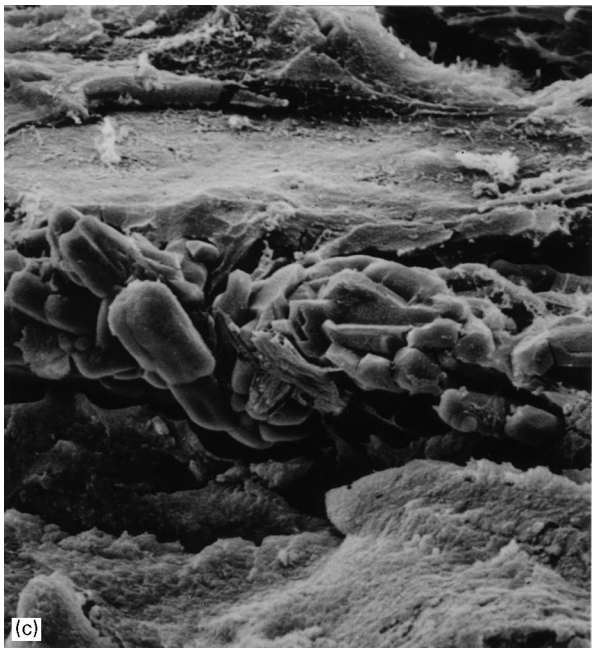


Figure 4 Scanning electron micrographs showing the scaffolds *in vivo* retrieved after 2 wk. The implantation site is illustrated in (a). Field width = 1.7 μm. The appearance of the CPP grain structure and its relationship with the biological surface is seen in (b). (Note also the osteocyte at the bottom left.) Field width = 43 μm. The interdigitation of the bone matrix with the surface of the scaffold is evident in (c). Field width = 32 μm. Individual CPP grains enveloped with collagen fibres are seen in (d). Field width = 18 μm.



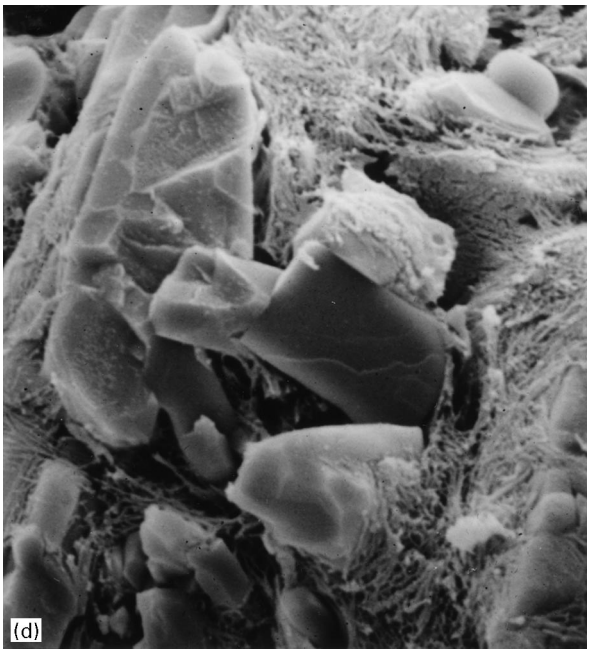


Figure 4 Continued.

5. Conclusion

These preliminary studies have demonstrated that the three-dimensional CPP employed can be successfully used as a bone tissue engineering construct.

Acknowledgments

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