Cell seeding into calcium phosphate cement

Carl G. Simon, Jr.,[1] William F. Guthrie,[2] Francis W. Wang[1]
[1]*Polymers Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8545*
[2]*Statistical Engineering Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899*

Received 31 January 2002; revised 26 June 2003; accepted 29 July 2003

Abstract: To improve the effectiveness of calcium phosphate cement (CPC), we have developed a method to seed osteoblasts into the cement. CPC powder is mixed with water to form a paste that can be shaped to fit a bone defect *in situ*. The paste hardens in 30 min, reacts to form hydroxyapatite, and is replaced with new bone. Reacted CPC is biocompatible but unreacted CPC paste was found to have toxic effects when placed on cell monolayers (MC3T3-E1 cells). In contrast, when cells were indirectly exposed to CPC paste using a porous membrane or by placing a coverslip containing adherent cells onto a bed of CPC paste, the unreacted CPC was nontoxic. These results suggested that gel encapsulation of the cells might protect them from the CPC paste. Thus, cells were encapsulated in alginate beads (3.6-mm diameter), mixed with CPC paste, and incubated overnight. Both vital staining (calcein-AM and ethidium homodimer-1) and the Wst-1 assay (measures dehydrogenase activity) showed that cell survival in alginate beads that were mixed with CPC was similar to survival in untreated control beads. These results suggest that gel encapsulation could be used as a mechanism to protect cells for seeding into CPC. © 2004 Wiley Periodicals, Inc.* J Biomed Mater Res 68A: 628–639, 2004

Key words: alginate; bone graft; calcium phosphate cement; cell seeding; tissue engineering

INTRODUCTION

It is estimated that approximately 1,000,000 bone grafts are performed each year to treat osseous defects resulting from trauma and disease in the United States.[1] Autografts are frequently used to treat these defects but available bone can be inadequate and difficult to shape. Allografts and xenografts must be processed to eliminate the risk of disease transmission which requires the removal of osteogenic cells and reduces the osteoinductivity of the grafts.[1] Thus, the development of a safe and effective alloplast for use as a bone graft would provide clinicians with an invaluable tool for treating patients.

A material that suits this purpose has been designed by our colleagues at the American Dental Association Health Foundation at the National Institute of Standards and Technology (NIST). They have developed a moldable, self-setting calcium phosphate cement (CPC) that is used clinically as a bone graft to treat non-load-bearing dental and craniofacial defects.[2] The cement powder, consisting of tetracalcium phosphate and anhydrous dicalcium phosphate, is mixed with water to form a workable paste that can be shaped during surgery to fit the contours of a bone defect. The cement, which reacts to form microcrystalline hydroxyapatite (HA), hardens within 30 min, is biocompatible, is resorbed by the body, and is replaced with new bone over several years.[2–5]

To improve the effectiveness of CPC, we have developed a method for seeding osteoblasts into the cement. Recent research has shown that human bone marrow contains pluripotential mesenchymal stem cells (MSCs) that can differentiate into bone *in vitro* and *in vivo*.[6,7] These cells can be purified and expanded *ex vivo* and retain their osteogenic potential. In addition, animal models have shown that isolated MSCs can be expanded and used to repair bone defects.[7–9] These advances suggest that it may soon be feasible to treat a patient's bone injuries using MSCs

Certain commercial materials and equipment are identified in this article to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or that the material or equipment identified is necessarily the best available for the purpose.

Correspondence to: F. W. Wang; e-mail: francis.wang@nist.gov

Contract grant sponsor: National Institutes of Health; contract grant number: YI-DE-7006-01

Contract grant sponsor: National Institute of Standards and Technology, Advanced Technology Program

© 2004 Wiley Periodicals, Inc. *This article is a US Government work and, as such, is in the public domain in the United States of America.

that have been isolated from their own marrow. Longer range, it may also be desirable to treat patients with embryonic stem cells because these cells also possess the potential to form bone and are being evaluated for cell therapies.[10,11] Toward this aim, we have developed a method for seeding cells into CPC using the MC3T3-E1 murine osteoblast-like cell line as a model. Because our initial studies suggested that unreacted CPC was toxic when mixed with cells, we have evaluated gel encapsulation as a protective measure for seeding cells into the cement.

MATERIALS AND METHODS

CPC

Tetracalcium phosphate [$Ca_4(PO_4)_2O$] was prepared according to Chow et al.[12] and anhydrous dicalcium phosphate ($CaHPO_4$) was obtained from J. T. Baker Co. (Phillipsburg, NJ). CPC powder was prepared by mixing equimolar amounts of ground $Ca_4(PO_4)_2O$ (72.9% mass fraction) and $CaHPO_4$ (27.1% mass fraction).[2,13] CPC paste was made by mixing four parts by mass of CPC powder (UV-sterilized) with one part of sterile-filtered water (0.25 g of CPC plus 0.063 mL of water) for 30 s with a spatula. When CPC discs were required, the CPC paste was placed in a steel mold to form a disc approximately 4.5 mm in thickness and 6.4 mm in diameter. A pressure of 0.7 MPa was applied to the mold using a device for applying a constant load.[13] The discs were incubated at 37°C and 100% relative humidity overnight before removal from the molds. CPC discs were sterilized by soaking them in 70% ethanol (mass fraction) for 15 min followed by an overnight wash in cell culture medium.

Cell culture

MC3T3-E1 cells were used because they are a well-characterized osteoblast-like cell line[14] which can serve as a model for endogenous osteoblasts. Established protocols for the culture and passage of MC3T3-E1 cells (murine osteoblast-like cells) were followed.[15] Cells were obtained from Riken Cell Bank (Hirosaka, Japan) and cultured in flasks (75 cm^2 surface area) at 37°C in a fully humidified atmosphere at 5% CO_2 (volume fraction) in α-modification of Eagle's minimum essential medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% (volume fraction) fetal bovine serum (Gibco, Rockville, MD) and kanamycin sulfate (Sigma, Inc., St. Louis, MO). Medium was changed twice weekly and cultures were passaged with 2.5 g/L trypsin (0.25% mass fraction) containing 1 mmol/L ethylenediaminetetraacetic acid (Gibco) once per week. Cultures of 90% confluent MC3T3-E1 cells were used for all experiments.

Tissue culture polystyrene (TCPS) experiments

Cells were trypsinized and plated (50,000/well) into 24-well plates (BD Labware, Franklin Lakes, NJ) of TCPS in 2 mL of medium. Cells were also plated onto CPC discs (prepared as described above) by adding 50,000 cells to wells containing a disc and medium. After an overnight incubation, cells were washed with media and "Live-Dead" stained in medium containing 2 μmol/L calcein-AM and 2 μmol/L ethidium homodimer-1 (both from Molecular Probes, Eugene, OR). After adding the stain to the cells, four treatments were started which lasted from 2 to 4 h:

1. three wells served as controls and were stained for 4 h;
2. CPC paste (0.25 g of CPC plus 0.063 mL of water) was gently added on top of the cells in three wells for 2 h;
3. cells that were plated for 24 h on top of three CPC discs were stained for 4 h; and
4. a CPC disc was gently placed on cells in three wells for 4 h.

After the treatments, the cells were observed by epifluorescence microscopy and photographed (Fig. 1).

Cell culture insert experiments

Cells were trypsinized and plated into a six-well plate (BD Labware) at 100,000 cells per well in 2 mL of medium. Cell culture inserts (0.4-μm pore size, BD Labware) were placed in each well and filled with 2 mL of medium. After an overnight incubation, CPC paste (0.5 g of CPC plus 0.125 mL of water) was added into the insert of three of the wells [see Fig. 2(A) for a diagram] and the remaining three wells were used as controls. After another 24-h incubation, the cells were Live-Dead stained, observed by epifluorescence microscopy, and photographed (Fig. 3). The inverse of this experiment was also performed where cells (75,000) were plated into the cell culture inserts (instead of onto the bottom of the six-well plate) and the CPC paste was placed in the bottom of the six-well plate [see Fig. 2(B) for a diagram].

Coverslip experiments

Cells (50,000/well) were plated into a 24-well plate (BD Labware) having round glass coverslips (12-mm diameter) and 2 mL of medium in each well. After an overnight incubation, three coverslips were placed in three separate wells of a six-well plate containing 4 mL of serum-free medium and Live-Dead stain (untreated controls). Three other coverslips were placed into separate wells of a six-well plate that had CPC paste (0.5 g of CPC plus 0.125 mL of water) and 4 mL of serum-free medium containing Live-Dead stain. The coverslips were gently placed on top of the CPC paste but were submerged under medium [see Fig. 2(C) for a diagram]. The cells on the coverslips were incubated for 4 h and then observed by epifluorescence microscopy and photographed (Fig. 3).

Determination of percent live cells

The Live-Dead images taken for the TCPS, cell culture insert, and coverslip experiments were analyzed to deter-

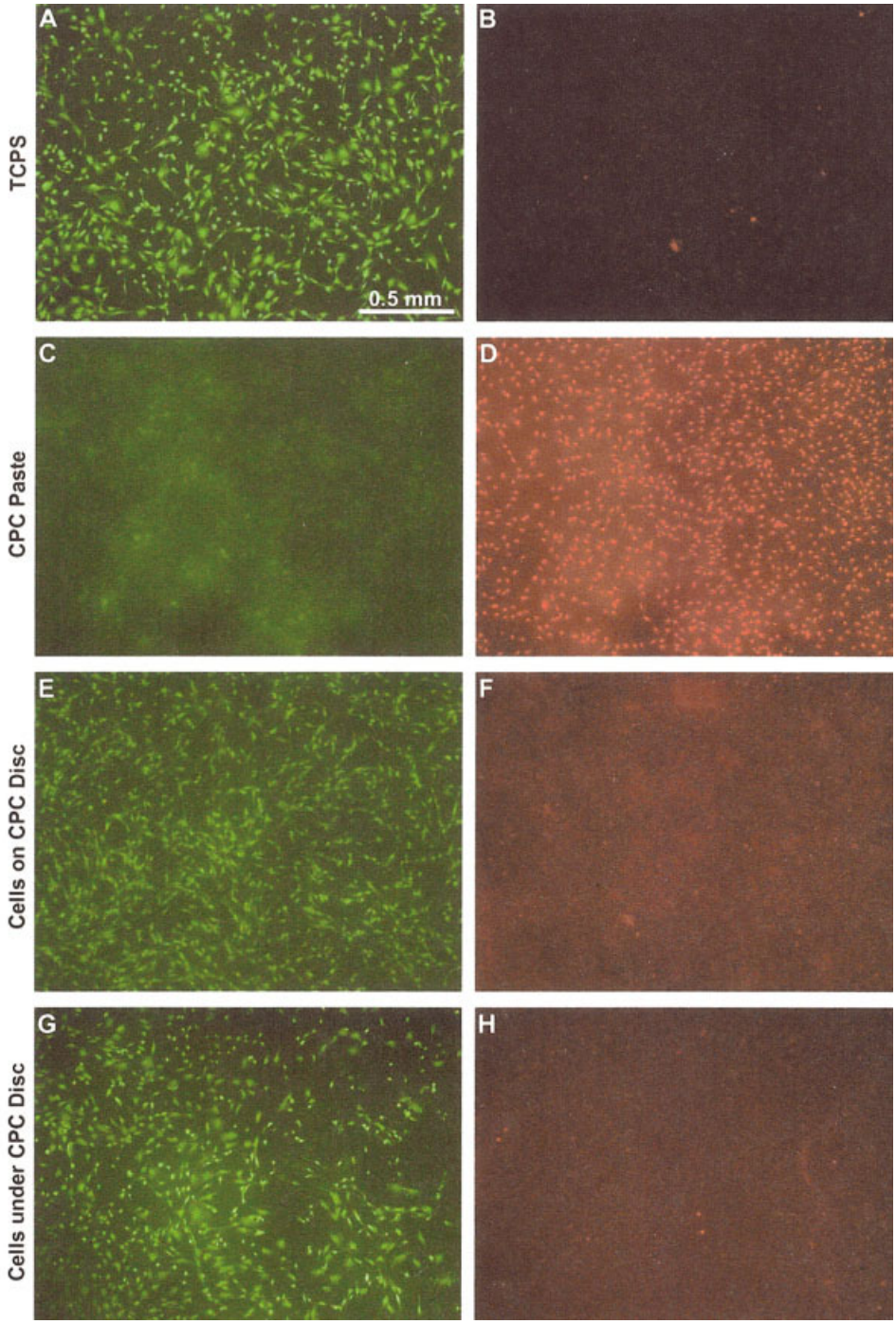

Figure 1. TCPS experiments, Live-Dead stain. Panels A and B (TCPS): cells plated on TCPS for 24 h. Panels C and D (CPC Paste): CPC paste placed on cells for 2 h. Panels E and F (Cells on CPC Disc): cells on a CPC disc for 24 h. Panels G and H (Cells under CPC Disc): a CPC disc placed on cells for 4 h. Pairs of green (left) and red (right) panels are the same field viewed with a green (live) or red (dead) filter. The size bar in Panel A applies to all panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mine the percent live cells present after each treatment (Fig. 4). Two hundred cells were counted for each well of each treatment and the percent live cells was determined by dividing the number of live cells (green) by the sum of the number of live cells (green) and dead cells (red).

Alginate encapsulation

Alginate was used as an encapsulating gel because it is biocompatible and easy to work with.[16,17] A 1.2% sodium alginate solution (mass fraction) was prepared by dissolving

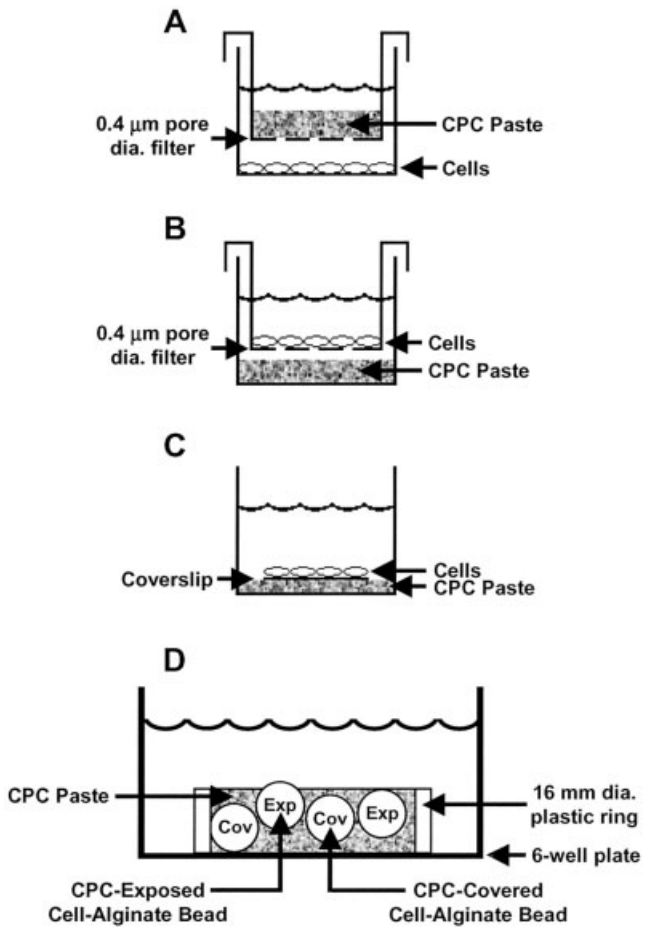

Figure 2. Diagram of cell culture insert, coverslip, and CPC-cell-alginate experiments.

1.2 g of Pronova UP LVG alginate (64% guluronic acid; MW = 75,000–220,000 g/mol; Pronova Biomedical, Oslo, Norway) in 100 mL of saline (155 mmol/L NaCl). Cultures of MC3T3-E1 cells (90% confluent) were trypsinized, washed, and resuspended in sterile-filtered alginate. The cell concentration varied from 10^5 to 3×10^6 cells/mL alginate depending on the experiment. Aliquots (0.5 mL) of the cell-alginate suspension were released in droplets from a 1-mL pipette into sterile-filtered $CaCl_2$ solution (100 mmol/L) in a six-well plate and allowed to gel for 5 min. After 5 min, the cell-alginate beads (3.6 mm in diameter) were removed, rinsed with medium, and used in experiments.

Seeding cell-alginate beads into CPC (Live-Dead stain)

A sterile plastic ring (16-mm diameter; 5-mm height) was placed in each well of a six-well plate and 12 cell-alginate beads (10^5 cells/mL alginate) were placed into each of the plastic rings. Three wells were used as controls while CPC paste (1 g of CPC plus 0.25 mL of water) was placed on top of and pressed around the beads in three of the wells to make a construct approximately 6-mm high [see Fig. 2(D) for a diagram]. The cell-alginate beads occupied approximately 40% of the volume of CPC-cell-alginate-bead mixture. Medium (8 mL) was added to the wells. After an overnight incubation, the beads were removed and Live-Dead stained (Figs. 5 and 6).

Preliminary experiments revealed a difference in cell survival between beads that were completely covered by CPC versus beads that were partially uncovered and had a portion of their surface directly exposed to the medium. Thus, as the cell-alginate beads were removed from the CPC, they were classified as either "CPC-Exposed" or "CPC-Covered" and analyzed separately. Exposed was defined as a bead with any portion of its surface visible by eye (not completely covered by CPC) at the time of removal [see Fig. 2(D) for a diagram]. The depth of coverage with CPC for CPC-Covered beads was $\leq$2 mm. A 1-mm-thick section was cut from the middle of each bead with a razor blade. The sections were Live-Dead stained and photographed using epifluorescence microscopy (Fig. 5). The micrographs were used to determine the percentage of live cells present in Control, CPC-Exposed, and CPC-Covered beads as described above. Three or more beads were analyzed for each well of each treatment. Because this experiment was performed three times, at least 27 beads were analyzed for each of the three treatments (Control, CPC-Exposed, and CPC-Covered).

Seeding cell-alginate beads into silicone grease

To determine whether cells in alginate can survive for 24 h without access to media, one cell-alginate bead (10^5 cells/mL alginate) was placed in the bottom of each of four sterile 1.5-mL microfuge tubes. The beads in the tubes were completely covered with 1 mL of autoclaved silicone grease such that they had no access to air. The tubes were left open and placed in a cell incubator for 24 h without any media present. The next day, the beads were sectioned, Live-Dead stained, and photographed using epifluorescence microscopy [Fig. 5(G,H)]. The micrographs were used to determine the percentage of live cells present in the beads as described above. Microfuge tubes were used for these experiments because it was much easier to completely cover a cell-alginate bead with silicone grease when it was in the bottom of a tube than when it was in a 16-mm plastic ring in the bottom of a six-well plate.

Seeding cell-alginate beads into CPC (Wst-1 assay)

Cell-alginate beads (3×10^6 cells/mL alginate) were incubated overnight in 16-mm rings in six-well plates as described above (four control wells and four CPC wells). The next day, the beads were analyzed for cell viability with the Wst-1 assay (Fig 7). The Wst-1 assay is a colorimetric assay of cellular dehydrogenase activity in which absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cells.[18] Six cell-alginate beads from each of the four control wells, six CPC-Exposed beads from each of the four CPC wells and six CPC-Covered beads from each of the four CPC wells were placed in separate wells of a 24-well plate and washed with 1 mL of Tyrode's HEPES buffer (140

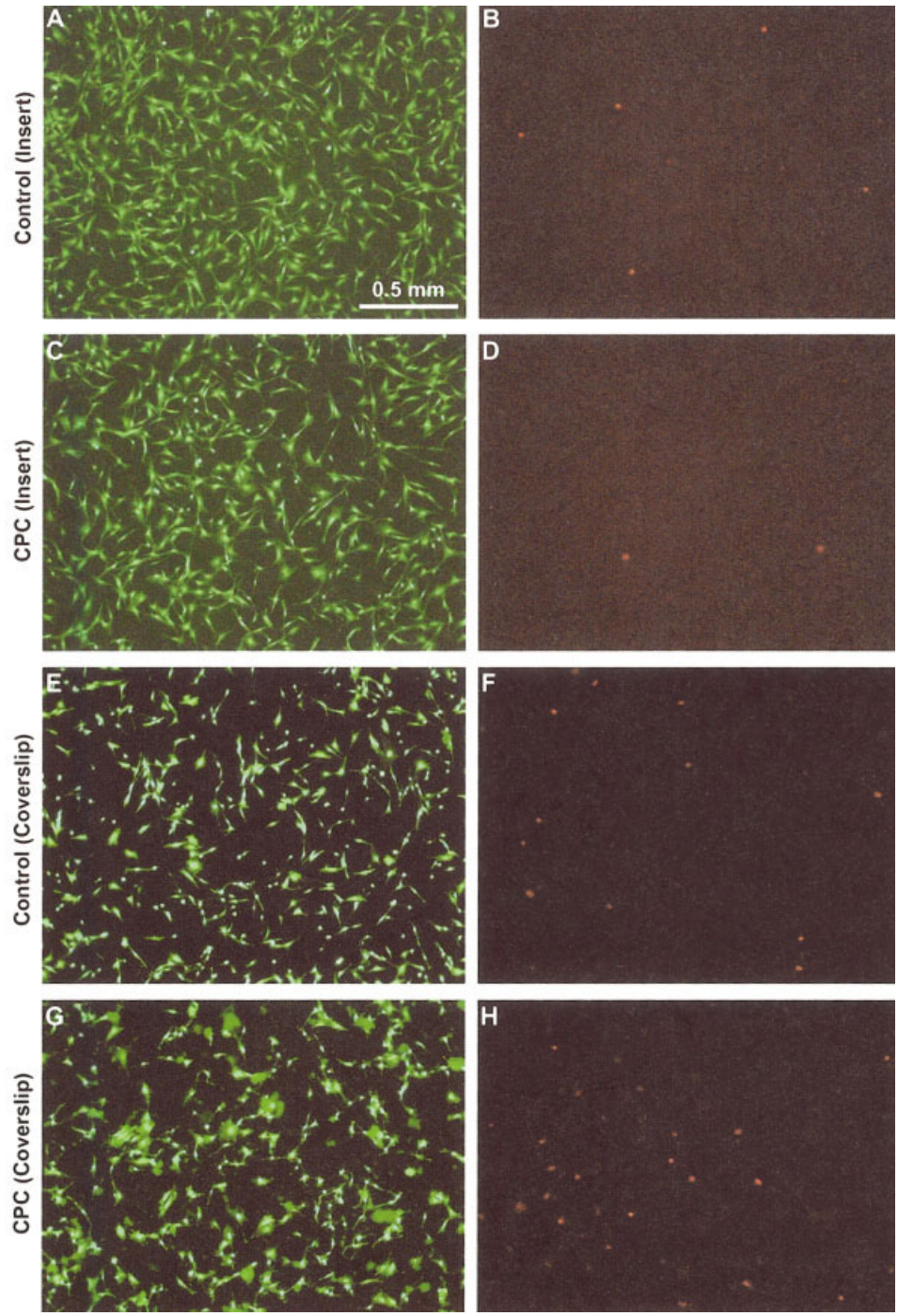

Figure 3. Cell culture insert and coverslip experiments, Live-Dead stain. Panels A and B [Control (Insert)]: an empty insert placed over cells on TCPS for 24 h. Panels C and D [CPC (Insert)]: an insert containing CPC paste placed over cells on TCPS for 24 h. Panels E and F [Control (Coverslip)]: a control coverslip covered by cells. Panels G and H [CPC (Coverslip)]: a coverslip covered by cells placed on CPC paste for 4 h. Pairs of green (left) and red (right) panels are the same field viewed with a green (live) or red (dead) filter. The size bar in Panel A applies to all panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mmol/L NaCl, 0.34 mmol/L Na_2HPO_4, 2.9 mmol/L KCl, 10 mmol/L HEPES, 12 mmol/L $NaHCO_3$, 5_mmol/L glucose, pH 7.4). One milliliter of Tyrode's HEPES buffer and 0.1 mL 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 a 2-h incubation at 37°C, 0.5 mL of each reaction mixture was clarified by centrifugation at 10,000 rcf for 30 s. A 0.2-mL aliquot from the supernatant of each tube was put in a 96-well plate and absorbance was measured with a platereader (Wallac 1420 Victor^2; PerkinElmer Life Sciences, Gaithersburg, MD). Experiments were repeated with alginate beads that did not contain any cells. These no-cell-

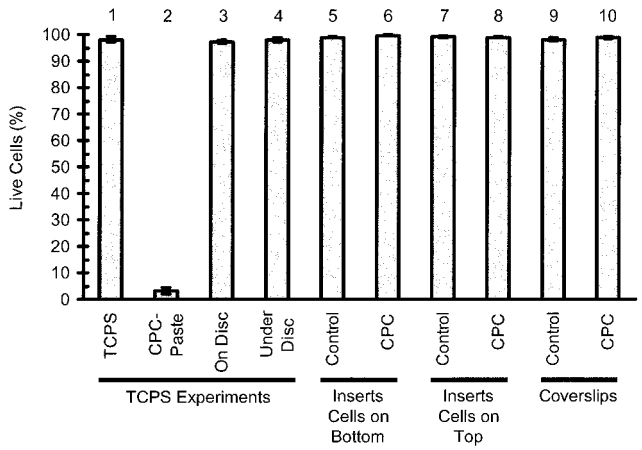

Figure 4. Percent live cells in the TCPS, cell culture insert, and coverslip experiments. The number of live and dead cells was counted in micrographs. (1) TCPS: cells plated on TCPS. (2) CPC Paste: CPC paste on top of cells. (3) On Disc: cells on a CPC disc. (4) Under Disc: a CPC disc placed on cells. (5) Cells on Bottom, Control: an empty cell culture insert in a well with cells on TCPS. (6) Cells on Bottom, CPC: a cell culture insert containing CPC paste in a well with cells on TCPS. (7) Cells on Top, Control: cells in an insert over an empty well. (8) Cells on Top, CPC: cells in an insert in a well with CPC paste. (9) Coverslip, Control: a control coverslip covered by cells. (10) Coverslip, CPC: a coverslip covered by cells placed on CPC paste for 4 h. Bonferroni t tests to compare the four TCPS treatments (bars 1–4) showed that CPC-Paste was different from the other three treatments ($p < 0.0083$). t Tests to compare the CPC to Control for "Inserts Cells on Bottom" (bars 5 and 6), "Inserts Cells on Top" (bars 7 and 8), and "Coverslips" (bars 9 and 10) showed that the CPC and Control treatments were the same for each of these experiments ($p > 0.05$).

alginate beads were used as a blank in the Wst-1 assay and their absorbance values were subtracted from the data. The absorbance value for Control was set at 100% and CPC-Exposed and CPC-Covered were normalized to Control. Wst-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) were obtained from Dojindo (Gaithersburg, MD).

Glass slide experiment

One milliliter of a cell-alginate solution (100,000 cells/mL) was gelled in 0.25 mL of $CaCl_2$ solution for 5 min. The cell-alginate gel was washed and placed in a trough formed by gluing together three glass slides (UV-sterilized) as depicted in Figure 8(A). CPC paste (1 g of CPC plus 0.25 mL of water) was placed in the trough against the cell-alginate gel such that it made direct contact with the gel across the length of the trough. A fourth slide was placed on top of the gel–CPC construct to prevent medium access to the gel–CPC interface (note that both ends of the trough were still open). The construct was placed in a large Petri dish and covered with medium (50 mL). After an overnight incubation, the top slide was removed and the construct was Live-Dead stained for epifluorescence microscopy and photography (Fig. 8).

Powder X-ray diffraction

Powder X-ray diffraction was used to confirm that CPC converts to HA in our alginate experiments. Reacted CPC (CPC paste mixed with cell-alginate beads and incubated overnight) was ground to a fine powder and characterized by powder X-ray diffraction. X-ray diffraction patterns were taken with monochromatized copper $K_{\alpha 1}$ radiation ($\lambda = 1.54$ nm) generated at 40 kV and 25 mA on a Rigaku powder diffractometer in the range 10–70° 2θ. Spectra were taken with a continuous scan at 1°/min and percent conversion to HA was estimated to be between 88 and 100% based on comparison of tetracalcium phosphate (29.2 and 29.8°) and HA (25.9°) peak intensities to previously published data.[19]

Statistics

When a "standard deviation" is shown in the text as an error bar, it refers to the "standard deviation of the mean," which is the same as the "combined standard uncertainty of the mean" for the purposes of this work. The data from the Live-Dead and Wst-1 assays were analyzed by computing simultaneous Student t tests using the Bonferroni inequality to guarantee a global confidence level of 95%.[20] When using the Bonferroni procedure, each of the individual t tests was performed at the 0.05/k significance level where "k" equals the number of comparisons being made. All experiments were performed three times and averages are presented in the figures.

RESULTS

Our first attempts at developing a method for seeding cells into CPC involved using a cell suspension as the liquid portion of the cement, mixing the cement, Live-Dead staining the cement, and then looking for the presence of cells by epifluorescence microscopy. Cells were also seeded onto freshly mixed CPC paste, incubated overnight, and stained. Nearly all of the cells observed with these approaches were dead with only an occasional live cell ever visible. These experiments were also very difficult to image so they were simplified to that shown in Figure 1 where freshly mixed CPC paste was placed on monolayers of cells adherent to TCPS [Fig. 1(C,D)].

In this experiment, control monolayers (TCPS) that were not exposed to CPC [Fig. 1(A,B)] had mostly live cells (green; panel A) and few dead cells (red; panel B). Cells plated onto [Fig. 1(E,F)][21] or under [Fig. 1(G,H)] set CPC discs were also nearly all live with few dead. In contrast, the monolayers that had CPC paste placed

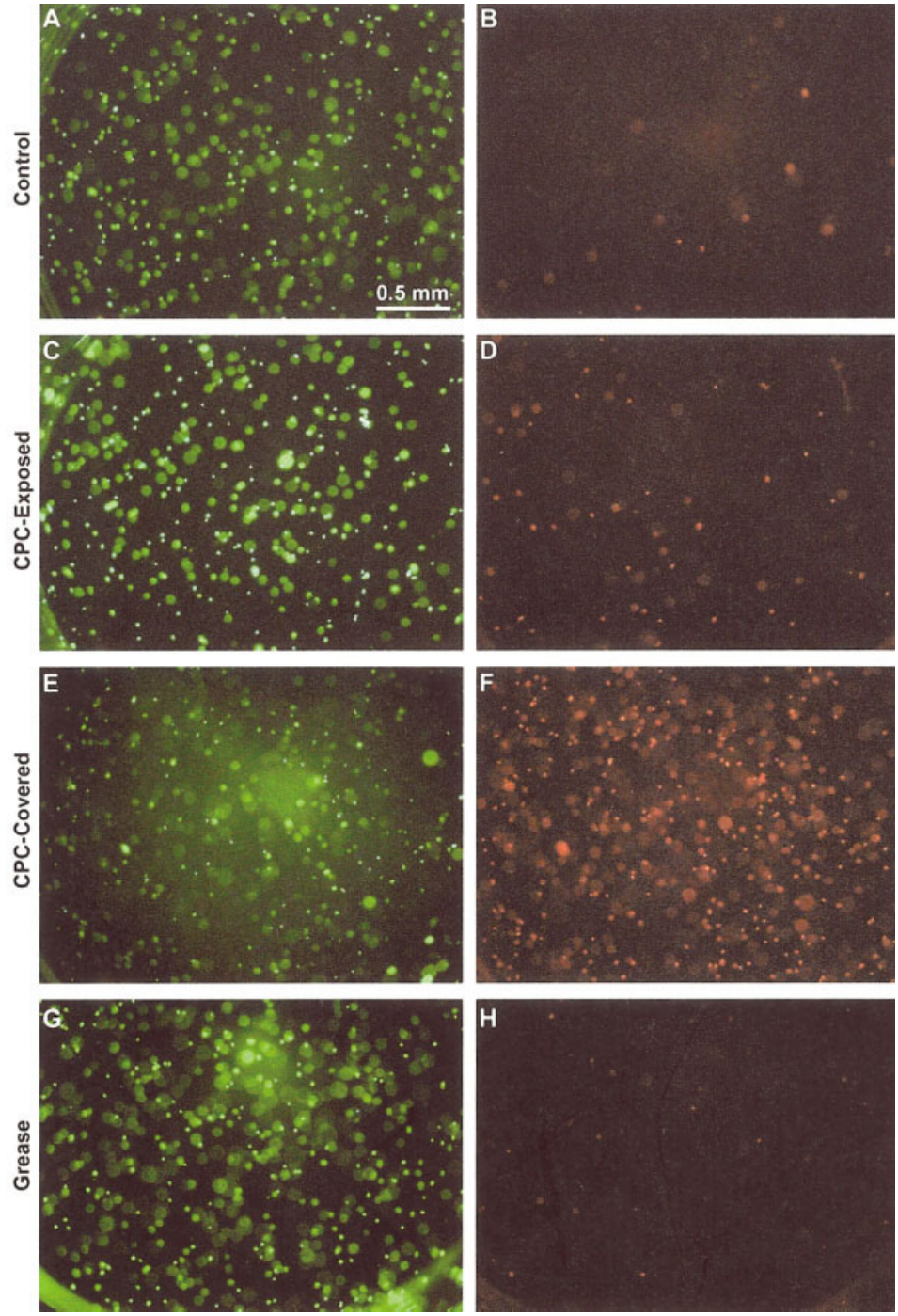

Figure 5. Micrographs of cell-alginate beads after seeding into CPC. Panels A and B (Control): a Control cell-alginate bead. Panels C and D (CPC-Exposed): a cell-alginate bead mixed into CPC paste having part of its surface exposed to media. Panels E and F (CPC-Covered): a cell-alginate bead mixed into CPC paste with none of its surface exposed to media (it was completely covered with CPC). Panels G and H (Grease): a cell-alginate bead that was coated in silicone grease. All panels are from overnight incubations and were Live-Dead stained. Pairs of green (left) and red (right) panels are the same field viewed with a green (live) or red (dead) filter. The size bar in Panel A applies to all panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

on them [Fig. 1(C,D)] contained nearly all dead cells (panel D) and very few live cells (panel C). The live and dead cells in micrographs from these experiments were counted to determine percent live cells for each of the treatments and these data are shown in the first four bars of Figure 4. The "TCPS," "CPC-Paste," "Cells on CPC Disc," and "Cells under CPC Disc" treatments had 98, 3, 97, and 98% live cells, respectively. These results suggest that unreacted CPC has a toxic effect on cells and that reacted CPC is nontoxic.

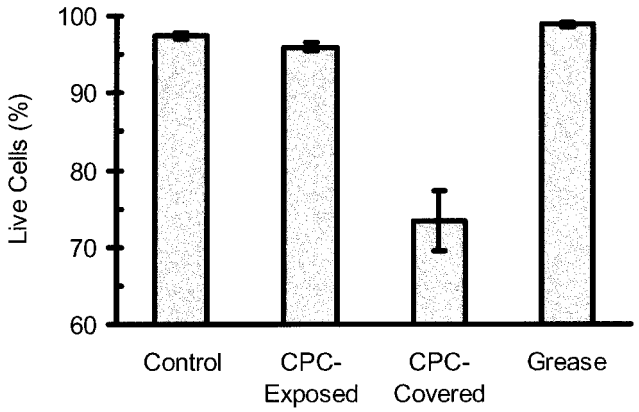

Figure 6. Percent live cells in cell-alginate beads after seeding into CPC or silicone grease. The number of live and dead cells was counted in micrographs from Control, CPC-Exposed, CPC-Covered, and Grease beads. Bonferroni t tests showed that CPC-Covered was different from the other three treatments and that CPC-Exposed was different than Grease ($p < 0.0083$).

Cell culture insert and coverslip experiments were performed to determine whether prevention of direct contact between cells and paste could protect the cells from unreacted CPC. Panels A–D of Figure 3 are from an experiment in which cells were seeded on the bottom of a six-well plate and CPC paste was placed above the cells in a cell culture insert [see Fig. 2(A) for diagram]. Cells in both Control [Fig. 3(A,B)] and CPC [Fig. 3(C,D)] wells were nearly all live with few dead. Counting of live and dead cells in micrographs revealed that the "Control" and "CPC" treatments had 99 and 100% live cells, respectively [Fig. 4 (bars 5 and 6)]. When the experiment was reversed with cells in the inserts and paste on the bottom of the wells [see Fig. 2(B) for diagram], the outcome was the same: cells exposed to unreacted CPC paste were as viable as control cells (data not shown) and 99% of the cells were live in both treatments [Fig. 4 (bars 7 and 8)]. Next, coverslips covered by a monolayer of cells were placed on CPC paste [see Fig. 2(C) for diagram]. Again, cells in both the Control [Fig. 3(E,F)] and CPC [Fig. 3(G,H)] treatments were nearly all live with 98 and 99% live cells, respectively [Fig. 4 (bars 9 and 10)]. These coverslip and cell culture insert experiments suggest that unreacted CPC is not toxic when it is exposed to cells by an indirect means.

Our next objective was to encapsulate MC3T3-E1 cells in a gel (alginate), seed the encapsulated cells into CPC, and determine whether the gel encapsulation protected the cells from the unreacted CPC. Control cell-alginate beads that had not been exposed to CPC contained mostly live cells [Fig. 5(A)] and few dead cells [Fig. 5(B)]. Comparison of panels A and B of Figure 1 to panels A and B of Figure 4 reveals that the ratio of live to dead cells on TCPS controls [Fig. 1(A,B)] is similar to that in the alginate beads [Fig. 5(A,B)]. These observations demonstrate that the alginate encapsulation procedure was not harmful to the MC3T3-E1 cells. The CPC-Exposed beads also contained primarily live cells [Fig. 5(C)] and only a few dead cells [Fig. 5(D)] showing that the alginate encapsulation protected cells from the unreacted CPC.

Comparison of panels B and F of Figure 5 reveals that the density of dead cells in beads that spent the night covered in CPC (with limited access to medium) was greater than that found in Control beads. Likewise, comparison of panels A and E (Fig. 5) reveals fewer live cells in CPC-Covered beads than in Control. These observations suggest that survival of the encapsulated cells is better if the cell-alginate beads are not completely enclosed by CPC and have access to medium.

To test whether a cell alginate bead contains enough nutrients to sustain cell viability for 24 h, cell-alginate beads were covered with silicone grease and incubated overnight (in the absence of any media). Beads that were covered overnight in grease ("Grease") contained mostly live cells and few dead cells [Fig. 5(G,H)], similar to Control [Fig. 5(A,B)]. These results show that there are enough media and nutrients in the beads to sustain cell viability for 24 h and they also suggest that a lack of media or nutrients is not the cause of the decreased cell viability in the CPC-Covered beads [Fig. 5(E,F)].

The percent live cells present in cell-alginate beads after the various treatments shown in Figure 5 was determined by counting live and dead cells in the

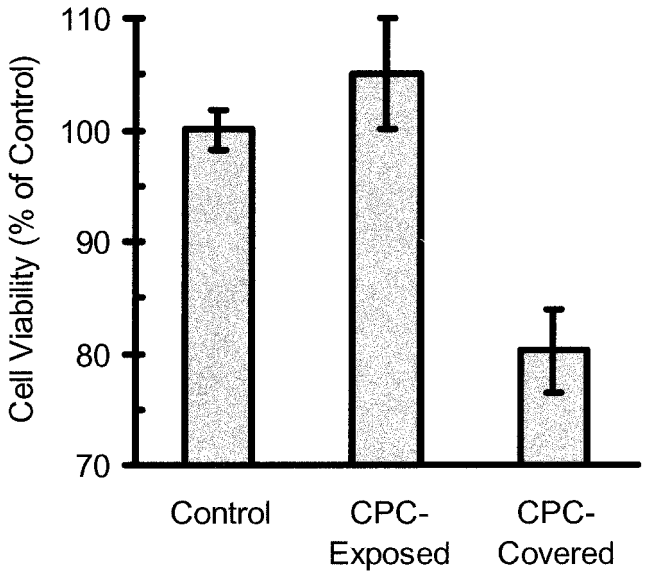

Figure 7. Wst-1 assay of cell-alginate beads after seeding into CPC. The viability of Control, CPC-Exposed, and CPC-Covered cell-alginate beads was quantified with the Wst-1 assay (dehydrogenase activity). Bonferroni t tests showed that CPC-Covered was different from Control and CPC-Exposed ($p < 0.017$).

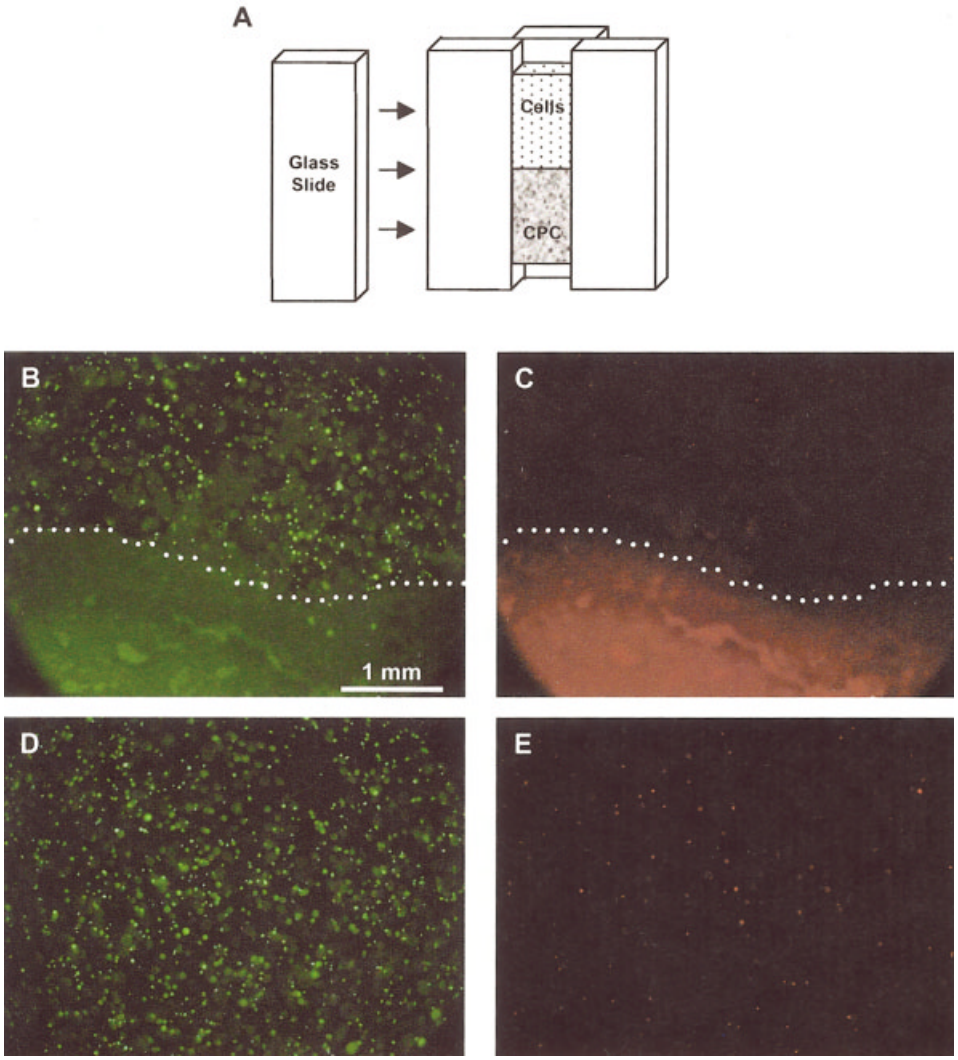

Figure 8. Glass slide experiment. A cell-alginate gel was placed in contact with CPC paste in a trough formed by gluing together glass slides as depicted in Panel A. The construct was incubated overnight and Live-Dead stained. Panels B and C are micrographs of the interface between the CPC and the cell-alginate gel. The white dots indicate the interface between CPC and gel. Panels D and E are micrographs of an area of the cell-alginate gel located 2 mm from the interface with the CPC. Pairs of green (left) and red (right) panels are the same field viewed with a green (live) or red (dead) filter. The size bar in Panel B applies to all panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

micrographs from these experiments. Control, CPC-Exposed, CPC-Covered, and Grease beads contained 97, 96, 74, and 99% live cells, respectively (Fig. 6). Cell viability was also quantitated by assessing dehydrogenase activity using the Wst-1 assay. CPC-Exposed and CPC-Covered beads had 105 and 80% of the dehydrogenase activity in Control beads, respectively (Fig. 7). These quantitative Live-Dead (Fig. 6) and Wst-1 (Fig. 7) results agree with the qualitative microscopy results presented in Figure 5 and they indicate: 1. that gel encapsulation can protect cells from unreacted CPC, and 2. that isolation of the cell-alginate beads from the medium with CPC reduces cell survival.

To better characterize the ability of alginate to protect cells, we determined the physical depth to which the alginate is able to provide protection from the unreacted CPC. When taking the micrographs for the experiments presented in Figure 5(C,D) there was not a "zone" of dead cells near the surface of the cell-alginate beads after an overnight incubation in unreacted CPC. The few dead cells present in these beads were evenly distributed throughout the bead and were not concentrated at the edges where the CPC was in direct contact with the alginate. Even so, we devised the system depicted in Figure 8(A) in which a cell-alginate gel is placed against freshly mixed CPC paste to provide a clearer view of the interface between the CPC and the cell-alginate gel. Again, no zone of dead cells was visible along the interface between the CPC and the alginate gel and the few dead cells present in the gel were uniformly dispersed throughout the alginate [Fig. 8(C)]. In addition, an

examination of the cells located 2 mm from the CPC–gel interface [Fig. 8(D,E)] reveals that the density of live and dead cells in this section of the gel is equivalent to that found in the section juxtaposed to the CPC [Fig. 8(B,C)]. These results show that cells are protected from unreacted CPC at any depth in the gel and that there is not a visible region at the CPC–gel interface where cells are affected by the CPC.

DISCUSSION

The TCPS experiments presented in Figure 1 showed that unreacted CPC had toxic effects on cells but that reacted CPC was nontoxic. Unreacted CPC paste killed osteoblast-like cells (MC3T3-E1) within 2 h when it was placed on top of cell monolayers [Fig. 1(C,D)]. The CPC setting reaction may be the cause of the cell death because this reaction involves the dissolution of calcium phosphate particles that may cause fluctuations in pH and ionic strength underneath the paste.[3] These local fluctuations may contribute to the toxic effects of the unreacted CPC, especially because the calcium phosphate particles and the cells make close contact under these conditions. Nutrient deprivation might also contribute to cell death because the cells were blanketed by a thin layer (<1 mm) of CPC paste which may restrict diffusion. However, preliminary work showed that cells seeded on top of CPC paste also died, suggesting that the toxic effect of the paste results from more than just blockage of diffusion.

Although previous studies have not examined the effects of placing CPC paste on cell monolayers, the effects of placing other calcium phosphate powders on osteoblasts have been studied. Grégoire et al.[22] observed that HA particles (<0.05 mm) or β-tricalcium phosphate (TCP) particles (<0.05 mm) caused an increase in protein synthesis when added to MC3T3-E1 cells or primary human osteoblasts. In contrast, Alliot-Licht et al.[23] found that HA particles (<0.01 mm) inhibited the growth and alkaline phosphatase expression of MC3T3-E1 cells, ROS 17/2.8 osteosarcoma cells, and primary rat osteoblasts. Both investigations[22,23] also reported that the osteoblasts phagocytosed the calcium phosphate particles.

Sun et al.[24] also exposed cells to calcium phosphate particles and reported that HA particles (0.1 μm) or β-TCP particles (0.1 μm) inhibited the growth of primary rat osteoblasts while causing an increase in their expression of alkaline phosphatase. In addition, Pioletti et al.[25] observed a decrease in growth, viability, and synthesis of extracellular matrix in primary rat osteoblasts that were exposed to β-TCP particles (1–10 μm) or dicalcium dihydrate particles (1–10 μm). A 90% decrease in viability was observed by Pioletti et al.[25] when evaluated using a Live-Dead assay similar to what we have used in the present study. These effects are similar to the decrease in viability observed when we placed CPC paste on osteoblast monolayers [Fig. 1(C,D)]. However, it is difficult to draw any conclusions from this study[25] or any of the others[22–24] in regard to our own work because different materials as well as different particle sizes, particle concentrations, cell types, and assays were used in each study. Although disparate observations were made in these four studies, an obvious conclusion is that calcium phosphate powders can affect osteoblast function and we have clearly observed an effect of CPC paste on osteoblasts in the present experiments.

Regardless of whether pH, ionic strength, or nutrient deprivation was responsible for the cell death observed in our experiments, CPC paste was not toxic to cells when direct contact between the cells and the CPC paste was prevented (Figs. 2–4). This was tested by exposing cells to CPC paste through porous membranes or by placing coverslips covered by cells on beds of CPC paste (Figs. 2–4). CPC paste was not toxic when exposed to cells via these indirect means, which led to the supposition that gel encapsulation could protect cells from unreacted CPC during seeding.

Many gel systems have been used for cell encapsulation but we chose alginate because of its biocompatibility, ease of use, and gentle encapsulation procedure.[16,17,26] Cells were encapsulated into alginate beads, mixed into CPC paste, and incubated for 24 h. Cell viability in beads that were mixed into CPC was similar to control beads that were not mixed with CPC (Figs. 5–8) suggesting that gel encapsulation can protect cells from unreacted CPC.

In addition, cell survival in the beads whose surfaces were partially exposed to the medium was the same as in control beads and survival was slightly lessened in beads that were completely covered by CPC (Figs. 5–7). The slight reduction in cell viability in CPC-Covered beads may have been caused by CPC inhibiting diffusion of media or nutrients to the cells. However, this possibility can be eliminated because there was enough media and nutrients in a cell-alginate bead to maintain cell viability during an overnight incubation of the bead in silicone grease in the absence of any media [Fig. 5(G,H)]. Taken together, these results suggest that the fluctuations in pH and ionic strength during the CPC setting reaction may become toxic to cells when washout of the ionic components is restricted by the presence of CPC paste. Thus, CPC-Exposed beads may be viable because they have access to media and washout of the ionic components of the setting reaction is not completely obstructed by paste, whereas viability is slightly lessened in CPC-Covered beads because diffusion and washout may be restricted by the covering of CPC.

The cell seeding system we have explored in the

present study could be optimized by imparting macroporosity to the CPC and by using a fast-degrading gel for encapsulation. Macroporosity would enhance survival of seeded cells by improving the diffusion of media and nutrients into the implant as well as the washout of ionic components from the setting reaction.[27,28] Second, alginate gels typically persist for months *in vivo*[29–31] yet the CPC setting reaction is complete by 24 h.[19] Thus, use of a gel that degrades after 24 h could safely free the encapsulated osteoblasts to begin bone regeneration as soon as the cement had set.[16,32–34]

Previous studies have documented that osteoblasts can produce bone when suspended in a gel[33,35,36] and dressings composed of alginate-encapsulated osteoblasts have been put forth as a material for bone tissue engineering.[33] In addition, it has been shown that alginate encapsulation can protect microorganisms from acidic conditions during seeding into yogurt.[37] Thus, osteoblasts can behave normally when encapsulated in a gel and there are precedents for using hydrogels to protect cells from their surroundings.

Previous studies have also shown that ceramics loaded with osteogenic cells make effective bone grafts.[8,9,38] Notably, cell-seeded HA/TCP implants established unions in critical-sized segmental defects (21 mm) created in the femora of dogs. In contrast, nonunion occurred in femora of untreated control defects or defects treated with cylinders that were not seeded with cells.[9] These results are promising for our own system in which gel-encapsulated cells are mixed into CPC paste. However, an advantage of our system is that CPC paste can be easily sculpted to fit the contours of a wound whereas the hardened ceramic blocks used in these other studies are preformed and difficult to shape.

In summary, we have developed a method to seed cells (MC3T3-E1) into CPC paste. CPC is currently used as a bone graft material and starts as a paste that can be shaped during surgery. The paste then hardens into HA and is resorbed over several years. In light of recent advances in autologous MSC therapy, our technique for seeding cells into CPC cement could potentially be used to seed a patient's *ex vivo* expanded stem cells into the cement to create an osteoinductive bone graft substitute that could be used to treat that same patient. Using MC3T3-E1 osteoblast-like cells as a model system, we found that unreacted CPC paste killed cells, reacted CPC was nontoxic, and encapsulation of cells in a hydrogel protected them from the unreacted CPC. Thus, gel encapsulation could be used as a mechanism to protect cells for seeding into CPC.

The authors thank C. Khatri (NIST) and S. Frukhtbeyn (NIST-ADA) for kindly providing the CPC; G. Balian (University of Virginia) and H. Wilson (University of Virginia) for helpful advice regarding alginate; E. Parry (NIST-ADA) for machining the molds; and B. Fowler (NIST-NIDCR) and N. Eidelman (NIST-ADA) for taking the X-ray spectra.

References

1. Buddecke DE Jr, Lile LN, Barp EA. Bone grafting: principles and applications in the lower extremity. Clin Podiatr Med Surg 2001;18:109–145.
2. Friedman CD, Costantino PD, Takagi S, Chow LC. Bonesource™ hydroxyapatite cement: a novel biomaterial for craniofacial skeletal tissue engineering and reconstruction. J Biomed Mater Res 1998;43:428–432.
3. Chow LC, Takagi S, Costantino PD, Friedman CD. Self-setting calcium phosphate cements. Mater Res Soc Symp Proc 1991; 179:3–24.
4. Yoshikawa MS, Toda T. Reconstruction of alveolar bone defect by calcium phosphate compounds. J Biomed Mater Res 2000; 53:430–437.
5. Yuasa T, Miyamoto Y, Ishikawa K, Takechi M, Nagayama M, Suzuki K. *In vitro* resorption of three apatite cements with osteoclasts. J Biomed Mater Res 2001;54:344–350.
6. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. Bone 1992;13:81–88.
7. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop 1998;355(Suppl):S247–S256.
8. Kadiyala S, Jaiswal N, Bruder SP. Culture-expanded, bone marrow-derived mesenchymal stem cells can regenerate a critical-sized segmental bone defect. Tissue Eng 1997;3:173–185.
9. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998;80:985–996.
10. Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 1998;95:13726–13731.
11. Thomson JA, Itskovit-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147.
12. Chow LC, Takagi S, Ishikawa K. Formation of hydroxyapatite in cement systems. In: Brown PW, Constantz B, editors. Hydroxyapatite and related minerals. Boca Raton, FL: CRC; 1994. p 124.
13. Chow LC, Hirayama S, Takagi S, Parry E. Diametral tensile strength and compressive strength of a calcium phosphate cement: effect of applied pressure. J Biomed Mater Res 2000; 53:511–517.
14. Sudo H, Kodama H-A, Magai Y, Yamamoto S, Kasai S. *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983;96: 191–198.
15. Attawia MA, Uhrich KE, Botchwey E, Langer R, Laurencin CT. *In vitro* bone biocompatibility of poly(anhydride-*co*-imides) containing pyromellitylimidoalanine. J Orthop Res 1996;14: 445–454.
16. Lanza RP, Jackson R, Sullivan A, Ringeling J, McGrath C, Kühtreiber W, Chick WL. Xenotransplantation of cells using biodegradable microcapsules. Transplantation 1999;67:1105–1111.
17. Becker TA, Kipke DR, Brandon T. Calcium alginate gel: a biocompatible and mechanically stable polymer for endovascular embolization. J Biomed Mater Res 2001;54:76–86.
18. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He P-G. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 1993;41:1118–1122.

19. Fukase Y, Eanes ED, Takagi S, Chow LC, Brown WE. Setting reactions and compressive strengths of calcium phosphate cements. J Dent Res 1990;69:1852–1856.
20. Glantz SA. Primer of bio-statistics. 4th ed. New York: McGraw-Hill; 1997. p 89–90.
21. Simon CG Jr, Khatri CA, Wight SA, Wang FW. Preliminary report on the biocompatibility of a moldable, resorbable, composite bone graft consisting of calcium phosphate cement and poly(lactide-*co*-glycolide) microspheres. J Orthop Res 2002;20:473–482.
22. Grégoire M, Orly I, Menanteau J. The influence of calcium phosphate biomaterials on human bone cell activities. J Biomed Mater Res 1990;24:165–177.
23. Alliot-Licht B, Gregoire M, Orly I, Menanteau J. Cellular activity of osteoblasts in the presence of hydroxyapatite: an *in vitro* experiment. Biomaterials 1991;12:752–756.
24. Sun J-S, Tsuang Y-H, Liao C-J, Liu H-C, Hang Y-S, Lin F-H. The effects of calcium phosphate particles on the growth of osteoblasts. J Biomed Mater Res 1997;37:324–334.
25. Pioletti DP, Takei H, Lin T, Landuyt PV, Ma QJ, Kwon SY, Sung K-LP. The effects of calcium phosphate cement particles on osteoblast functions. Biomaterials 2000;21:1103–1114.
26. Strand BL, Mørch YA, Skjåk-Bræk. Alginate as immobilization matrix for cells. 2000;12:223–233.
27. Klawitter JJ, Hulbert SF. Application of porous ceramics for the attachment of load bearing internal orthopedic applications. J Biomed Mater Res 1971;2:161–229.
28. Markovic M, Takagi S, Chow LC. Formation of macropores in calcium phosphate cements through the use of mannitol crystals. Key Eng Mater 2001;192–195:773–776 [Bioceramics: Proceedings of the 13th International Symposium on Ceramics in Medicine, Bologna, Italy, 2000].
29. Lanza RP, Kühtreiber WM, Ecker D, Staruk JE, Chick WL. Xenotransplantation of porcine and bovine islets without immunosuppression using uncoated alginate microspheres. Transplantation 1995;59:1377–1384.
30. Al-Shamkhani A, Duncan R. Radioiodination of alginate via covalently bound tyrosinamide allows monitoring of its fate *in vivo*. J Bioact Compat Polym 1995;10:4–13.
31. Klöck G, Pfeffermann A, Ryser C, Gröhn P, Kuttler B, Hahn H-J, Zimmermann U. Biocompatibility of mannuronic acid-rich alginates. Biomaterials 1997;18:707–713.
32. Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. Biotechnol Prog 2001;17:945–950.
33. Lee KY, Alsberg E, Mooney DJ. Degradable and injectable poly(aldehyde guluronate) hydrogels for bone tissue engineering. J Biomed Mater Res 2001;56:228–233.
34. Mann BK, Gobin AS, Tsai AT, Schmedlen RH, West JL. Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering. Biomaterials 2001;22:3045–3051.
35. Majmudar G, Bole D, Goldstein SA, Bonadio J. Bone cell culture in a three-dimensional polymer bead stabilizes the differentiated phenotype and provides evidence that osteoblastic cells synthesize type III collagen and fibronectin. J Bone Miner Res 1991;6:869–881.
36. Nishimoto SK, Stryker WF, Nimni ME. Calcification of osteoblast-like rat osteosarcoma cells in agarose suspension cultures. Calcif Tissue Int 1987;41:274–280.
37. Lee K-Y, Kim J-Y, Yu W-K, Lee Y-J, Yoon S-S, Heo T-R. Survival of *Bifidobacterium breve* in acidic solutions and yogurt, following immobilization in calcium alginate beads. J Microbiol Biotech 2001;11:412–417.
38. Cong Z, Jianxin W, Huaizhi G, Bing L, Xingdong Z. Repairing segmental bone defects with living porous ceramic cylinders: an experimental study in dog femora. J Biomed Mater Res 2001;55:28–32.