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Strong, Macroporous and In-Situ Hardening Hydroxyapatite Scaffold for Bone Tissue Engineering

Feature

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Bone fracture and damage result in more than 1.3 million surgical procedures every year in the United States alone, and this number is predicted to increase dramatically as the life expectancy of the population increases. Hydroxyapatite (HA) has found wide use as a bone replacement material due to its chemical and crystallographic similarity to the apatite in human bone. However, to fit into a bone cavity, sintered HA involves machining, which is difficult due to its brittleness. The surgeon needs to modify the surgical site in the tissue to fit the implant, or to carve the hard and brittle implant to the desired shape. This leads to increases in bone loss, trauma to the surrounding tissue, and longer surgical time. A self-hardening calcium phosphate cement (CPC) can adapt to complex cavity shapes without machining. CPC is comprised of a mixture of fine particles of tetracalcium phosphate [TTCP, or $Ca_4(PO_4)2O$] and dicalcium phosphate anhydrous [DCPA, or $CaHPO_4$]. The CPC powder can be mixed with water or body fluids to form a paste, which can be placed in the bone cavity, molded to the desired geometry, and hardened *in situ* to form resorbable HA. However, the low strength of CPC limits its use to only non-stress-bearing orthopaedic applications. In addition, macropores need to be built into CPC to enhance cell infiltration and tissue ingrowth.

To increase the fracture resistance of CPC, biocompatible and absorbable suture fibers were incorporated to develop strong CPC for bone repair in stress-bearing locations. The polymer suture consisted of individual fibers braided into a bundle with a diameter of 322 μm. The suture was cut to 8 millimeters length and randomly mixed with the CPC paste at 30 percent volume fraction. The composite paste was placed in 3 millimeters x 4 millimeters x 25 millimeters molds to make flexural specimens. The paste in each mold was allowed to set at 100 percent relative humidity at 37 ∞C for 4 h. The hardened specimens were immersed in a simulated physiological solution (1.15 mmol/L Ca, 1.2 mmol/L P, 133 mmol/L NaCl, 50 mmol/L HEPES, buffered to a pH of 7.4) at 37 ∞C for 20 h prior to mechanical testing. Fracture toughness of cement specimens was measured by using a single-edge-notched beam method in flexure with a span of 20 millimeters on a computer-controlled Universal Testing Machine. One standard deviation (sd) was given in this paper for comparative purposes as the estimated standard uncertainty of the measurements. These values should not be compared with data obtained in other laboratories under different conditions. The fracture toughness results are listed in Table I.

Materials	CPC Control	CPC Composite	Sintered Porous HA	Cortical Bone
Fracture Toughness K_{IC} ($MPa \cdot m^{1/2}$)	0.23 ± 0.03	1.44 ± 0.30	0.1 to 0.25	2 to 12

Table 1. *Fracture Toughness (mean ± sd; n = 6) of CPC-Suture Fiber Composite, CPC Control without Fibers, and Literature Values* of Sintered Porous Hydroxyapatite and Cortical Bone*

** Suchanek W, Yoshimura M. Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants. J Mater Res 1998;13:94-117. Hing KA, Best SM, Bonfield W. Characterization of porous hydroxyapatite. J Mater Sci: Mater in Med 1999;10:135-145. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: A review of current technology and applications. J Appl Biomater 1991;2:187-208. O'Kelly K, Tancred D, McCormack B, Carr A. A quantitative technique for comparing synthetic porous hydroxyapatite structure and cancellous bone. J Mater Sci: Mater in Med 1996;7:207-213.*

Although significant reinforcement was achieved using suture fibers, a concern was raised on reproducibility when different operators with varied experience were mixing the fibers with the paste and making the specimens. Therefore, three operators each made six specimens: Operator A with extensive experience in CPC and fiber mixing; Operator B with some experience in CPC and fiber mixing; and Operator C with no prior experience in CPC mixing. Operator C was given a 1-minute verbal instruction and watched Operator A making one specimen (which took about one minute). Operator C was then left alone to make specimens. The specimens were 3 millimeter x 4 millimeter x 25 millimeter bars without notches, and were used for flexural strength measurement. The

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Strong, Macroporous...

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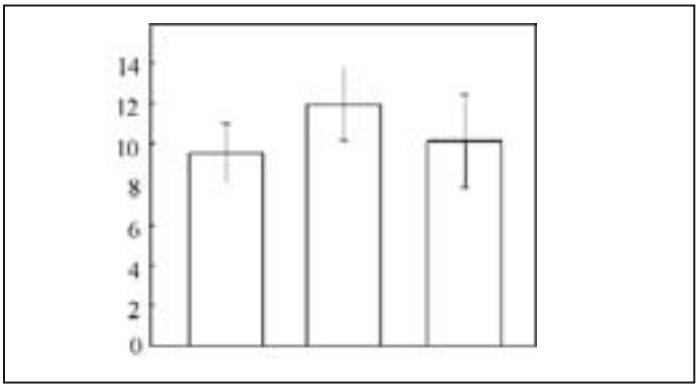

Figure 1. *Strength of CPC-suture fiber composite made by three different operators to examine operator sensitivity. The three operators produced cements with similar strengths, indicating that the composite fabrication method was reproducible. Horizontal line indicates values not significantly different (p>0.05).*

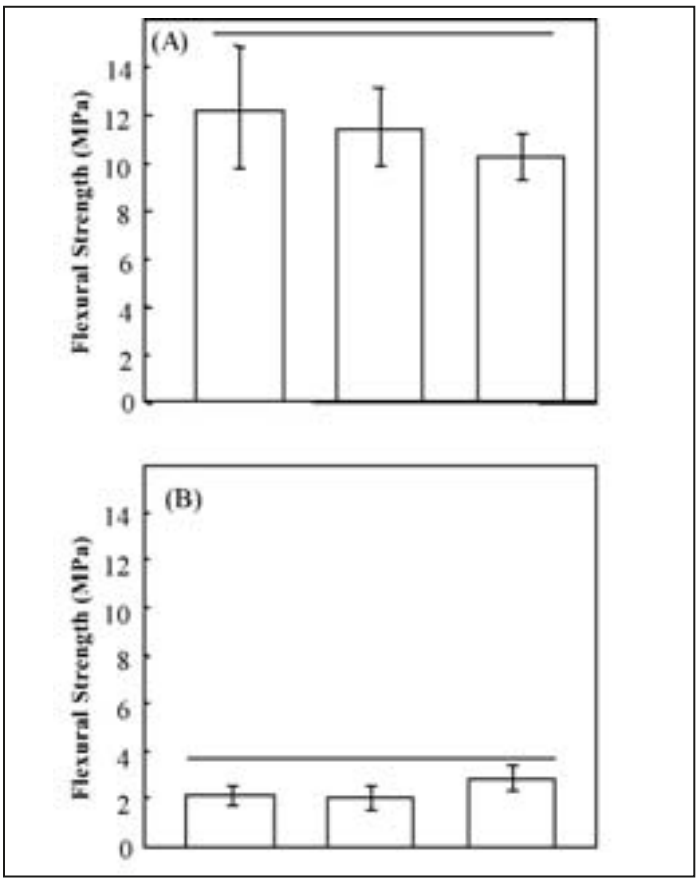

Figure 2. *Flexural strength of (A) CPC-mesh, and (B) CPC control without mesh, made by three different operators to examine the operator sensitivity. Mesh reinforcement may have potential for craniofacial and thin bone repairs. Horizontal line indicates values that are not significantly different from each other (p > 0.05).*

flexural strengths of these specimens after 1-day immersion are plotted in Fig. 1. Error bar shows one sd, n = 6. The three operators produced strengths that are not significantly different from each other (one-way analysis of variance [ANOVA]; p = 0.10).

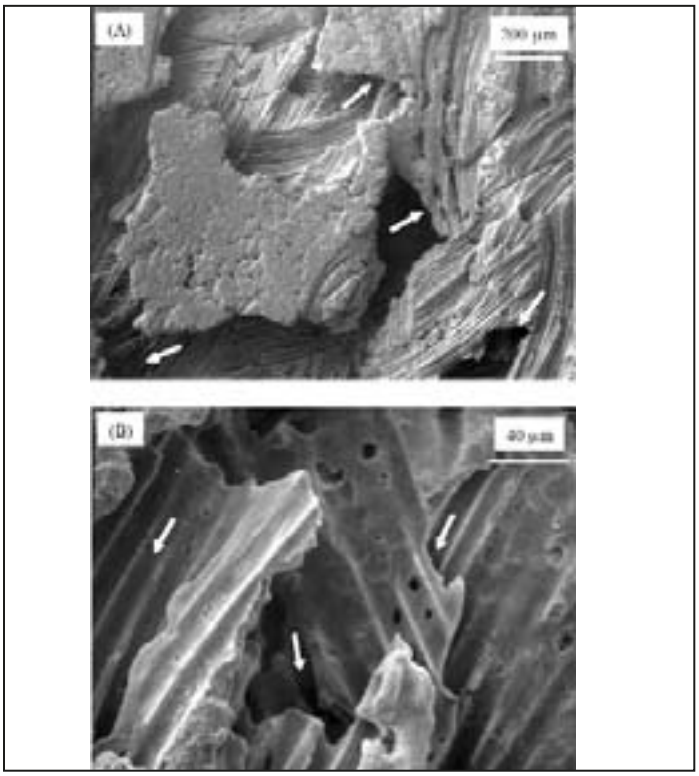

Figure 2. *SEM of pore channels in CPC-suture fiber composite scaffold after suture fiber dissolution. Arrows indicate the flow of the interconnected pore channels beneath the CPC surface.*

The above random suture fiber-CPC paste may be useful to fill a volume of bone loss, for example, due to trauma to a long bone. On the other hand, two-dimensional fiber mesh reinforcement may have potential for craniofacial and thin bone repairs, such as the reconstruction of parietal bone in the skull or other shell structures. To test the operator sensitivity, again the three operators each made six specimens. For each specimen, six sheets of an absorbable polymer fiber mesh of 4 millimeters wide and 25 millimeters long were placed into the same mold, and the CPC paste was placed on top of the mesh to fill the mesh pores and the mold, and set to form a solid specimen. Flexural strengths of mesh specimens at 1-day immersion are plotted in Fig. 2 (A), with no significant difference between each other (One-way ANOVA; p = 0.17). To show the extent of reinforcement, the three operators also made CPC control specimens without fibers or meshes. The strengths (mean ± sd; n = 6) of CPC control specimens after one day immersion in the physiological solution were shown in Fig. 2 (B). Error bar shows one sd, n = 6. The strengths of the reinforced CPC were about 4 to 5 fold higher than those of the unreinforced CPC control.

Examples of macropore channels in CPC after suture fiber dissolution are shown in Fig. 3. In (A), the arrows indicate the flow of the interconnected pore channels beneath the CPC surface. The higher magnification in (B) shows the imprints of the individual fibers of the suture bundle in the CPC,

suggesting an intimate contact between the fibers and the CPC paste. The random orientations of the pores are visible as indicated by the flow of pore channels in (A) and the pores from individual fibers in (B).

Because cell culture toxicity assays are the international standard for biocompatibility screening, *in-vitro* cell culture was performed to evaluate the biocompatibility of the new CPC formulation. MC3T3-E1 osteoblast-like cells were cultured following established protocols. Cells were cultured in flasks at 37 ∞C and 100 percent humidity with 5 percent CO2 in α modified Eagle's minimum essential medium. The medium was supplemented with 10 percent volume fraction of fetal bovine serum and kanamycin sulfate and changed twice weekly. The cultures were passaged with 2.5 g/L trypsin containing 1 mmol/L EDTA once per week. Cultures of 90 percent confluent cells were trypsinized, washed and suspended in fresh medium. Fifty thousand cells diluted into 2 mL of medium were added to each well containing a cement specimen.

A scanning electron microscope (SEM) was used to examine the specimens and the cells. Cells cultured for 1 d on cement specimens were rinsed with saline, fixed with 1 percent volume fraction of glutaraldehyde, subjected to graded alcohol dehydrations, rinsed with hexamethyldisilazane, and then sputter-coated with gold. Interactions between osteoblast cells and macroporous scaffold are shown in Fig. 4 with cells cultured for 1 day. This CPC scaffold contained a biopolymer chitosan, and the pores were formed by the dissolution of water-soluble mannitol crystals. In (A), osteoblast cells (O) attached to the scaffold near the edge of a macropore. The cell developed long cytoplasmic extensions (E) across the opening of the pore and anchored on the other side of the CPC. Cytoplasmic extensions (also termed "filopodial extensions") are regions of the cell plasma membrane that contain a meshwork or bundles of actin-containing microfilaments, which permit the movement of the migrating cells along a substratum. In (B), cells appeared to have migrated into two macropores that were interconnected with each other. In (C), cells colonized and anchored on the pore bottom, established cell-cell junctions and interactions, and formed a three-dimensional cell web.

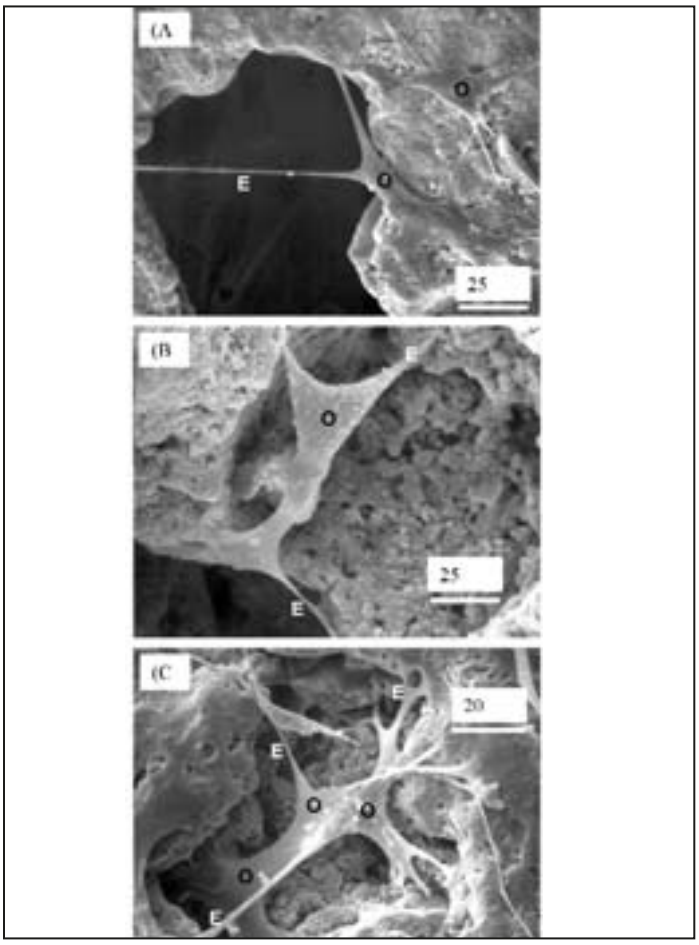

Figure 4. *SEM showing osteoblasts migrating into the macropores of a CPC scaffold. "O" = osteoblasts, "E" = cytoplasmic extensions. (A) Osteoblast cell attached near the edge of a macropore. (B) Cells migrated into macropores. (C) Cells colonized on the pore.*

The composite methods described in the present study imparted substantial reinforcement and macroporosity to a moldable, *in-situ* setting and resorbable hydroxyapatite graft. The mechanical strength and fracture resistance of the new CPC scaffold were increased to exceed those of sintered porous hydroxyapatite implants and approach that of natural bone. Compared to sintered hydroxyapatite, CPC had the advantage of self-hardening *in-situ* without machining, intimately conforming to complex cavity shapes, and being able to be resorbed and replaced by new bone. The new compositions were non-cytotoxic and supported the adhesion and spreading of osteoblast-like cells. Compared to the conventional CPC without macropores, the increased macroporosity of the new apatite scaffold may help facilitate bone ingrowth, implant fixation, and more rapid new bone formation. The CPC-suture fiber composite may be useful in moderate stress-bearing applications. When implanted *in vivo*, the suture fibers would provide strength and then dissolve to form macropores. The strengthening of CPC from new bone ingrowth should offset the weakening of CPC due to fiber degradation. In addition, the long cylindrical macropore channels in CPC should be beneficial in facilitating cell infiltration and bony ingrowth deep into the implant. These features are expected to expand the use of CPC in orthopedic repairs. The method of using absorbable fibers in grafts for strength and then formation of long cylindrical macropores for tissue ingrowth may have applicability to other tissue engineering materials.

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