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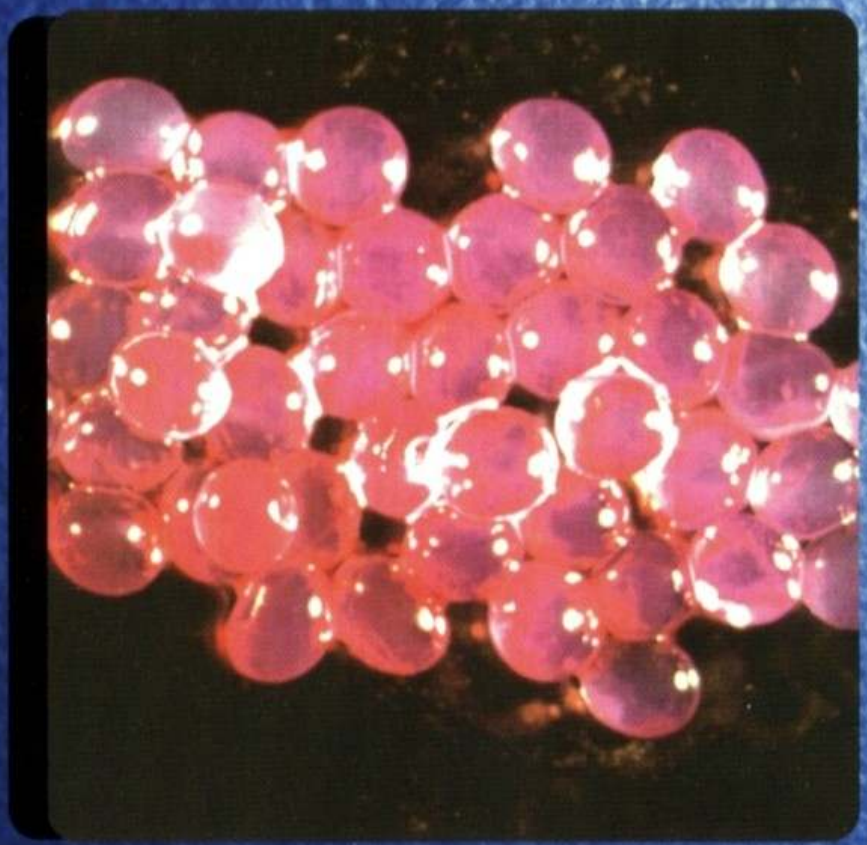

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Strong calcium phosphate cement-chitosan-mesh construct containing cell-encapsulating hydrogel beads for bone tissue engineering

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Abstract: Calcium phosphate cement (CPC) can conform to complex cavity shapes and set *in situ* to form bioresorbable hydroxyapatite. The aim of this study was to introduce cell-encapsulating alginate hydrogel beads into CPC and to improve the mechanical properties using chitosan and fiber mesh reinforcement. Because the CPC setting was harmful to the MC3T3-E1 osteoblast cells, alginate was used to encapsulate and protect the cells in CPC. Cells were encapsulated into alginate beads, which were then mixed into three pastes: conventional CPC, CPC-chitosan, and CPC-chitosan-mesh. After 1 day culture inside the setting cements, there were numerous live cells and very few dead cells, indicating that the alginate beads adequately protected the cells. Cell viability was assessed by measuring the mitochondrial dehydrogenase activity, using a Wst-1 colorimetric assay. Absorbance at 450 nm (arbitrary units) (mean ± SD; $n = 5$) was 1.36 ± 0.41 for cells inside conventional CPC, 1.29 ± 0.24 for cells inside CPC-chitosan composite, and 0.73 ± 0.22 for cells inside CPC-chitosan-mesh composite. All three values were similar to 1.00 ± 0.14 for the control with cells in beads in the cell culture medium without any CPC (Tukey's at $p = 0.05$). Flexural strength for conventional CPC containing cell-encapsulating beads was 1.3 MPa. It increased to 2.3 MPa when chitosan was incorporated. It further increased to 4.3 MPa with chitosan and the reinforcement from one fiber mesh, and 9.5 MPa with chitosan and three sheets of fiber mesh. The latter two strengths matched reported strengths for sintered porous hydroxyapatite implants and cancellous bone. In summary, cell-encapsulated-alginate-CPC constructs showed favorable cell viability. The use of chitosan and mesh progressively improved the mechanical properties. These strong, *in situ* hardening, and cell-seeded hydroxyapatite cements may have potential for bone tissue engineering in moderate stress-bearing applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 487–496, 2006

Key words: calcium phosphate cement; alginate hydrogel; macroporous; mesh reinforcement; cell encapsulation; cytotoxicity; bone tissue engineering

INTRODUCTION

It is estimated that bone tissue is the second most transplanted tissue, with an estimated 1 million procedures performed annually to repair bone defects caused by trauma, disease, or congenital defects.[1,2] Autologous grafts have drawbacks, including limited availability and donor site morbidity. Allogenic grafts, where bone is harvested from another person's body, suffer from the risk of disease transmission and, in reducing that risk, compromising the osteoinductivity of the grafts.[1,2] It is evident that the development of a biocompatible, osteoinductive biomaterial with physical properties similar to that of cancellous bone is important. Hydroxyapatite has been used for hard tissue repair because of its similarity to the apatite in teeth and bones.[3–10] While sintered hydroxyapatite implants require machining to fit a cavity, calcium phosphate cements (CPC) can be molded and self-harden in the bone cavity.[11–14] The CPC powder can be mixed with an aqueous liquid to form a thick paste that can be placed into a defect site. Once hardened, CPC converts to microcrystalline hydroxyapatite, which is biocompatible and can be replaced by a new bone.[11–13]

Disclaimer: Certain commercial materials and equipment are identified to specify experimental procedures. In no instance does such identification imply recommendation by NIST or the ADA Foundation or that the material identified is necessarily the best available for the purpose. One standard deviation was used as the estimated standard uncertainty of the measurements. These values should not be compared with data obtained in other laboratories under different conditions.

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Although moldability and osteoconductivity make CPC an excellent candidate for a wide range of orthopedic applications, its poor strength has limited its use to only nonstress-bearing locations.[12,13] Fibers are known to increase the strength of biomaterials.[15–17] Accordingly, in recent studies, strong and macroporous CPC scaffolds were developed.[18–20] For example, CPC with absorbable fibers possessed a high initial strength, and the degradation of the fibers created macroporous channels in the implant suitable for bone ingrowth.[20] In addition to the use of fibers, the incorporation of a biopolymer chitosan also increased the strength of CPC.[19]

Inherent in any design of biomaterials for tissue engineering is the incorporation of living cells inside the biomaterial construct. Recent work suggested that cells mixed with the CPC paste had a limited survival rate.[21] To overcome this limitation, researchers encapsulated cells in hydrogel beads composed of alginate.[21] However, in the previous study,[21] no investigation was made on the degradation of CPC strength by the incorporation of hydrogel beads. Furthermore, no attempt was made to incorporate cells into the CPC-chitosan composite and the CPC-chitosan-fiber mesh composite.

The aim of this study was to incorporate MC3T3-E1 osteoblast cells into the pastes of CPC-chitosan and CPC-chitosan-fiber mesh composites. The following concepts were investigated: (1) encapsulation would protect the cells from the setting reactions of CPC-chitosan paste and CPC-chitosan-fiber mesh composite paste; and (2) while CPC containing cell-encapsulated beads would be extremely weak mechanically, chitosan and mesh reinforcement would increase the mechanical properties of the cell-seeded CPC construct to match reported strengths for sintered porous hydroxyapatite implants and natural cancellous bone.

MATERIALS AND METHODS

Powder, liquid, and fiber mesh

The CPC powder consisted of a mixture of tetracalcium phosphate (TTCP:$Ca_4[PO_4]_2O$) and dicalcium phosphate anhydrous (DCPA:$CaHPO_4$). TTCP was synthesized from a solid-state reaction between DCPA and calcium carbonate ($CaCO_3$) (Baker Chemical, Phillipsburg, NJ), then ground and sieved to obtain TTCP particle sizes of 1–80 mm, with an average diameter of 17 μm. The DCPA powder was ground to obtain particles with sizes ranging from 0.4 to 3.0 μm, with an average diameter of 1 μm. The TTCP and DCPA powders were mixed at a molar ratio of 1:1 to form the CPC powder, which was sterilized under ultraviolet light for 48 h prior to experiments.

Chitosan and its derivatives are natural biopolymers that are biocompatible and biodegradable.[22] Hence chitosan lactate (referred to as chitosan in this paper) was incorporated into the CPC-cell constructs for reinforcement. Chitosan powder (VANSON, Redmond, WA) was dissolved in sterile distilled water at chitosan/(chitosan + water) = 15% mass fraction to form the chitosan liquid. The 15% fraction was selected following the results of a previous study.[23] Prior to use, the liquid was sterilized under ultraviolet light for 48 h. An absorbable fiber mesh (Vicryl, Ethicon, Somerville, NJ), which is a copolymer of glycolic and lactic acids, was used to reinforce the CPC-cell construct. This mesh was selected because it was used clinically and possessed a relatively high strength.[20]

Cell culture with freshly-mixed CPC paste

Cells were cultured with freshly-mixed CPC paste to examine the cytotoxicity of the CPC setting reaction. Clonal murine calavarial cells, MC3T3-E1 subclone 4 (American Type Culture Collection, Manassas, VA) were cultured at 37°C with 100% relative humidity, and 5% CO_2 in α-modified Eagle's minimum essential medium (BioWhittaker, Walkersville, MD).[20] The medium was supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate (Invitrogen, Carlsbad, CA).

Fifty thousand cells diluted into 2 mL of media were added to each of 12 wells of tissue culture polystyrene (TCPS) and incubated for 1 day. Each well had an inner diameter of 22.1 mm and a height of 17.5 mm. The CPC powder was mixed with distilled water at 3:1 mass ratio to form a paste of 0.4 g. Within 2 min from mixing, the 0.4 g CPC paste was placed in each of six wells over the monolayer of cells. The CPC paste was allowed to set while submerged in the cell culture medium. The other six wells had no CPC and served as control. After 24 h, the cells were stained by adding 3.0 mL of medium containing 0.002 mmol/L calcein-AM and 0.002 mmol/L ethidium homodimer-1 (Molecular Probes, Eugene, OR).[20,21] The cells were observed by epifluorescence microscopy (Eclipse TE300, Nikon, Melville, NY), in which the live cells displayed green fluorescence and the dead cells displayed red fluorescence. The principle of this live/dead double staining assay is that membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence.

Cell culture with set CPC

Cells were cultured on hardened CPC, in which the setting reaction was complete to examine the cytotoxicity of set CPC. Two materials were made, one with water as liquid (conventional CPC), and the other using the chitosan liquid (CPC-chitosan composite). Each paste was placed into $3 \times 4 \times 25$ mm^3 molds and incubated in a humidor with 100% humidity at 37°C. After 4 h, the set specimens were de-

molded and immersed in distilled, deionized water for 20 h. The CPC setting reaction was largely complete in 1 day.[23]

Fifty thousand cells diluted into 2 mL of media were added to each well containing a CPC specimen or a CPC-chitosan specimen. A previous study already performed live/dead staining on set CPC and showed numerous live cell with few dead cells.[20] Hence the live/dead staining on the set CPC was not repeated in the present study. Instead, scanning electron microscopy (SEM) was used to examine cell attachment to the set specimens at a higher magnification than the epifluorescence microscopy. Cells cultured for 1 day on the set specimens were rinsed with saline, fixed with 1% glutaraldehyde, subjected to graded alcohol dehydrations, rinsed with hexamethyldisilazane, and sputter coated with gold. SEM (JEOL 5300, Peabody, MA) was used to examine the specimens.

Cell encapsulation in alginate beads

The above experiments showed that the setting of the CPC paste was harmful to the cells, but the set CPC was noncytotoxic. Hence, alginate was used as an encapsulating gel to protect the cells. Alginate is biocompatible and can form a crosslinked gel under mild conditions.[24,25] A 1.2% (mass fraction) sodium alginate solution was prepared by dissolving 0.3 g alginate (UP LVG, 64% guluronic acid, MW = 75,000–220,000 g/mol, ProNova Biomedical, Oslo, Norway) in 25 mL of saline (155 mmol/L NaCl). Cells were encapsulated in alginate at a density of 100,000 cells/mL of alginate solution for the live/dead staining experiment, and 500,000 cells/mL for the Wst-1 experiment, following a previous study.[21] This resulted in the number of encapsulated cells being 558 cells/bead and 2790 cells/bead, respectively. Bead formation was accomplished by extruding alginate/cell droplets through a sterile syringe fitted with a 25-gauge needle into wells containing 7 mL of 100 mmol/L calcium chloride solution. The alginate droplets crosslinked and formed beads in the calcium chloride solution. The beads were then collected and washed with sterile α-modified Eagle's minimum essential medium.

Seeding cell-alginate beads into CPC composite pastes

A sterile Teflon ring (16 mm diameter and 3 mm height) was placed in the well to contain the alginate beads and facilitate the subsequent removal of the set CPC for analysis.[21] Fifty-seven cell-encapsulating alginate beads were placed into each ring. The remaining volume of the ring was filled with a CPC paste at powder:liquid of 3:1 to completely cover all the beads. This resulted in the beads and the CPC paste each occupying approximately half of the volume of the ring. This was based on preliminary results in making flexural specimens with the requirement of incorporating as many beads into CPC as possible without rendering the specimens too weak mechanically. The cell-alginate-CPC construct was allowed to set at 37°C for 30 min. Then, fresh culture medium was added to each well until the construct was completely submerged.

Four materials were tested: conventional CPC (using water as liquid); CPC-chitosan (using the chitosan liquid); CPC-chitosan-mesh (a circular mesh sheet of 16-mm diameter was placed on the cell-encapsulating alginate beads, with the CPC-chitosan paste impregnating the mesh and filling the rest of the ring). The fourth material, serving as the control, consisted of cell-encapsulating alginate beads in the culture medium without any CPC.

Live cell density and viability quantification

The above four materials were submerged in the culture medium for 24 h. Then, the constructs were carefully broken and the alginate beads were harvested following a previous study.[21] The cells in beads were stained and observed by epifluorescence microscopy. Live cell density was measured as the number of live cells/(the number of live cells + the number of dead cells).[26] To estimate the live cell density, four randomly-chosen fields of view were photographed from each specimen. Each field of view was photographed through a green filter and red filter to yield eight pictures from each specimen. With $n = 5$, this yielded 40 images/material and a total of 160 images for the four materials.

The same four materials were tested using the Wst-1 assay, which is a colorimetric assay where the absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cells.[20] Larger absorbance values indicate increased production of the formazan product that is correlated to cell viability. Wst-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt], and 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) were obtained from Dojindo (Gaithersburg, MD). Specimens were washed with 1 mL of Tyrode's Hepes buffer.[20] One milliliter of Tyrode's Hepes buffer and 100 μL of Wst-1 solution (5 mmol/L Wst-1 and 0.2 mmol/L 1-methoxy PMS in water) were then added to each well. After 2-h incubation at 37°C, a 0.2 mL aliquot from each well was placed in a 96-well plate and the absorbance was measured with a microplate reader (PerkinElmer, Gaithersburg, MD).[20]

Mechanical properties

The CPC powder and liquid were mixed at powder:liquid of 3:1. The cell-alginate beads were mixed into the paste, and the composite paste was placed into a $3 \times 4 \times 25$ mm^3 mold. Twenty-nine beads in each specimen gave an alginate bead volume fraction (volume of alginate beads/specimen volume) of ~54%. This volume fraction was selected based on the need to incorporate as many beads into CPC as possible without rendering the specimen too weak to be demolded. With a cell density of 10^5 cells/mL and 29 beads, each specimen contained an average of 16,182 cells. The specimen in the mold was incubated in the humidor for 4 h at 37°C, then demolded and immersed in water for 20 h.[18] In making mesh-composite specimens, the mesh was cut into sheets of

$\sim 4 \times 25$ mm^2 and placed into the mold. Then, the CPC paste containing the cell-alginate beads was placed with a spatula on the top of the mesh and lightly pressed to fill the pores of the mesh and to fill the rest of the mold.[20] The fiber mesh had a layer thickness of $\sim$230 μm and pores of 100–300 μm; the CPC paste impregnated the pores of the mesh and then hardened to form a cohesive specimen.

Four formulations were tested: (1) conventional CPC containing cell-alginate beads (no mesh); (2) CPC-chitosan containing cell-alginate beads (no mesh); (3) CPC-chitosan-mesh containing cell-alginate beads; (4) CPC-chitosan-3mesh containing cell-alginate beads. "CPC-chitosan-mesh" contained one sheet of mesh on the prospective tensile side of the specimen. "CPC-chitosan-3mesh" contained three sheets of mesh on top of each other in the tensile side of the specimen. These numbers of mesh were selected following a previous study.[20] During the flexural test, the side of the specimen containing the mesh was placed in tension, and the mesh plane was perpendicular to the applied load. A three-point flexural test with 20-mm span was used to fracture the specimens at a crosshead speed of 1 mm/min on a computer-controlled Universal Testing Machine (5500R, MTS, Cary, NC). Flexural strength, elastic modulus, and work-of-fracture were measured.[27]

One-way ANOVA was performed to detect significant effects of material compositions. Tukey's multiple comparison test was used at $p = 0.05$ to compare the data.

RESULTS

Fresh CPC paste

Figure 1(A) shows numerous live cells (stained green), while (B) shows few dead cells (stained red) for the TCPS control. Figure 1(C) shows no visible live cells, and Figure 1(D) shows numerous dead cells after culture with freshly-mixed CPC paste for 24 h. Figure 1(E,F) shows the same features for the CPC-chitosan paste.

Set CPC

Figure 2 shows SEM micrographs of cells cultured for 1 day on hardened specimens: (A) osteoblasts "O" attaching to set CPC, (B) higher magnification showing the tip of the cytoplasmic extension "E" anchoring onto CPC, and (C) cytoplasmic extensions attaching to the CPC-chitosan surface. "HA" indicates the nano-sized hydroxyapatite crystals. The cells exhibited a normal, polygonal, and spread morphology consistent with osteoblast-like cells.

Cell encapsulation

Figure 3 shows optical photos of (A) cell-encapsulating alginate beads, (B) a bead at a higher magnification, and (C) cell-alginate beads mixed into a CPC paste. The bead diameter (mean ± SD; $n = 10$) was measured by magnification to be 2.2 ± 0.1 mm.

Live/dead staining of cells inside beads

Fluorescence microscopy in Figure 4 shows cells in alginate beads cultured for 1 day: (A) live cells (stained green) in beads in cell medium without any CPC; (B) dead cells (stained red) in beads in cell medium without CPC; (C) live cells in the beads inside the conventional CPC paste; (D) live cells in the beads inside the CPC-chitosan paste; and (E) live cells in the beads inside a paste of CPC-chitosan-mesh. Live cells were numerous for all the four materials. Dead cells were few for all the four materials. Live cell density is plotted in Figure 4(F). Cells in beads cultured in cell medium without CPC had a significantly higher live cell density (mean ± SD; $n = 5$) of (90.1 ± 1.6)% (Tukey's at 0.05). The live cell density of the CPC-chitosan composite and the CPC-chitosan-mesh composite was (81.5 ± 4.9)% and (81.6 ± 3.6)%, respectively, not significantly different from (81.8 ± 4.6)% of the conventional CPC ($p > 0.1$). All specimens exhibited live cell densities above 80%.

Quantitative cell viability

The cell viability (mean ± SD; $n = 5$) was measured using the Wst-1 assay (Fig. 5). The absorbance at 450 nm (arbitrary units) was 1.36 ± 0.41 for conventional CPC and 1.29 ± 0.24 for CPC-chitosan composite, similar to 1.00 ± 0.14 for the control with the beads in the culture medium without any CPC ($p > 0.1$). The viability for the CPC-chitosan-mesh composite was 0.73 ± 0.22; although lower than the conventional CPC and the CPC-chitosan composite, it was similar to the 1.00 ± 0.14 of the control ($p > 0.1$).

Mechanical properties

Figure 6 plots mechanical properties for conventional CPC, CPC-chitosan composite, CPC-chitosan-mesh composite with one mesh on the tensile side of the specimen, and CPC-chitosan-3mesh composite with three sheets of mesh on the tensile side. All specimens contained cell-alginate beads with a bead volume fraction of 54%. Flexural strengths (mean ± SD; $n = 6$) of conventional CPC containing beads and CPC-chitosan containing beads were 1.3 ± 1.7 MPa

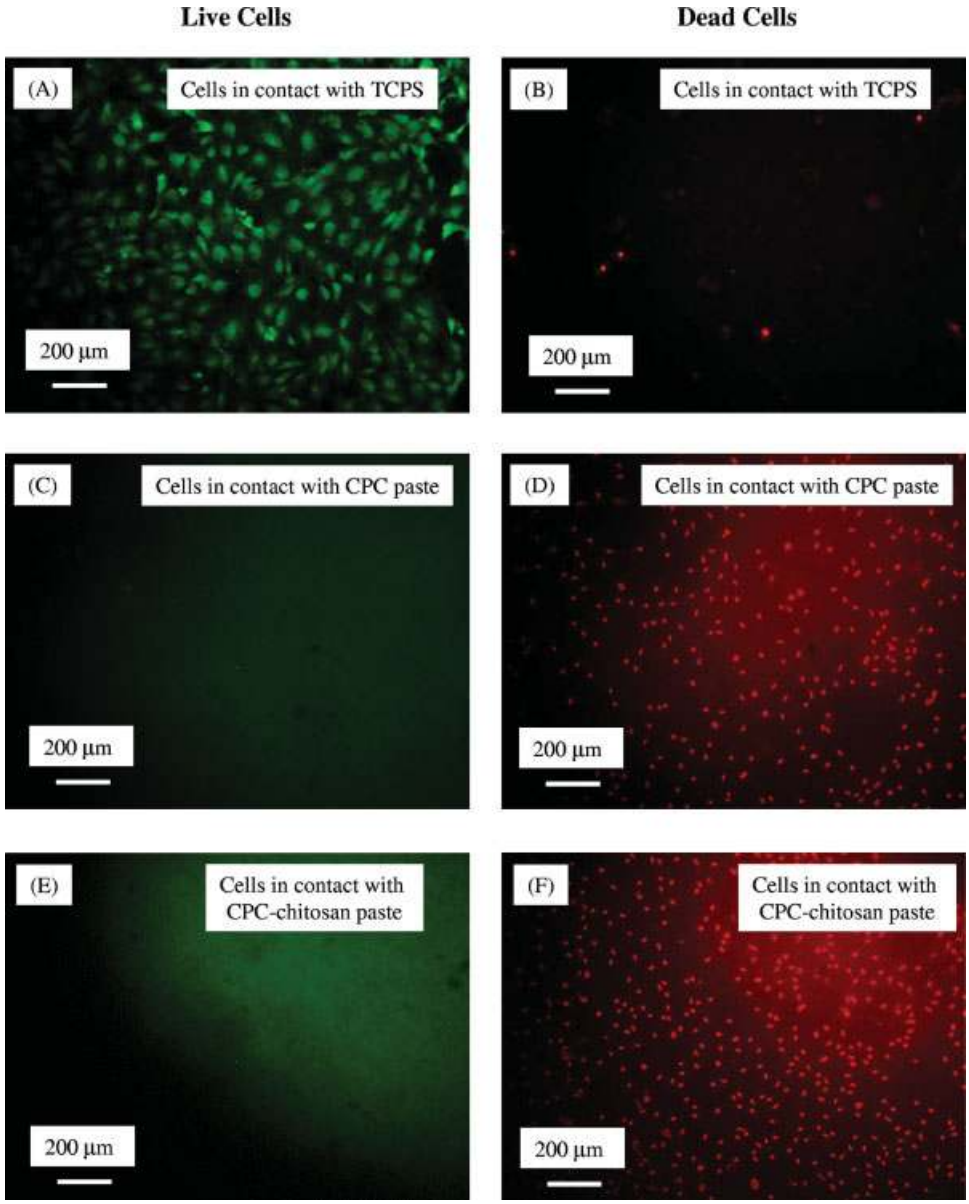

Figure 1. MC3T3-E1 cells were seeded onto tissue culture polystyrene (TCPS), incubated for 24 h and double-stained to be green for live cells and red for dead cells. A and B: Cells treated with cell culture medium. C and D: Cells in contact with freshly-mixed CPC paste (using water as the cement liquid) were compromised. E and F: cells in contact with the CPC-chitosan paste, using the chitosan-water liquid. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com]

and 2.3 ± 0.8 MPa, respectively, not significantly different from each other ($p > 0.1$). The addition of 3 meshes to the CPC-chitosan-bead specimens increased the strength to 9.5 ± 3.6 MPa, a 7-fold increase over conventional CPC and a 4-fold increase over CPC-chitosan. Work-of-fracture was increased even more markedly, from 5.9 ± 6.6 J/m^2 for conventional CPC to 1912 ± 710 J/m^2 for the CPC-chitosan-3mesh composite, a 300-fold increase. The increase in elastic modulus for the CPC-chitosan-3mesh composite over the other materials, while noticeable, was not statistically significant ($p > 0.1$).

DISCUSSION

Cell protection via alginate beads

Freshly-mixed CPC paste caused cell death (Fig. 1). In Figure 1, the CPC paste was placed on the cells. This was because culturing the cells on the top of a fresh CPC paste also showed cell death, but it was very difficult to take images because of the microscopic unevenness and roughness of the cement surface. In addition, the specimen weight did not appear to affect

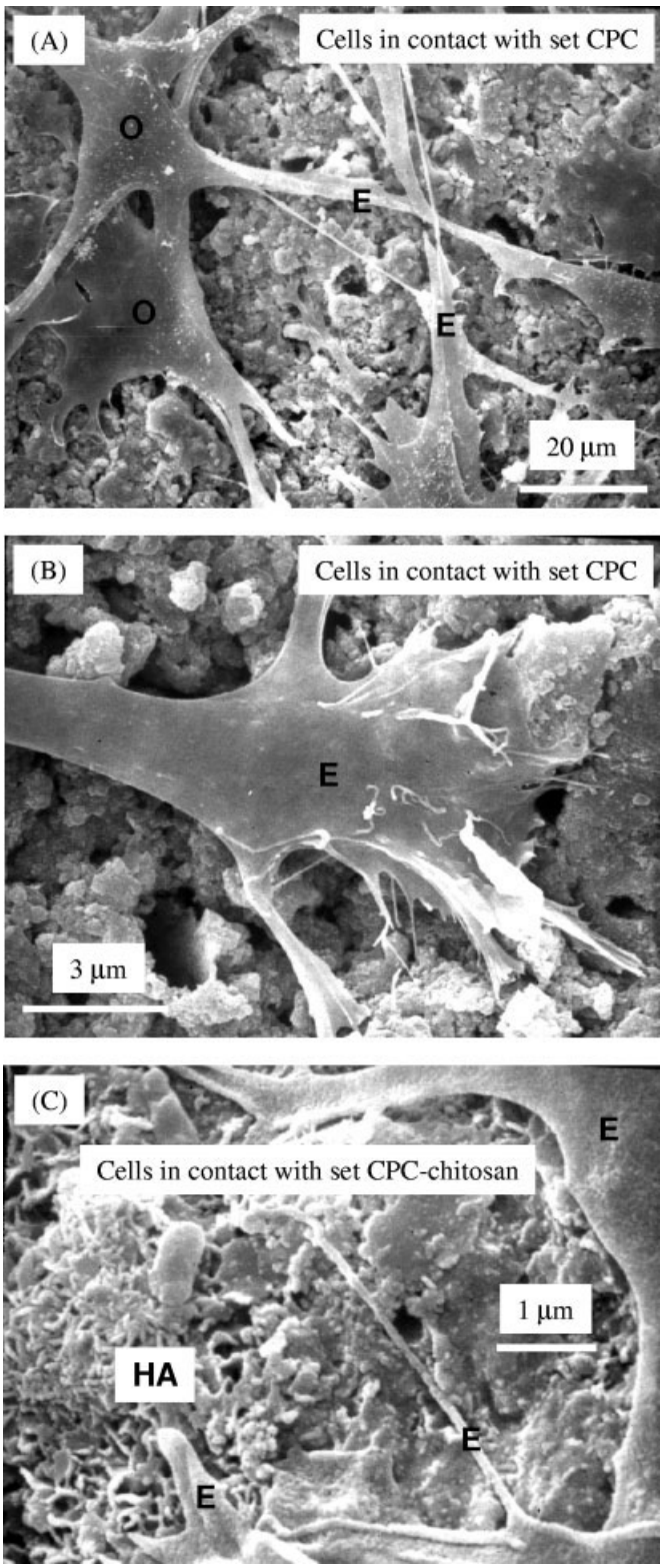

Figure 2. SEM of cells 1 day after being seeded onto a conventional CPC (A and B) and CPC-chitosan composite (C). Both the conventional CPC and CPC-chitosan pastes were allowed to set for 1 day before cell culture. Cells exhibited a healthy polygonal and spread morphology on both CPC and CPC-chitosan once the cements were set.

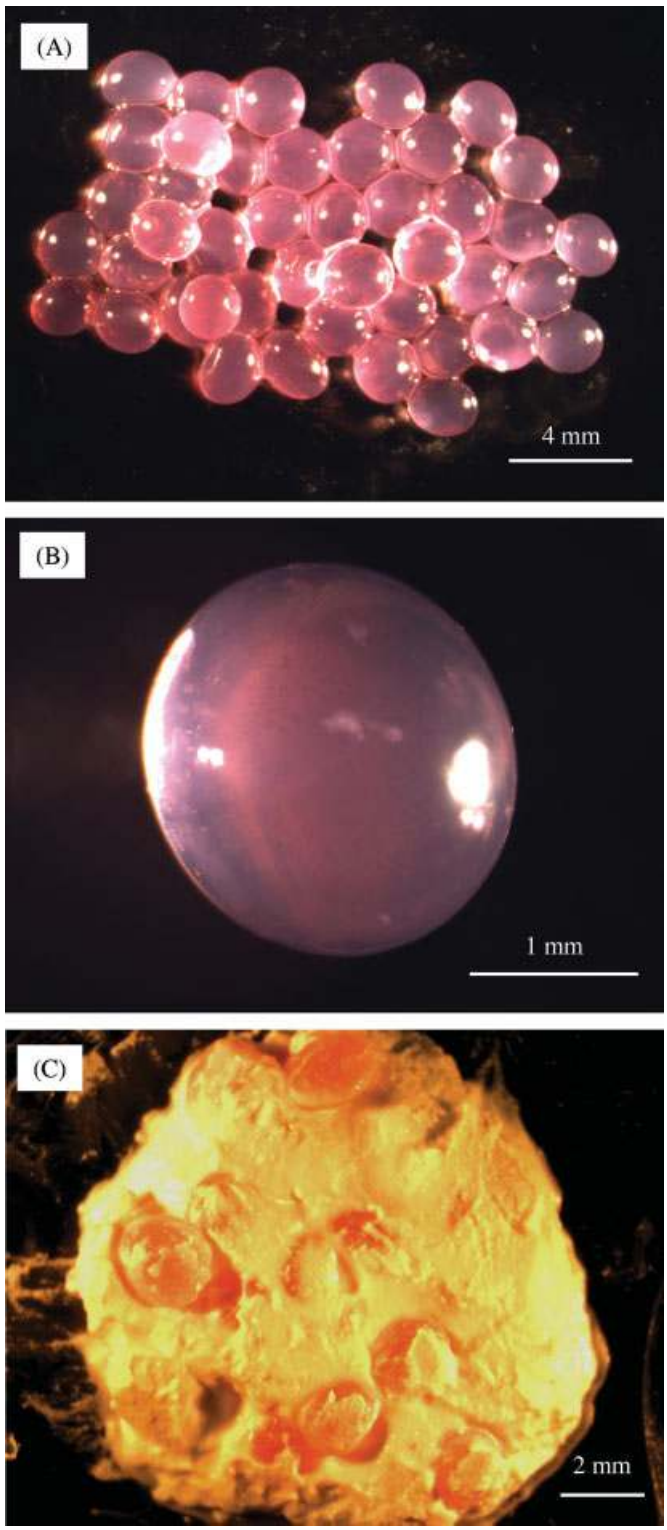

Figure 3. Cells were encapsulated with alginate hydrogel beads by dropping the cell-alginate mixture into an agitated bath of calcium chloride using a syringe. A: Low magnification, and (B) higher magnification. C: Cell-alginate beads were mixed into a CPC paste at a 54% volume fraction of alginate beads. A cell density of 10^5 cells/mL resulted in the number of cells in the beads to be 558 cells/bead. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com]

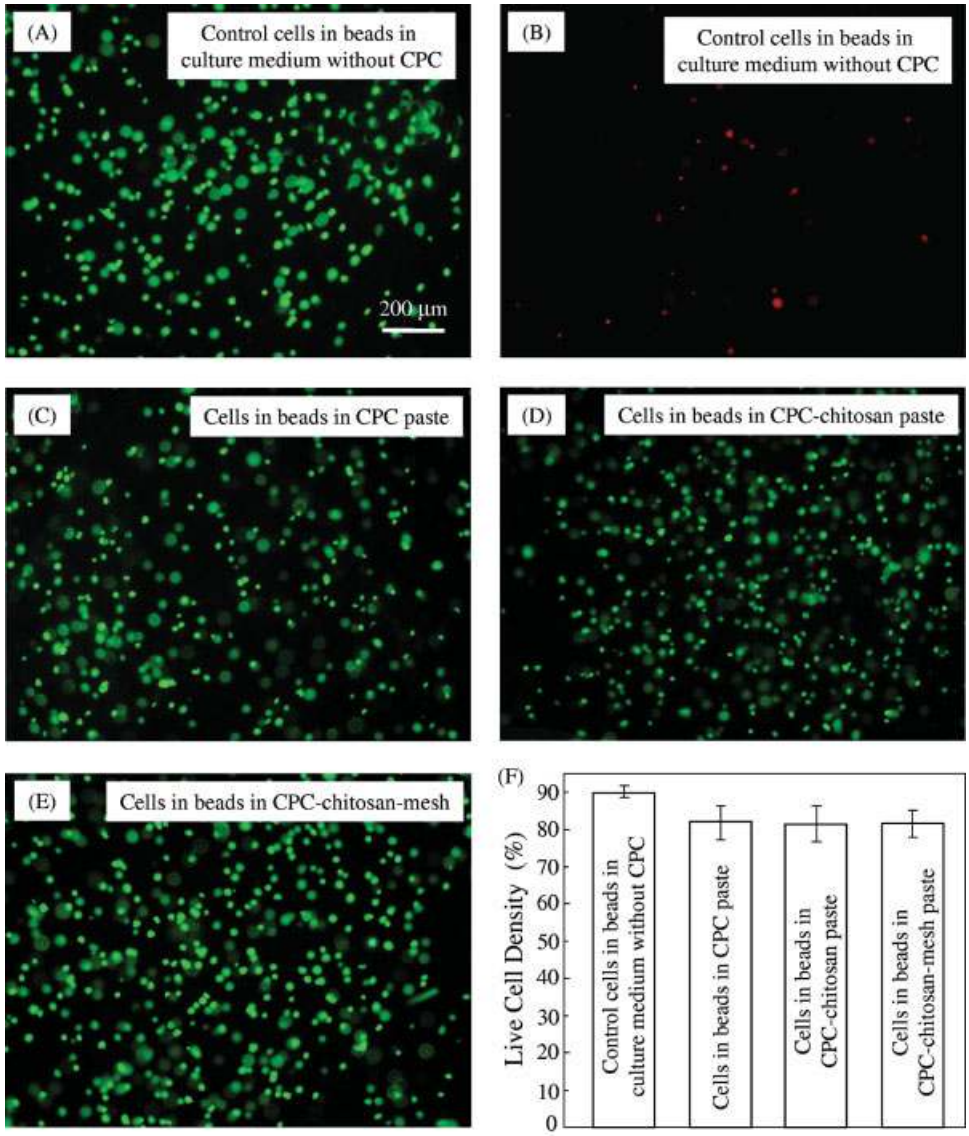

Figure 4. Cells encapsulated in alginate hydrogel beads inside fresh pastes were cultured for 1 day. (A) Live (green) and (B) dead (red) cells in alginate beads in culture medium control without any CPC. C: Live cells in alginate beads in conventional CPC paste without chitosan. D: Live cells in alginate beads in CPC-chitosan paste. E: Live cells in alginate beads in a paste of CPC-chitosan-mesh. F: Live cell density = number of live cells/(number of live cells + number of dead cells). Each value is the mean of five measurements with the error bar showing one standard deviation (mean ± SD; $n = 5$). All photos had the same magnification. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com]

the cell viability in a previous study, in which the cells were cultured both under a set CPC specimen and on the top of the specimen.[21] In both cases, there were numerous live cells and few dead cells[21]; this shows that the weight of the CPC did not cause cell death. During CPC setting, TTCP ($Ca_4(PO_4)_2O$) and DCPA ($CaHPO_4$) dissolved in the liquid as Ca^{2+}, PO_4^{3-}, and OH^- ions, which then reprecipitated to form hydroxyapatite: $2Ca_4(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2$.[11] It is likely that the ionic activities and pH changes during setting (the pH could increase to as high as 10 and last for a few hours)[28] were responsible for the cell death. However, once CPC had set, it was noncytotoxic and supported cell attachment (Fig. 2), consistent with a previous study showing cells attaching to set CPC with few dead cells.[20] Therefore, there was a need to protect the cells during the CPC setting reaction. Alginate beads (Fig. 3) adequately protected cells from the setting of a conventional CPC paste, a CPC-chitosan paste, and a composite paste of CPC-chitosan-mesh (Figs. 4 and 5). Alginate served as a protective material to buffer the cells against acute environmental changes. In addition, the buffered media solution in the beads also contributed in absorbing and normalizing fluctuations in the microenvironments within CPC.

Alginate is the most abundant marine biopolymer in the world and is a popular material for tissue engineering investigation, such as cartilage repair and regenera-

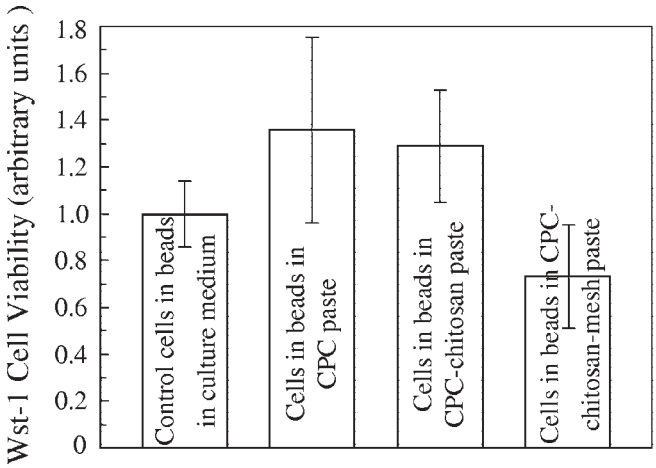

Figure 5. Wst-1 assay for cell-alginate beads in four different environments: cell culture medium without any CPC as control; conventional CPC paste without chitosan; CPC-chitosan paste; and CPC-chitosan-mesh paste. The viability of cells was quantified by measuring the dehydrogenase activity. Each value is mean ± SD; $n = 5$.

tion.[24,25,29,30] The major source of alginate is found in the cell, walls and the intracellular spaces of brown seaweed. In the present study, the alginate from the manufacturer was ionically crosslinked with calcium chloride to form a hydrogel. A hydrogel is a three-dimensional polymer network, crosslinked chemically, physically or ionically, with water as the predominant dispersal medium. The alginate hydrogel beads were used in the present study: (1) as a vehicle to deliver cells and nutrients into CPC-chitosan and CPC-chitosan-mesh composites; (2) to protect the cells from environmental changes during cement setting; and (3) to generate a porous structure in CPC via subsequent degradation of the hydrogel beads. To fulfill these purposes, the hydrogel degradation rate and bead diameter need to be characterized and optimized.

Hydrogel bead degradation rate

After the CPC paste is placed into a bone cavity *in vivo*, the cement setting reaction is largely complete after 1 day. Therefore, it would be desirable for the alginate beads to quickly degrade, thereby releasing the cells from the beads and concomitantly creating a porous CPC scaffold implant. Thus, the live cells could be dispersed throughout the scaffold to begin the process of proliferation, differentiation, and matrix production to form a mineralized bone-like tissue. Significant work has been performed in modulating the degradation rate of alginate by partial periodate oxidation and gamma irradiation, yielding alginate degradation over the course of days or weeks.[29,30] In addition, recent work has suggested that encapsulating cells in gelatin had potential for short-term protection of cells.[31] Furthermore, rapidly

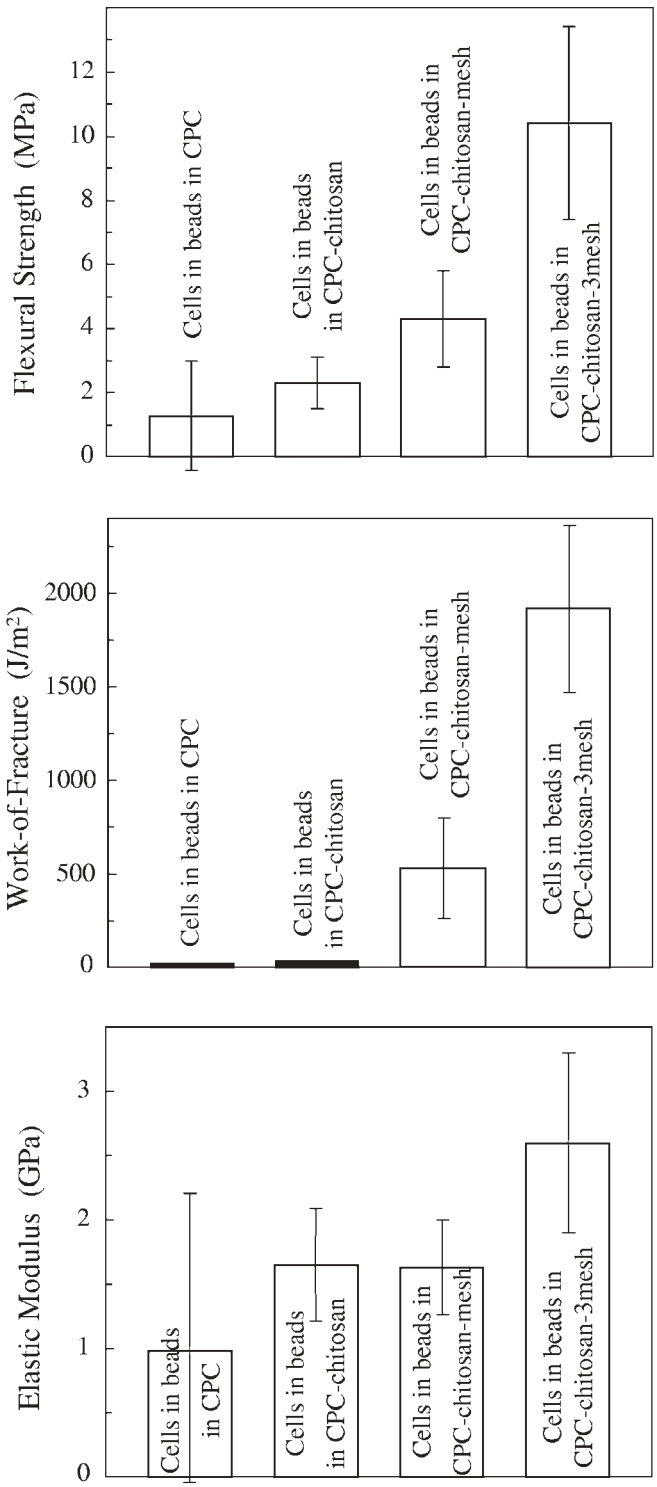

Figure 6. Flexural strength, work-of-fracture, and elastic modulus of four different composites containing the same 54% volume fraction of cell-encapsulating alginate beads. With a cell density of 10^5 cells/mL and 29 cell-alginate beads in each flexural specimen, there were 16,182 cells/specimen. The incorporation of chitosan, one mesh, and three sheets of mesh progressively increased the mechanical properties. Each value is mean ± SD; $n = 5$.

degradable hydrogels can also be formed from copolymers of poly(lactide) and poly(ethylene glycol) (PEG).[32] Studies have shown that photocrosslinkable hydrogels such as poly(ether-anhydride) dimethacrylates can be synthesized, and when PEG was used as the base ether group, complete degradation occurred at 2 days.[33] Therefore, further studies should apply these formulations to the cell seeding into CPC-chitosan pastes with the cells encapsulated in fast-dissolving hydrogels for maximal cell function at the early stages post-operation.

Hydrogel bead diameter

In a previous study, alginate beads were produced by releasing droplets from a pipette into calcium chloride, resulting in a mean bead diameter of 3.6 mm.[21] In the present study, a syringe was fitted with a 25-gauge needle, producing smaller beads of 2.2 mm in diameter. The smaller beads of the present study would be suitable for future injectability studies, for example, by using a 10-gauge needle that has an inner diameter of 2.69 mm. The 3.6-mm beads of the previous study[21] would be too large to be injected. There are several benefits in reducing the bead size: (1) small beads are injectable in minimally-invasive techniques; (2) small beads can result in a more homogeneous dispersion throughout the CPC matrix compared with large beads assuming the same volume fraction; (3) CPC containing small beads should have improved mechanical properties compared with larger beads because larger particles in a matrix tend to create larger flaws with lower strength[34]; and (4) small beads increase the surface area between cell-encapsulating beads and CPC, which could help encourage a more uniform growth of new bone throughout CPC.

Another benefit of incorporating beads is that, after bead dissolution, macropores can be created in CPC. Pore sizes of 100–600 μm were shown to promote cell infiltration and bone ingrowth.[2,3,9,35] Therefore, while the present study focused on cell protection in CPC-chitosan and strength improvement of cell-CPC-mesh constructs, further studies are needed to develop cell-encapsulating beads with diameters <1 mm and preferably around 500 μm.

Mechanical properties

A previous study showed that the conventional CPC at the same powder:water ratio of 3:1 had a flexural strength of ~10 MPa.[18] In the present study, the strength of the conventional CPC was degraded to only 1.3 MPa because of the addition of 54% volume fraction of alginate beads. Such a low strength may result in implant fracture. To overcome this deficiency, chitosan and a resorbable fiber mesh were incorporated into the CPC-alginate system. The flexural strength of CPC-chitosan-3mesh containing 54% volume fraction of cell-encapsulating hydrogel beads was increased to 9.5 MPa. This value overlapped the reported flexural strength of 2–11 MPa for sintered porous hydroxyapatite implants and a tensile strength of about 3.5 MPa for cancellous bone.[36,37]

Compared with sintered porous hydroxyapatite, the material of the present study could (1) be seeded with live cells before CPC hardening, thus ensuring cell dispersion throughout the implant, and (2) set *in situ* with intimate contacts to neighboring bone. The cells could be obtained from the patient and proliferated *in vitro*. Recent studies showed that human bone marrow contained pluripotential mesenchymal stem cells that could differentiate to form bone tissue.[38] These cells could be purified, expanded, and used to repair bone defects.[38] Potential applications of the cell-CPC-chitosan-mesh construct include craniofacial repairs such as the reconstruction of defects in parietal skull or in other shell structures. One mesh could be placed on the prospective tensile side of the cell-seeded CPC paste. One mesh improved the implant strength to 4.3 MPa and the work-of-fracture by two orders of magnitude (Fig. 6). Alternatively, two meshes could be used with one mesh on each side of the cell-seeded CPC-chitosan paste. For filling bulk cavities in bone, the cell-seeded CPC-chitosan paste could be placed to fill the majority of the cavity, and then three sheets of mesh impregnated with the CPC-chitosan paste could be placed to cover the cavity and provide strength and protection to the cell-seeded cavity. The seeding of cells inside the CPC paste before implant hardening could disperse the cells throughout the implant, while the fiber meshes provide the needed strength. Once new bone had formed thus increasing the implant strength,[36] the absorbable meshes could then be dissolved to create highly-interconnected macropores for further bone ingrowth.[19,20]

SUMMARY

In situ hardening cell-CPC-chitosan-mesh constructs were developed that showed composite strength matching the strengths of sintered porous hydroxyapatite and cancellous bone. While freshly-mixed CPC paste evoked a cytotoxic response, alginate hydrogel beads adequately protected the cells from the cement setting reaction. Osteoblast cells encapsulated in alginate beads inside a conventional CPC paste, a CPC-chitosan paste and a CPC-chitosan-mesh paste had cell viability similar to that of cells in beads in culture medium without CPC. The incorporation of cell-encapsulating beads into CPC severely degraded the mechanical strength. However, the use of chitosan, one mesh, and three sheets of mesh progressively increased the mechanical properties. Po-

tential applications include reconstruction of defects in parietal skull or in other shell structures, as well as in filling bulk cavities in bones using the cell-CPC-chitosan paste, with the meshes covering the paste and providing the needed strength. While the new biomaterial system of the present study shows promise for bone tissue engineering, further studies are needed to reduce the alginate bead size and tailor the hydrogel degradation rate.

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