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Bioactive Ca-P scaffolds used for bone reconstruction①

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Abstract: Bioactive ceramic scaffolds HA¶TCP, aimed to be applied in clinic, were evaluated both in vitro and in vivo models. HA¶TCP was supposed as a completely biodegradable material and designed as a scaffold to be used for bone reconstruction or regeneration. Materials processing was proposed and physical properties as well as microstructure feature were characterized. Biological postulation of the relationship between seeding density and proliferation, and viability of human osteoblasts cultured on the porous HA¶TCP were quantitatively measured. Bone reconstruction was investigated both in vitro and in vivo by using these biodegradable scaffolds with pore sizes ranged in 200-400 μm in diameter. The degradable scaffold supported cellular proliferation of seeded osteoblasts on the scaffold and shown normal differentiated function in vitro. Seeding density is an important factor for cell attachment and proliferation expression and has been considerably discussed. Suitable pore size of the scaffolds is required if promotion of bone reconstruction is desired. Clinical trials show that HA¶TCP scaffolds are successful applied for bone reconstruction and regeneration and can be completely degraded in human body in 12 months. This approach suggests the feasibility of using porous HA¶TCP scaffold materials for the transplantation of autogenous osteoblasts to regenerate bone tissue.

Key words: Ca-P bioactive scaffold; processing and characterization; biological evaluation in vitro; clinical trails
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1 INTRODUCTION

Skeletal reconstruction or regeneration is required in cases involving large defects created by tumor resection, trauma, and skeletal abnormalities. Grafts and flaps of autogenous tissue are two of the most successful means of reconstruction because they allow the transplantation of bone containing bioactive molecules, live cells, and frequently, a vascular supply that allow the transplant to survive and remodel even in hostile radiated environments. However, only a minimal amount of tissue can be harvested for autografts, and it is very difficult to form in the desired shapes. Therefore surgeries are looking for some allograft bone or special materials with the features similar to autografts, meanwhile with plenty of resources. There are a lot of options for artificial materials including allograft bone, nondegradable bone cement, metals, and ceramics. All of these options have their associated shortcomings and limitation. These shortcomings have inspired a research for improved methods of repairing skeletal defects. Although metallic biomaterials display the ideal mechanical properties, the chemical feature is different from that of human being[1,2]. Many applications revealed that after being implanted into human body, various detrimental reactions are unable to be eliminated. Interface corrosions each other between prosthesis and human tissue are always presented[3-6]. As a result, the host's tissue is likely to respond through the rejection phenomenon of immuno-response, which endangers the host's body and the fibrous capsule formed at interface[7,8]. In order to obtain biomaterial which can be efficiently applied in clinic, excellent physio-chemical characteristics paralleled with some particular biologic functions must be carefully considered together. Bioactive ceramic material used to repair and substitute human hard tissue is a new branch of material field and is to be gradually noticed in the field of medicine and clinic[9-12]. Ca-P bioactive ceramic may meet the biological requirements for its composition and microstructure can be similar to that of hard tissue of human beings. Even though the mechanical performance of Ca-P bioactive ceramic can not be matched to human hard tissue(bone and tooth), Ca-P bioactive ceramic can be taken as scaffolds to allow cells ingrowth, and is helpful to promote bone reconstruction or regeneration in the way of tissue engineering[13,14]. In many cases, compared with biological features, mechanical properties are much less important. For example, due to excellent mechanical properties, metallic implants or prostheses are used to connect broken bone. However, stress that must be

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transformed by bone is impaired by the metallic implants, consequently, there is no stress to stimulate tissue growth at the broken bone ends, and the bone reconstruction would stop soon[15,16]. On the other hand, if biodegradable implants such as Ca-P bioactive ceramic are used as scaffolds, cellular tissue can grow into the pores in the scaffolds and partial stress will pass through the broken ends, which may result in a stimulation action to accelerate bone reconstruction. For this reason, Ca-P biodegradable scaffolds are fabricated and modulated by cell culture before the scaffolds are moved into human body. Some biological performances related to both material feature and cell characters are revealed in this study.

2 EXPERIMENTAL

2.1 Material design and scaffolds fabrication

Based on analysis of the fundamental construction, and chemical element of human bone, Ca, P, O, H, C, Mg elements which form the main part of hard tissue in human bone were elected as chemical compositions of bioactive ceramic materials to produce HA•TCP bioactive ceramic scaffold. Firstly, HA•TCP powder was made by sol-gel solution reactions[17-19]. It is critical to control correctly the original composition and process parameters of making powder. Most of all, pH value of sol-gel solution and reaction temperature of sol-gel solution are essential[20,21]. Depending on the research result, the compositions and parameters may be adjusted. As the secondary step, HA•TCP powder was blended with foams and polyvinyl solution to obtain slurry, which was soaked by porous organic material mould with the biological specimen or the expecting scaffold prosthesis shape. Finally, the specimen was sintered at elevated temperature ranged in 1 000-1 350 ℃. Sintered specimens for cell culture in vitro were approximately 3.0 mm thick and 10 mm × 10 mm square, and scaffolds for clinical trials were depended on practical application in clinic without exactly morphological sizes. Pore size in specimen was controlled by pore size in the porous organic material mould and the viscosity of the slurry. X-ray diffractogram (Sieffert XRC-2 diffractometer) was recorded to investigate phase structure of the scaffold materials to compare with that of human ilium. The mole ratio of Ca to P and the mass fraction of HA in sintered materials were also determined by X-ray diffractometer. For SEM characterization of the scaffold surfaces, the porous samples were mounted on carbon adhesive discs on aluminum stubs and sputter coated with gold. Pore size and pore shape were observed by scanning electron microscopy(Jeol JSM 840A SEM).

2.2 Biological test on materials

Biological tests were carried out both in vitro and in vivo. Prior to cell seeding, the scaffold specimen was prewetted with ethanol for 2 h to sterilize and enhance its water uptake. The ethanol was removed by soaking with agitation for 1 h in three changes of phosphate-buffered saline(PBS) and then for 3 h in two changes of media. Stroma osteoblastic cells were obtained from the marrow of young adult male. Cell clumps were broken up by repeatedly pipetting the cell suspension. The cells were then centrifuged at 6 000 $r \cdot min^{-1}$. The resulting cell pellets were resuspended in DMEM and plated in T-75 flasks. After 10 d, other un-attached cells were removed from the flasks by repeatedly washing with PBS. With confluent monolayers reached, cells were enzymatically lifted from the flasks by trypsin. Aliquots of 50 μL of cell suspensions with cell seeding densities of 5×10^3, 1×10^4, 5×10^4, 1×10^5, 1×10^6 and $5 \times 10^6/cm^2$ were seeded on the top surface area of the HA•TCP scaffolds(specimen) which were mounted in the 24 well polystyrene culture plats, respectively. The scaffolds were left undisturbed in an incubator for 45 min to allow the cells to attach to the scaffolds, after which time an additional 2 mL culture medium containing 20 μL dexamethasone was added to each well. Medium was changed every 2 d. At time intervals of 1, 2, 3, 4, 5, 6 week, cells in the scaffolds were washed three times by PBS, and de-attached by trypsin solution. Cell numbers were determined both by heamocytometer counter and by a fluorometric quantification of DNA by an assay adapted from West. Cells were visualized in the confocal laser scanning microscope(CLSM) studied by staining the sample with ethdium and phalloidin(Southern Biotechnology Associate, Inc, USA). Cell morphology and cellular mitosis on the scaffolds was observed by CLSM. Function to differentiate extrocellular matrix collagen was evaluated by collagen immunoflorescence analysis. At day 42, the culture medium was discarded and the cells were washed with sterilized PBS three times followed by fixing the cells on the scaffold by using methanol-acetone solution with the volume ratio of 1∶1. Samples were fixed over night at 4 ℃. Primary antibody-goat anti-type I collagen(Southern Biotechnology Associate, Inc, USA) was applied to samples. This was left over night for 20 h. After carefully washing the samples with PBS, neutral avidin-FITC (Southern Biotechnology Associate, Inc, USA) was covered on samples and incubated at room temperature for 3 h. Examination was performed by CLSM. On the ground of those experiments, scaffolds made by these materials were used to fill up bone defects where necrotic hard tissue was cut away during operation or to connect the broken bone.

2.3 Clinical trials

Stromal marrow containing osteogenic cells of patients was obtained by drilling a small hole on the healthy hip bone and primary cultured in the medium containing 250 mg/L gentamicin sulfate (GS). This antibiotic was used to avoid contamination during the period of cell culture in vitro condition. 10 d later, osteoblasts(partial osteoblasts together) were harvested and then seeded on the scaffolds, resulting in seeding density of $5 \times 10^6/cm^2$. The scaffolds were left undisturbed in an incubator for 45 min to allow the cells to attach to the scaffolds, then culture medium was added to the wells. After 28 d culture in 14 changes of the culture medium, the scaffolds were moved into an operational hole on the thigh bone of the patient where necrotic hard tissue was cut away. The operational hole sized 4 cm in diameter roughly and about 20 cm^3 scaffold was filled in. Reconstruction procedure of the new bone in the operational site was observed by X-ray diffractometer at the time of 3, 6, 9, 12 and 15 months after the operation.

2.4 Statistical analysis

All measurements were collected and expressed as means +/− standard deviations. Single factor analysis of variance was employed to assess the statistical significance of results for all biological experiments. In addition, a two-tailed unpaired test was used to evaluate the significance of the cell seeding density effect on percent of cell attachment after 2 d of culture and the culture time effect on cell proliferation.

3 RESULTS AND DISCUSSION

3.1 Characteristics of materials

XRD patterns of HA-TCP sintered at 1 250 ℃ and human ilium are shown in Fig. 1. Compared with the XRD pattern of human ilium, HA-TCP material has the phase structure similar to that of natural human bone, which consists of HA containing partial TCP. In agreement with change of processing parameters and controlling the original composition concentrates in solution, the ideal phase and elements levels in harmony with the need of practices could be approached. Generally, the mole ratio of Ca and P in material should be equal to 1.50−1.67. Calcium-phosphate with the ratio being less than 1.5 expresses too easy to be solved in the liquid of human body and calcium-phosphate with the ratio being larger than 1.67 expresses bioinert[12, 13]. The mole ratio of Ca to P was measured by X-ray diffractometer and the

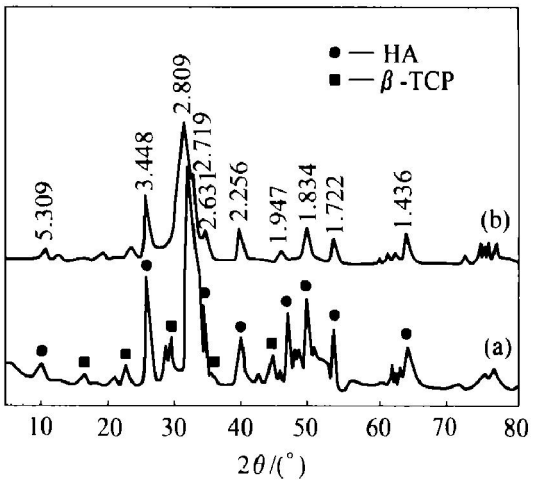

Fig. 1 XRD Patterns of (a) HA-TCP and (b) human ilium

datum is 1.62, corresponding hydroxyapatite in the sintered material being equal to 70% and tricalcium phosphate equal to 30% around. SEM observation in Fig. 2(a) shows that most pore size are ranged within 200−400 μm, which is 10−20 times of the diameter of human osteoblast[8]. Pore size in scaffold is very important because cell growth in pores requires enough nutrition and metabolism. Too small the pore size will limit cellular metabolism and also limit the cells to move into the pores. For cell growth in the pores, cell number must be enough, because the environmental condition must be met for cell proliferation which depends on cell communication each other[22−24]. The less the cell density, the less the information, the lower the proliferation rate.

3.2 Biological characteristics

Osteoblasts seeded onto HA-TCP scaffolds were attached to the pore surface and continued to proliferate over 6 weeks in vitro culture period on all the samples. Scanning electron micrographs reveal the pore morphology before osteoblast seeding. CLSM depth projection micrographs demonstrate the initial attachment of osteoblasts on pore bottom surface (Fig. 3(a)). By phalloidin stained cellular mitosis can also be observed under CLSM observation (Fig. 3(b)). Only a fraction of the seeded cells remained attached to the scaffolds. The initial seeding density of $5 \times 10^6/cm^2$ resulted in only $3.28 \times 10^6/cm^2$, which gives 65.2% after this 24 h period. The proliferation results determined by quantification of DNA in the scaffolds, indicate that a tendency between culture time and cell proliferation. Osteoblast proliferation greatly developed between week 2 and week 4 culture in vitro(Fig. 4). Basically, cell proliferation will stop after 35 d culture in vitro.

Cell viability were significantly influenced by seeding density, as shown in Fig. 5. For cell healthy

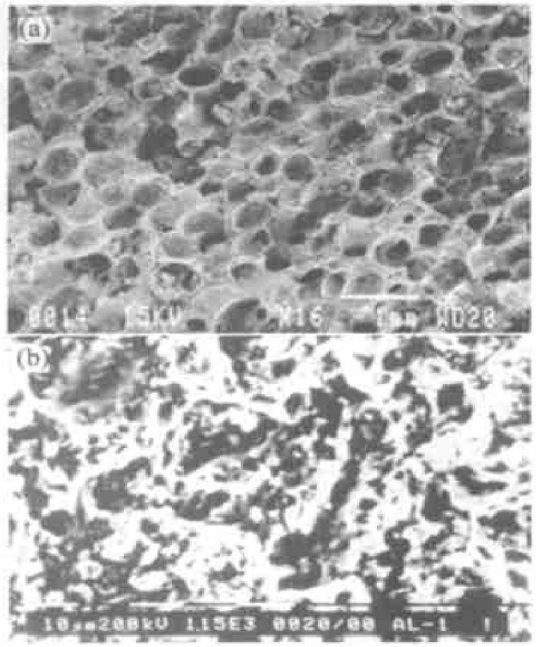

Fig. 2 SEM images of sintered Ca-P porous scaffolds with pore size equal to 200-400 μm(a), extrocellular matrix (collagen fibre) synthesized on surface of Ca-P scaffolds during cell culture(b)

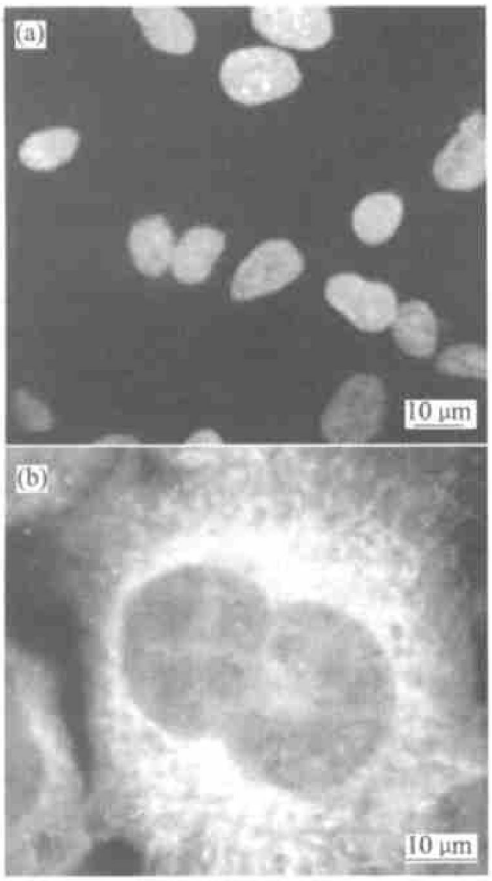

Fig. 3 CLSM images of cells in pores (a) —Depth projection micrograph of initial attachment of cells on pore bottom surface (200 μm depth from material surface); (b) —Mitosis of cells on pore bottom surface during osteoblast culture

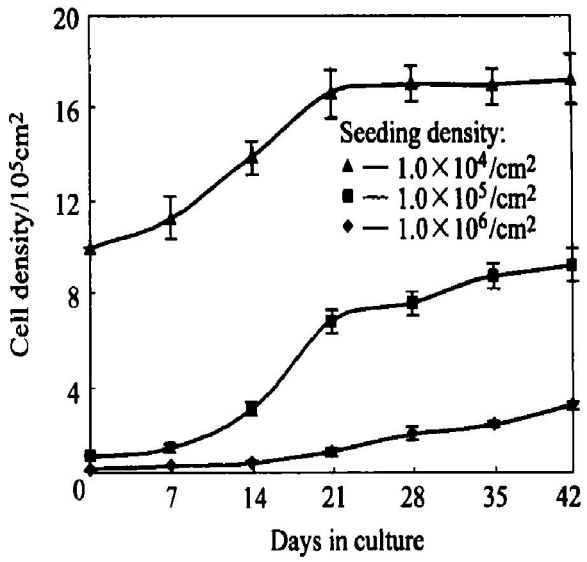

Fig. 4 Cellular proliferation on Ca-P Scaffolds

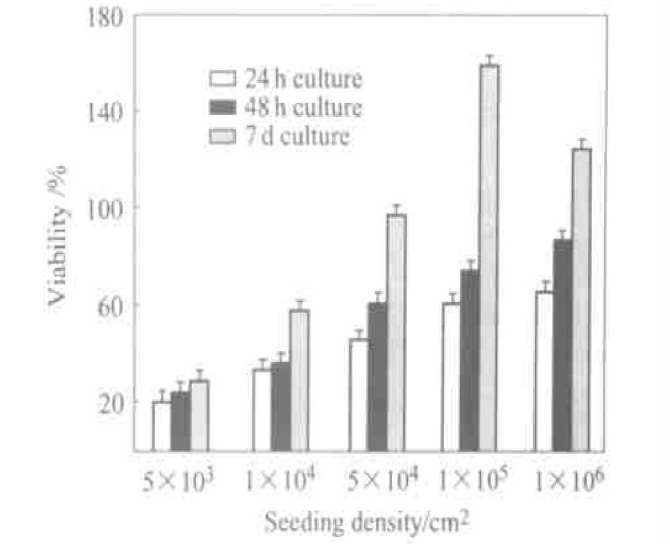

Fig. 5 Viability of osteoblasts cultured on Ca-P

growth, seeding density at least of $1 \times 10^4/cm^2$ is required, otherwise, cells in pores will die due to less information between cells each other. Observation of stained extrocellular matrix (collagen fibers) is shown in Fig. 2(b). Collagen fibers are differentiated on the scaffold surface and cellular mitosis processed in pores, indicating that human osteogenic cells have a normally functional expression on the Ca-P scaffolds.

3.3 Discussion

Defects in bone caused by disease, trauma or congenital reason are usually treated or implanted with bone graft, which is, in most cases, autogenous bone. Autogenous bone is not enough for use and the method involves another incision when it is cut from the body. The ideal bone substitute would be approximated to the autograft and requires minimally that it be biocompatible and osteoconductive, or contain osteoinductive factors to enhance new bone ingrowth, or contain osteogenic cells to begin secreting new ex-

tracellular matrix. By primary cell culture in vitro before operation, and followed by cell remolding during cell culture, the Ca-P scaffolds seem to have these features, and are suitable to be used as osteoconductive scaffolds. Tissue engineering original since 1980s is progressing now. Bioactive Ca-P, together with biodegradable polymers, could be the best materials used as scaffolds in tissue engineering. During bone regeneration by autogenous osteoblast sterilizes from stem marrow cells, the scaffolding materials used in this approach must allow the attachment of osteblasts because they are anchorage-dependent cells that require a supportive matrix in order to survive and proliferate[25-28]. This approach shows that osteoblast can attach to the Ca-P scaffolds, even mitoses in the pores. Subsequently, human osteoblasts populated the constructs by the proliferation of the transplanted cells and the migration of cells into the construct from the surrounding tissue while the HA•TCP gradually degrades or partly degrades. Eventually the construct is filled with calcified extracellular matrix secreted by the osteoblasts and is devoid of the synthetic biodegradable HA¶TCP. HA¶TCP scaffolds practically demonstrate that osteoblasts have normal proliferation and differentiation on the scaffold by regulating both the material feature and culture condition. Up to now the porous HA¶TCP bioceramic has been implanted into various bone defects more than 200 cases in our practice. Among that about 1/10 is used as scaffolds, and before the scaffolds are moved into human bodies, hostile cell culture, proliferation and differentiation on the scaffolds are performed. The required degradable time is statistically 3-5 months short than that of the same HA¶TCP materials without primary cell culture before being implanted into human body. Fig. 6 shows the typical radiograph that the process of HA•TCP scaffolds takes place biodegradation. After the osteoma on the thigh bone of the patient is cleaned off, the HA•TCP scaffolds with cell culture for 4 or 5 weeks fill into the operation vacancy and 12 months later the scaffold biodegrades fairly, and new bone forms perfectly.

Ceramic materials based on calcium phosphate are known to be the most suitable material for the implantation in the human bone because of their ideal biological performances. In the traditional sense, calcium phosphate is unable to induce the growth of newly generated bone cells and does not degrade in short time[29, 30]. However, in the present work they do express the special characteristics and the biological function. This seems that two factors affect the results, e g crystal/chemical composition and material pore structure. Many researches also show that excessive TCP in HA is useless[31, 32]. Too fast degradation velocity is very dangerous as new bone cell formation might be out of step, so the level of TCP in HA must

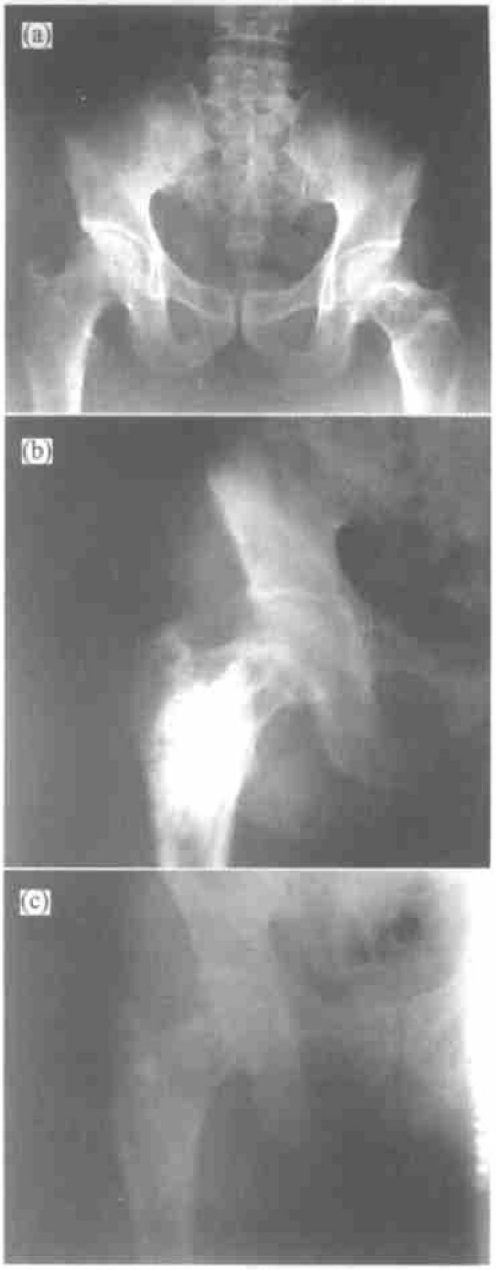

Fig. 6 Clinical trials of Ca-P scaffolds degradation and bone reconstruction
(a) —Before operation; (b) —After operation; (c) —12 months after operation

be optimally controlled. Another reason for the material degradation and new bone cell formation so rapid is the optimal pore structure and high pore ratio. The connecting pore size ranged from 200 to 400 μm is suitable for the ingrowth of soft tissue and nutrition system.

4 CONCLUSIONS

Bioactive ceramic Ca-P scaffolds display an excellent biological characteristics. The components and phase structure of bioactive ceramic HA¶TCP are similar to those of human hard tissue. Seeding density plays pivotal role in cell viability and cell proliferation. The scaffolds provide an appropriate environment for the proliferation and differentiation of os-

teoblasts and allow the ingrowth of vascular tissue to ensure the survival of the transplanted cells. An investigation of the effects of HA¶TCP scaffolds morphology and culture conditions on cell proliferation and function is needed to elucidate the important parameters in the design of an in vitro osteoblast culture system before osteoblasts transplantation could be attempted in vivo. Such an investigation is the focus of the present approach. Following this approach, several clinical trials were performed. Finally, we hope that HA¶TCP scaffolds be degradable with a controllable rate of degradation into molecular that can easily be metabolized or excreted. The HA•TCP scaffolds basically satisfy many, if not all, of these requirements. The ability of HA¶TCP scaffolds to support osteoblast anchoring, proliferation, and function already has been tested.

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