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Preliminary report on the biocompatibility of a moldable, resorbable, composite bone graft consisting of calcium phosphate cement and poly(lactide-co-glycolide) microspheres ☆

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Abstract

We have assessed the biocompatibility of a new composite bone graft consisting of calcium phosphate cement (CPC) and poly(lactide-co-glycolide) (PLGA) microspheres (approximate diameter of 0.18–0.36 mm) using cell culture techniques. CPC powder is mixed with PLGA microspheres and water to yield a workable paste that could be sculpted to fit the contours of a wound. The cement then hardens into a matrix of hydroxyapatite microcrystals containing PLGA microspheres. The rationale for this design is that the microspheres will initially stabilize the graft but can then degrade to leave behind macropores for colonization by osteoblasts. The CPC matrix could then be resorbed and replaced with new bone. In the present study, osteoblast-like cells (MC3T3-E1 cells) were seeded onto graft specimens and evaluated with fluorescence microscopy, environmental scanning electron microscopy and the Wst-1 assay (an enzymatic assay for mitochondrial dehydrogenase activity). Cells were able to adhere, attain a normal morphology, proliferate and remain viable when cultured on the new composite graft (CPC–PLGA) or on a control graft (CPC alone). These results suggest that our new cement consisting of CPC and PLGA microspheres is biocompatible. This is the first time that a 'polymer-in-mineral' (PLGA microspheres dispersed in a CPC matrix) cement has been formulated that is moldable, resorbable and that can form macropores after the cement has set.

Introduction

Bone grafts have been used for decades to repair osseous defects resulting from trauma and disease and over 100,000 operations are performed each year in the US [43]. Autografts are widely used by surgeons, but the amount of bone available for autografts is limited and available material is difficult to shape. In addition, autografts come with the small but existent risk of infection or pain at the donor site. Alternatives, such as allografts and xenografts, require extensive processing to minimize disease transmission. This processing of the grafts destroys the bone cells and many of the proteins present in the grafts that promote healing, while infection and transmission of disease are still infrequent but potential outcomes. Thus, the development of a safe and effective alloplast for use as bone graft is a worthy objective that would be met by patients and clinicians with great enthusiasm.

Our colleagues at the American Dental Association Health Foundation at NIST have developed a moldable, self-setting calcium phosphate cement (CPC) that suits this purpose [13]. The cement powder is mixed with water to form a workable paste which can be shaped during surgery to fit the contours of a wound. The cement precipitates as microcrystalline hydroxyapatite which is resorbed by the body and replaced with new bone [7,44,45]. The cement sets within 30 min and extensive testing has found the cement to be biocompatible [13]. The mechanical strength of the CPC is not suitable for load-bearing applications. However it is used clinically to treat non-load-bearing dental and craniofacial defects [13].

In order to enhance the rate of CPC resorption and bony ingrowth into the implant [27,37], our colleagues have created macropores in the CPC by particulate

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leaching techniques using salt or sugar crystals as porogens [28,29,40]. However, when the crystals are included in a high volume fraction (0.6), the grafts become too weak to adequately maintain their shape for use in vivo. Instead, we have used biodegradable poly(lactide-co-glycolide) (PLGA) microspheres (approximate diameter of 0.18–0.36 mm) to impart macroporosity to the cement. Pores of this size range have been found to be optimal for bony ingrowth [21] and when randomly mixed into a matrix at a volume fraction of greater than 0.4 they should form interconnected pores [15,20]. When implanted in vivo, the polymer microspheres should degrade to expose macropores for bony ingrowth. The strengthening of the graft from bony ingrowth and the deposition of new bone should offset the weakening of the graft due to polymer degradation.

PLGA degrades by random hydrolysis into lactic and glycolic acids, both of which are harmless physiological metabolites [1]. PLGA is used clinically in suture materials and is considered to be non-toxic and biocompatible both in vitro and in vivo [1,12,17,33,36,41,47]. Herein, we use cell culture techniques to characterize the initial biocompatibility of the new bone graft consisting of PLGA microspheres dispersed in a hydroxyapatite matrix (CPC–PLGA).

Materials and methods

Preparation of PLGA microspheres. PLGA copolymer with a lactide to glycolide molar ratio of 1, carboxyl end groups and an average relative molecular mass of 91,200 (91.2 kDa) was obtained from [1] Birmingham Polymers (Birmingham, AL). PLGA microspheres were made by a solvent evaporation method [23]. PLGA (4 g) was dissolved in 20 ml of dichloromethane and added to 1.6 l of 1% polyvinyl alcohol [88% mole fraction hydrolyzed; average relative molecular mass of 25,000 (25 kDa); Polysciences, Warrington, PA]. The solution was stirred at 375 revolutions per minute with an overhead stirrer at room temperature for 4.5 h in a hood to allow the solvent to evaporate. After stirring, the solution was allowed to stand for 30 min and the liquid was decanted. The microspheres were washed four times with 250 ml of water and lyophilized. Microspheres having an approximate diameter of 0.18–0.36 mm were isolated using sieves and were stored at −20 °C until use.

Preparation of CPC and CPC–PLGA discs. Tetracalcium phosphate ($Ca_4(PO_4)_2O$) was prepared according to Chow et al. [8] and anhydrous dicalcium phosphate ($CaHPO_4$) was obtained from J.T. Baker (Phillisburg, NJ). CPC and CPC–PLGA discs were prepared as described [9,13]. 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) in a blender. Each CPC disc was prepared by mixing four parts by mass of CPC powder with one part of water (0.25 g of CPC powder plus 63 μl of water) for 30 s with a spatula. The paste was placed in a stainless 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 [9]. The discs were incubated at 37 °C and 100% relative humidity overnight before their removal from the molds. CPC PLGA discs were made in a similar manner, except that the CPC powder was mixed with PLGA microspheres. PLGA microspheres were mixed with the cementious powder at a volume fraction of 0.6 to 0.4 (mass fraction of PLGA to CPC was 0.4:0.6). Four parts by mass of the mixture of cement powder and PLGA microspheres were mixed with one part of water (0.15 g of CPC powder mixed with 0.10 g of PLGA microspheres plus 63 μl of water) for 30 s with a spatula and cured in molds as described above. CPC and CPC–PLGA discs were sterilized by soaking them in 70% ethanol (mass fraction) for 15 min and then incubating them in cell culture media overnight.

Cell culture. Established protocols for the culture and passage of MC3T3-E1 cells were followed [5]. 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, Walkersville, MD) supplemented with 10% (volume fraction) fetal bovine serum (Gibco, Rockville, MD) and kanamycin sulfate (Sigma, 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 EDTA (Gibco, Rockville, MD) once per week. Cultures of 90% confluent MC3T3-E1 cells were trypsinized, washed and suspended in fresh media. CPC–PLGA discs or CPC discs were placed one each into the wells of a 24-well plate (BD Biosciences, Bedford, MA). Fifty thousand cells diluted into 2 ml of media were added to wells containing the discs or to empty wells (TCPS controls), incubated overnight or for 2 weeks (2 ml of fresh media every 2 days) and then prepared for fluorescence, electron microscopy or for the Wst-1 viability assay.

Fluorescence microscopy. After overnight or 2-week incubations of the cells on the CPC–PLGA, CPC or TCPS, the media was removed and the cells were washed two times in 2 ml of Tyrode's Hepes buffer (140 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). Cells were stained for 1 h with 2 ml of Tyrode's Hepes buffer containing 2 μmol/l calcein-AM and 2 μmol/l ethidium homodimer-1 (both from Molecular Probes, Eugene, OR) and viewed by epifluorescence microscopy (Olympus BH-2, Melville, NY). Calcein-AM is a non-fluorescent, cell-permeant fluorescein derivative, which is converted by cellular enzymes into cell-impermeant and highly fluorescent calcein. Calcein accumulates inside live cells having intact membranes causing them to fluoresce green. Ethidium-homodimer-1 enters dead cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to their DNA causing the nuclei of dead cells to fluoresce red. Double-staining cells anchored on the bone graft discs allow simultaneous examination of both live and dead cells on the discs. Cells grown on TCPS served as a control and should possess few dead cells and many live cells. Images were captured with a digital camera from three CPC discs, three CPC–PLGA discs and three TCPS control wells.

Environmental scanning electron microscopy of cells on discs. Cell morphology was also examined using environmental scanning electron microscopy (ESEM). Cells grown overnight on cement discs were fixed overnight in cacodylate-buffered 2.5% glutaraldehyde (volume fraction) at 4 °C, rinsed and observed directly at ≈7 Torr (≈900 Pa) in a hydrated state in an ESEM operated at 20 keV [35]. Images were captured from three CPC and three CPC–PLGA discs.

Wst-1 viability assay. Cells grown on CPC–PLGA or CPC for 2 weeks were analyzed for viability using the Wst-1 assay which measures mitochondrial dehydrogenase activity [18]. Discs with cells were transferred to clean wells in a 24-well plate and rinsed two times with Tyrode's Hepes buffer. One ml of Tyrode's Hepes buffer was added to each well, 100 μl of Wst-1 solution (5 mmol/l Wst-1 and 0.2 mmol/l 1-methoxy PMS in water) was added to each well and the discs incubated at room temperature. After a 2-hour incubation at room temperature, 200 μl of each reaction mixture was transferred to a 96-well plate and the absorbance at 450 nm was measured with a microplate reader (Wallac 1420 Victor[2], PerkinElmer Life Sciences, Gaithersburg, MD). The assay was performed with cells on three CPC and three CPC–PLGA discs. 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).

[1] Certain commercial equipment, instruments or materials are identified in this paper in order to specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment specified is necessarily the best available for the purpose.

Photographs and scanning electron micrographs of degraded discs. Three CPC and three CPC–PLGA discs were prepared, sterilized as described above and photographed (Nikon Coolpix 990, Melville, NY). The discs were then placed in 10 ml of cell media and incubated in a cell incubator (37 °C; 5% CO_2, volume fraction) to allow the PLGA microspheres to degrade. After 90 days, the discs were photographed. Two of the CPC and CPC–PLGA specimens were split evenly in half to allow examination of the interior of the discs by scanning electron microscopy (SEM). The remaining CPC and CPC–PLGA discs were left intact for surface examination by SEM. The samples were prepared for SEM by rinsing them in distilled water and then dehydrating them in 100% ethanol followed by hexamethyldisilazane. The dehydrated samples were sputter-coated with gold (Model #22179, Denton Vacuum, Morrestowne, NJ) and observed in a scanning electron microscope (JEOL JSM-5300, Peabody, MA; 15 kV, 50 μA, 50× magnification).

Statistics. When the 'standard deviation' (S.D.) is given in the text of the manuscript or as an error bar in a figure (Fig. 4), it refers to the 'standard deviation of the mean', also known as the 'combined standard uncertainty of the mean'. The results for CPC–PLGA and CPC from the Wst-1 assay (Fig. 4) were compared using the unpaired Student's *t* test with a confidence level of 95%.

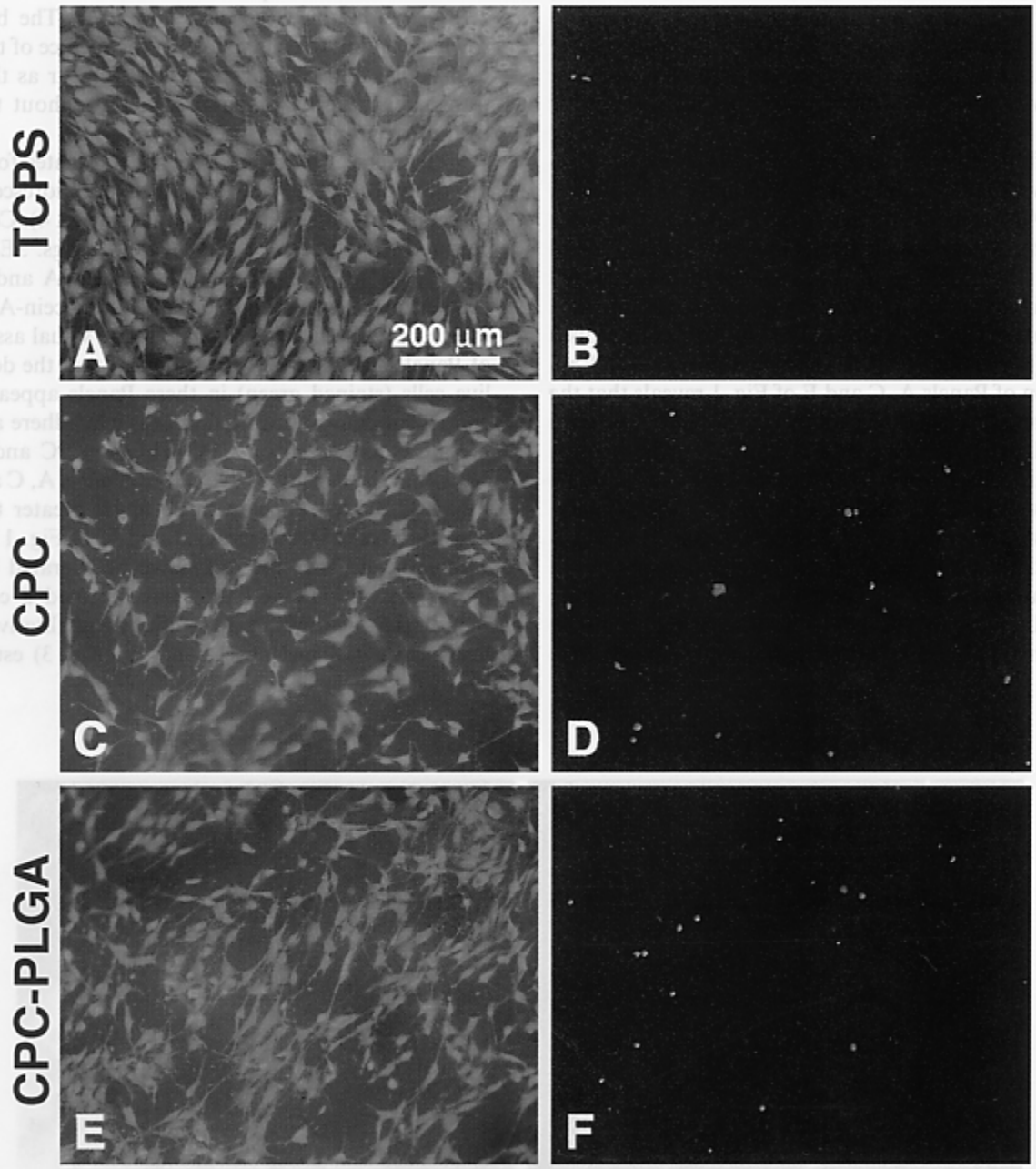

Fig. 1. MC3T3-E1 cells were seeded onto TCPS, CPC or CPC–PLGA, incubated overnight and prepared for fluorescence microscopy. Cells were double-stained with calcein-AM and ethidium homodimer-1 and observed using epifluorescence. Panels A and B are images of the same field of cells on TCPS viewed with a green (A, live cells) or red (B, dead cells) filter. Panels C and D are images of the same field of cells on a CPC disc viewed with a green (C, live cells) or red (D, dead cells) filter. Panels E and F are images of the same field of cells on a CPC–PLGA disc viewed with a green (E, live cells) or red (F, dead cells) filter.

Results

Cell culture toxicity assays are the international standard for the initial screening of biomaterials for biocompatibility since they are more rapid, reproducible, sensitive and less expensive than animal testing [19]. Thus, we have used in vitro cell culture to evaluate the biocompatibility of our new cement formulation that contains PLGA microspheres (CPC–PLGA). MC3T3-E1 cells, a murine osteoblast-like cell line [38], were plated onto CPC–PLGA discs, CPC discs or TCPS, cultured overnight and observed by microscopy to evaluate adhesion and morphology (Fig. 1). Since the cement discs do not transmit light, cells growing on their surface were viewed with fluorescence microscopy or ESEM.

For fluorescence experiments, cells were double-stained with calcein-AM and ethidium homodimer-1. Calcein-AM stains live cells green (Figs. 1A, C and E) while ethidium homodimer-1 stains the nuclei of dead cells red (Figs. 1B, D and F). Live cells (green) adhered and attained a normal, polygonal morphology [5] when seeded onto CPC–PLGA discs (Fig. 1E), CPC discs (Fig. 1C) or TCPS (Fig. 1A). A qualitative visual examination of Panels A, C and E of Fig. 1 reveals that the density of live cells (green) adherent to each substrate (CPC–PLGA, CPC and TCPS) appears to be equivalent. This observation shows that cells can adhere equally well to CPC–PLGA, CPC and TCPS. A qualitative visual examination of Panels B, D and F of Fig. 1 reveals that the density of dead cells (red spots are stained nuclei of dead cells) adherent to each substrate (CPC–PLGA, CPC and TCPS) also appears to be equivalent. This observation demonstrates that CPC–PLGA is not toxic, or is not more toxic to the cells than is CPC or TCPS. Thus, after overnight culture, cell adhesion and viability on CPC–PLGA are the same as those on CPC or TCPS.

Higher resolution images of cells cultured overnight on the discs were obtained using ESEM (Figs. 2A and B). We chose ESEM over conventional SEM since hydrated samples can be viewed in an ESEM which simplifies sample preparation and eliminates the artifacts that can arise from sample dehydration [10]. Cells were able to adhere and spread on CPC–PLGA discs (Fig. 2B) as well as on CPC discs (Fig. 2A). The black arrowheads point to cells visible on the surface of the discs. Cell bodies and cellular extensions appear as the semi-transparent 'wispy' material seen throughout the electron micrographs.

Cells were also cultured on the substrates for longer durations (2 weeks) and examined by fluorescence microscopy (Fig. 3) and the Wst-1 assay (Fig. 4). Cells were cultured for 2 weeks on CPC–PLGA (Figs. 3E and F), CPC (Figs. 3C and D) or TCPS (Figs. 3A and B) and then fluorescently double-stained (calcein-AM and ethidium homodimer-1). A qualitative visual assessment of Panels A, C and E (Fig. 3) reveals that the density of live cells (stained green) in these Panels appears to be equivalent demonstrating that cells can adhere and proliferate equally well on CPC–PLGA, CPC and TCPS. Note that the density of live cells in Panels A, C and E of Fig. 3 (2 weeks) appears to be much greater than the density of cells in Panels A, C and E of Fig. 1 (1 day). This indicates that the cells have proliferated between day 1 and day 14 and they have formed a confluent monolayer by day 14. In addition, qualitative visual assessment of Panels B, D, and F (Fig. 3) establishes

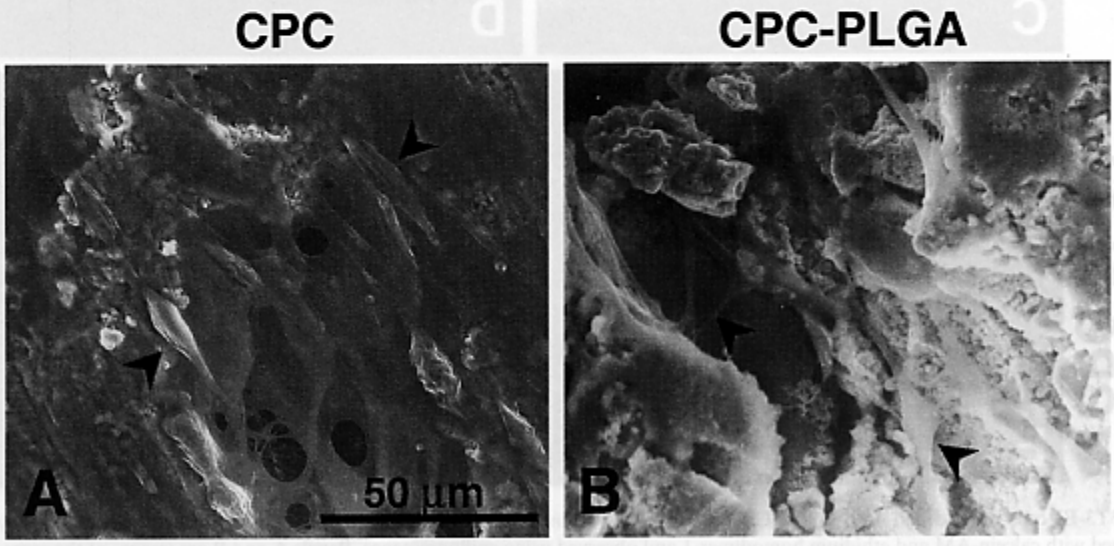

Fig. 2. MC3T3-E1 cells were seeded onto CPC or CPC–PLGA discs, incubated overnight and prepared for ESEM microscopy. Cells on the discs were fixed in glutaraldehyde and viewed directly in an ESEM while hydrated. Panel A is cells on a CPC disc and Panel B is cells on a CPC–PLGA disc. The black arrowheads point to cells or cell extensions.

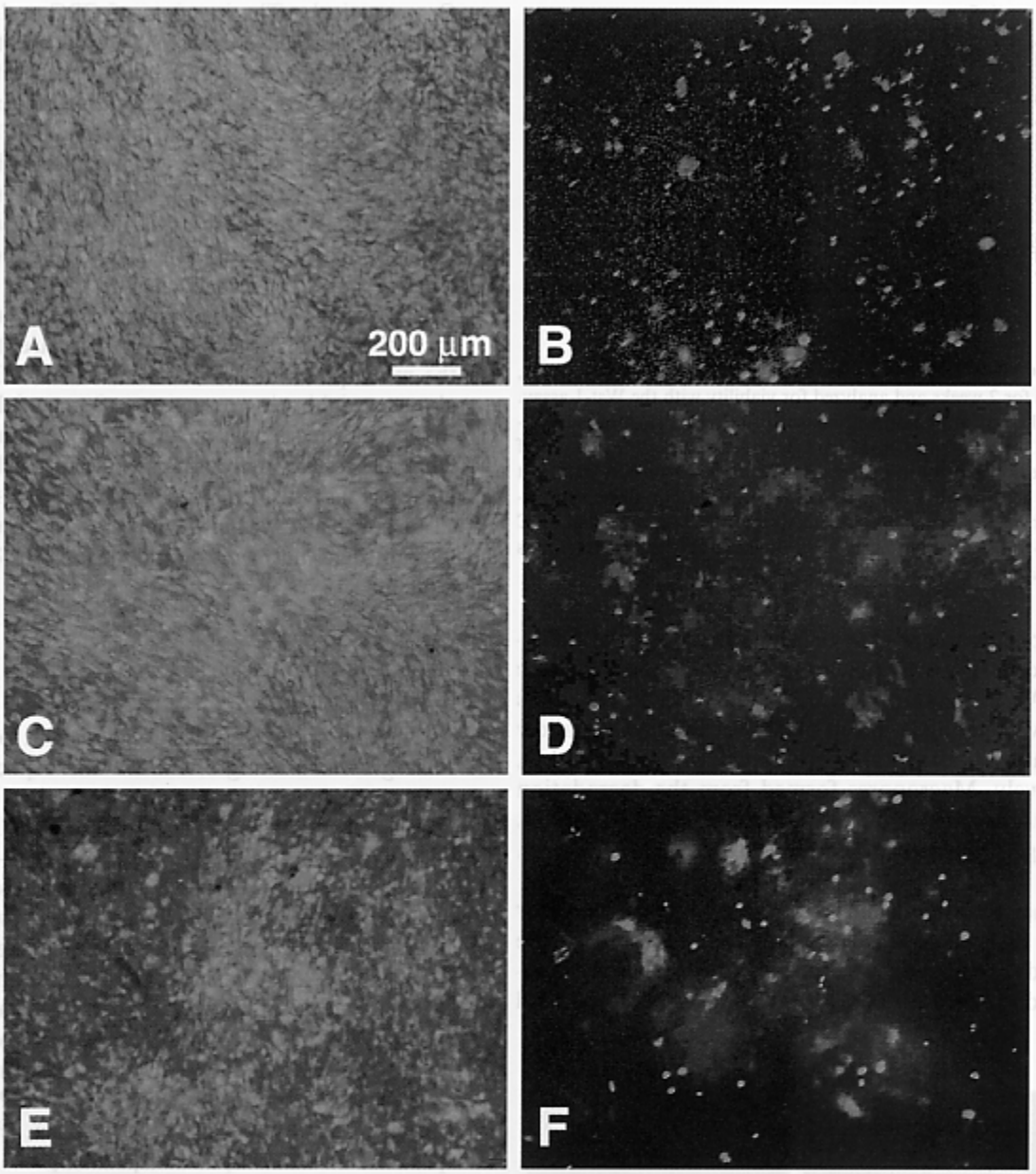

Fig. 3. MC3T3-E1 cells were seeded onto TCPS, CPC or CPC–PLGA, incubated 2 weeks and prepared for fluorescence microscopy. Cells were double-stained with calcein-AM and ethidium homodimer-1 and observed using epifluorescence. Panels A and B are images of the same field of cells on TCPS viewed with a green (A, live cells) or red (B, dead cells) filter. Panels C and D are images of the same field of cells on a CPC disc viewed with a green (C, live cells) or red (D, dead cells) filter. Panels E and F are images of the same field of cells on a CPC–PLGA disc viewed with a green (E, live cells) or red (F, dead cells) filter.

that the density of dead cells (red spots) on each of the substrates appears to be equivalent indicating that CPC–PLGA is not toxic to cells after 2 weeks, or at least not more toxic than is CPC or control TCPS. Thus, cell proliferation and viability after 2 weeks of culture on CPC–PLGA were the same as on CPC or control TCPS, demonstrating that CPC–PLGA is as biocompatible as is CPC or TCPS in these assays.

Next, the viability of cells cultured for 2 weeks on CPC–PLGA and CPC was quantitatively assessed with the colorimetric Wst-1 assay which measures mitochondrial dehydrogenase activity (Fig. 4). As shown in Fig. 4, the same amount of dehydrogenase activity was present in cells cultured on CPC–PLGA or CPC for 2 weeks. We did not perform the Wst-1 assay on cells cultured on TCPS since the growth area of the 24-well TCPS plates was not equivalent to the growth area on the cement discs and would not allow an accurate comparison. These results support the thesis that the new CPC–PLGA cement is as biocompatible as is CPC.

Photographs of freshly prepared CPC and CPC–PLGA discs are shown in Figs. 5A and C, respectively.

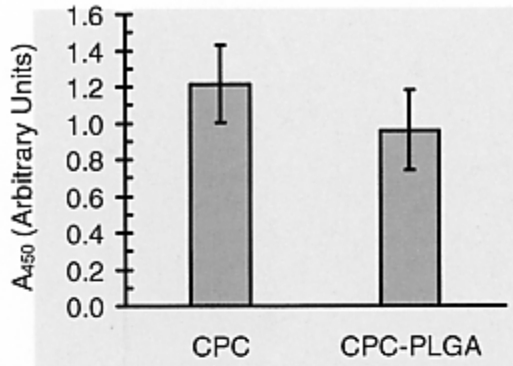

Fig. 4. MC3T3-E1 cells were seeded onto CPC or CPC–PLGA discs, incubated for 2 weeks and analyzed for viability with the Wst-1 assay. The Wst-1 assay is a colorimetric assay of cellular dehydrogenase activity and the absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cells on the discs. Error bars represent S.D. and cells on three CPC and three CPC–PLGA discs were assessed. There was no statistically significant difference between CPC and CPC–PLGA as determined by the unpaired Student's *t* test (*P* was greater than 0.05).

Microspheres are visible on the surface of the CPC–PLGA disc (Fig. 5C) that were absent on the CPC disc (Fig. 5A). Photographs of CPC and CPC–PLGA discs that have been aged in cell media for 90 days (to allow degradation of the PLGA) are shown in Figs. 5B and D, respectively. Macropores formed from the degradation of PLGA are visible on the surface of the CPC–PLGA disc (Fig. 5D) which were absent on the CPC disc (Fig. 5B).

Higher resolution images of the surface and interior of aged discs were taken to examine the microstructure of the discs (Fig. 6). The surfaces of CPC and CPC–PLGA discs aged for 90 days are shown in Figs. 6A and C, respectively. The interiors of CPC and CPC–PLGA discs that have been aged for 90 days and then split into half are shown in Figs. 6B and D, respectively. PLGA microspheres were not visible on the surface (Fig. 6C) or in the interior (Fig. 6D) of the aged CPC–PLGA discs indicating that all of the PLGA microspheres had degraded within 90 days. Instead, macropores were present in the CPC–PLGA (Figs. 6C and D) that were absent in the CPC (Figs. 6A and B). The size of the macropores (Figs. 6C and D) correlated with the size of the PLGA microspheres (180–360 μm in diameter) that degraded to form the pores. In Fig. 6D, it is apparent that each of the macropores in the interior of the CPC–PLGA disc abuts a neighboring macropore. Some of the pores seem to be separated from one another by a thin shell of CPC while others are interconnected (see arrowheads in Figs. 6C and D). Thus, the PLGA microspheres in the CPC–PLGA degrade within 90 days to form a partially interconnected network of macropores (180–360 μm in diameter).

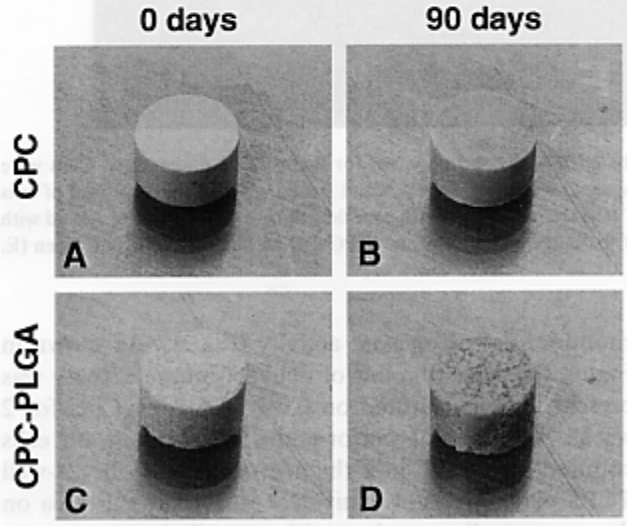

Fig. 5. Aged and non-aged CPC and CPC–PLGA discs were photographed. CPC and CPC–PLGA discs were prepared as described in the Materials and methods and either photographed without being aged (Panels A and C) or photographed after 90 days of aging in cell media in a cell incubator (Panels B and D). The discs are 6.4 mm in diameter.

Discussion

The cell culture studies suggest that our composite bone graft consisting of PLGA microspheres and CPC (CPC–PLGA) is biocompatible. During overnight incubations, osteoblast-like cells (MC3T3-E1) were able to adhere, spread and remain viable on CPC–PLGA, CPC or TCPS when observed by either fluorescence microscopy (Fig. 1) or ESEM (Fig. 2). After 2-week cultures, fluorescence microscopy (Fig. 3) and the quantitative Wst-1 assay (Fig. 4) showed that cell adhesion, proliferation and viability were equivalent on each of the substrates. Taken together, these in vitro cell culture results suggest that CPC–PLGA is biocompatible.

Our new composite bone graft, CPC–PLGA, is formed from a mix of CPC powder, PLGA microspheres and water. Specifically, four parts by volume of CPC powder (0.15 g or 50 μl; density = 3.02 g/ml) were mixed with six parts by volume of PLGA microspheres (0.10 g or 75 μl; density = 1.34 g/ml; 180–360 μm in diameter). The mixture of CPC powder was then mixed with 63 μl of water to make the specimens used in the present study. Since water is added, the final volume fraction of PLGA microspheres in the set CPC–PLGA specimens will not be exactly 0.6. Thus, the dimensions of CPC–PLGA specimens were measured to determine the final volume fraction of PLGA microspheres in the cured CPC–PLGA specimens. The average volume of a CPC–PLGA specimen prepared as described above (also in Materials and methods) was 141 μl. Assuming that the PLGA microspheres do not change their volume from 75 μl during the setting of the CPC–PLGA specimens, they occupy 53% of the final volume of the set CPC–PLGA specimens (0.53 volume fraction). CPC

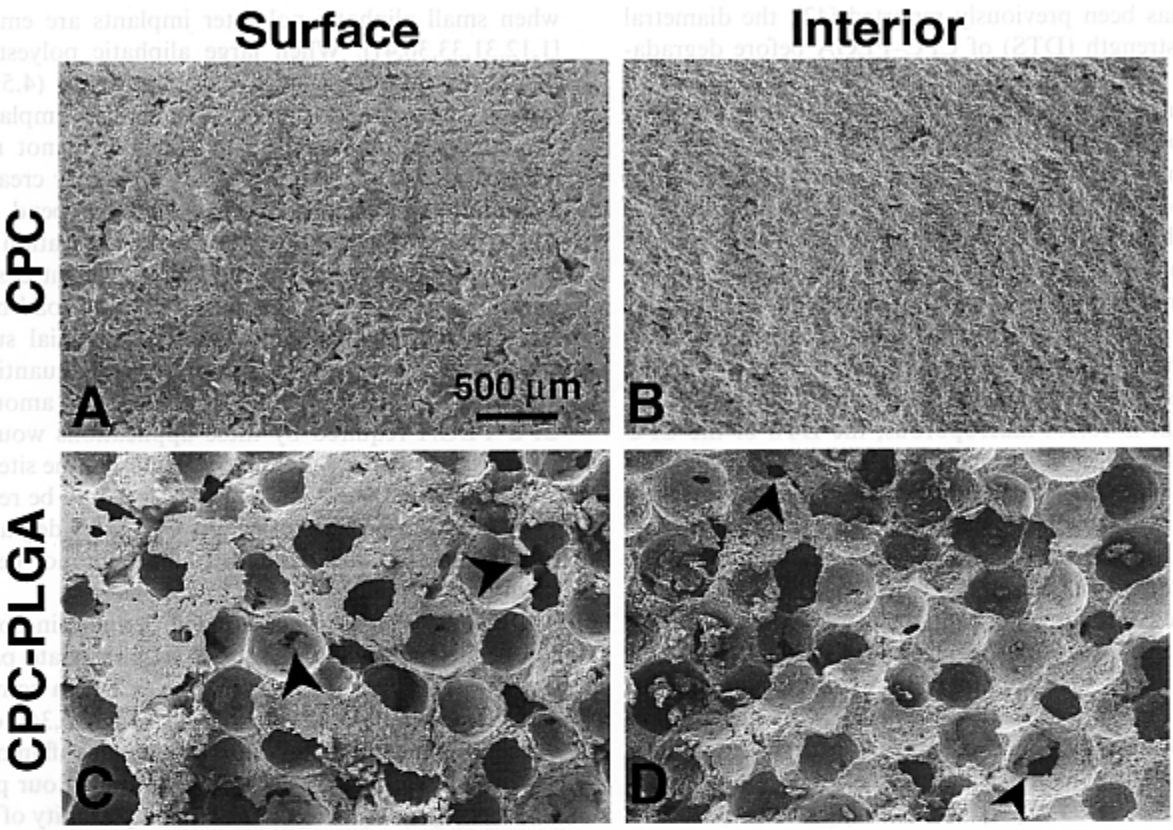

Fig. 6. The surface and interior of aged CPC or CPC–PLGA discs were examined by SEM. CPC and CPC–PLGA discs were prepared as described in the Materials and methods and aged for 90 days in cell media in a cell incubator. Ninety days was enough time for the PLGA microspheres contained in the CPC–PLGA discs to completely degrade. The samples were dehydrated, sputter-coated with gold and observed by SEM (15 kV, 50 µA, 50× magnification). The surface and interior of an aged CPC disc are shown in Panels A and B, respectively. The surface and interior of an aged CPC–PLGA disc are shown in Panels C and D, respectively.

itself is 38.6% microporous by volume [40], which means that the set CPC–PLGA is 53% by volume of PLGA microspheres, 18.0% by volume of micropores and 29% by volume of CPC.

When PLGA microspheres occupy 53% of the volume of a CPC–PLGA specimen, they should form infinitely long chains of connected particles, i.e. the macropores formed from degradation of the PLGA microspheres should be fully interconnected. Theoretical calculations and earlier studies have shown that spheres included in a solid matrix become fully interconnected when the volume fraction of the included spheres reaches 0.4 [15,20]. However, the SEMs of CPC–PLGA discs that were aged to allow complete degradation of the PLGA microspheres revealed that the macropores are only *partially* interconnected (Fig. 6). It appears that the CPC paste wets the contacts between the PLGA microspheres and then sets to form a thin shell of hydroxyapatite between the microspheres. In many cases the PLGA microspheres have broken through their CPC 'shell' to make direct contact with neighboring microspheres and have formed interconnections (see arrowheads in Figs. 6C and D). Conversely, in many cases the macropores formed by the degradation of the PLGA microspheres appear to be separated from neighboring macropores by this 'shell' of hydroxyapatite. Thus, the macropores formed by degradation of the PLGA microspheres create a partially interconnected network of macropores.

The lack of complete interconnectivity of the macropores might slow down the ingrowth of regenerative bony tissue into the CPC–PLGA implant in vivo. However, it may be that the thin shell of hydroxyapatite that separates many of the macropores from one another would be rapidly resorbed by osteoclasts after implantation to allow bony ingrowth.

Although the macropores formed by degradation of the PLGA microspheres do not form a fully interconnected network, CPC itself contains an interconnected microporous network (38.6% by volume [40] and ≈2 nm in diameter [7,39]) that allows small molecules, such as methylene blue, to penetrate into the cement [22]. The presence of these interconnected micropores must have allowed water to penetrate into the CPC–PLGA to facilitate the degradation of the PLGA microspheres in the interior of the specimens (Fig. 6D).

As has been previously reported [42], the diametral tensile strength (DTS) of CPC–PLGA before degradation of the PLGA microspheres (6.4 MPa; S.D. = 0.4 MPa; $n = 5$) is less than the DTS of CPC without microspheres (10.4 MPa; S.D. = 0.2 MPa; $n = 5$) [9]; however, strength is being sacrificed in order to impart macroporosity to the cement. In contrast, the DTS of CPC–PLGA (6.4 MPa before degradation of the PLGA microspheres), which has a 0.53 volume fraction of PLGA microspheres, is greater than the DTS of macroporous CPC formed by dissolution of water-soluble porogens such as sucrose or mannitol. When sucrose particles (125–250 μm) are used as a porogen to make CPC that is 18.9% macroporous, the DTS of the CPC after dissolution of the sucrose is 3.7 MPa (S.D. = 0.17; $n = 3$) [40]. When mannitol particles (100–400 μm) are used as a porogen to make CPC that is 29% macroporous, the DTS of the CPC after dissolution of the mannitol is 3.5 MPa (S.D. = 0.18; $n = 5$) [28,29]. We computed simultaneous confidence intervals comparing the DTS of each material [CPC, CPC–sucrose (after dissolution of sucrose) and CPC–mannitol (after dissolution of mannitol)] to CPC–PLGA (before degradation of PLGA) using the Bonferroni t test (based on the Bonferroni inequality) which guarantees a global confidence level of 95% [14]. This analysis confirmed that CPC was stronger than CPC–PLGA before degradation of the PLGA microspheres ($P < 0.05$). This analysis also confirmed that CPC–PLGA (before degradation of the PLGA microspheres) was stronger than CPC–sucrose and CPC–mannitol ($P < 0.05$) after dissolution of the sucrose or mannitol in the test of DTS [CPC > CPC–PLGA > (CPC–sucrose or CPC–mannitol)].

It is important to note that the DTS of the CPC containing these water-soluble porogens was measured after dissolution of the porogens while the DTS of the CPC–PLGA was determined before the PLGA had degraded. The water-soluble porogens would be expected to dissolve within several hours [28,29,40] while PLGA would be expected to degrade within 90 days (Figs. 5 and 6). However, the rationale for including PLGA microspheres as a porogen in the cement was to increase the *initial* mechanical strength of the cement with the expectation that osteoblasts could colonize and strengthen (through deposition of bony matrix) the cement as the PLGA microspheres degrade. Thus, the initial mechanical strength of CPC–PLGA is better than the mechanical strength of macroporous CPC formed by solvent leaching of water-soluble porogens.

As PLGA degrades by random hydrolysis of its polymer chains [3], the protons released can cause a local increase in acidity. Acidity could potentially cause inflammation or tissue necrosis if the capacity to clear degradation products at the site of the implant was not adequate. However, this is generally not a problem since clearance is generally sufficient to prevent this outcome when small aliphatic polyester implants are employed [1,12,31,33,36,41]. When large aliphatic polyester implants are employed, such as bone screws (4.5 mm × 30 mm), a sterile sinus may form over the implant, yet the outcomes are usually positive and do not require removal [3,6,32]. Thus, the effects of acidity created by degradation of aliphatic polyesters will depend on the size of the implant and clearance at the location of the implant. The composite CPC–PLGA cement described in the current study is designed for non-load-bearing applications, such as dental and craniofacial surgery, which generally would not require 'bulk' quantities of implant material. Hence, the relatively small amounts of CPC–PLGA required by these applications would not be expected to pose the threat of acidity at the site of the implant. Nevertheless, animal studies would be required to determine the effects of acidity caused by degradation of CPC–PLGA since wound drainage cannot be mimicked in vitro.

Other groups have constructed 'mineral-in-polymer' composites consisting of a calcium phosphate particulate dispersed in a polymer matrix and each composite was biocompatible in vitro [4,11,25,26,30,34] or enhanced bone growth in vivo without an inflammatory response [2,16,24,46]. These studies support our present findings regarding the in vitro biocompatibility of CPC–PLGA and they also hold promise that our formulation may be osteoconductive. Nevertheless, our CPC–PLGA differs from these 'mineral-in-polymer' composites in that ours is the first 'polymer-in-mineral' formulation where a polymer particulate (PLGA microspheres) is dispersed in a calcium phosphate matrix (CPC).

In summary, our CPC–PLGA cement is a moldable, resorbable, composite of a self-setting CPC and PLGA microspheres. Although our CPC–PLGA cement has not been tested in a clinical setting, the CPC–PLGA has been designed to form a paste when mixed with water which could be shaped to fit bone defects. In addition, the cement is designed such that the PLGA microspheres could initially stabilize the graft but then should degrade after implantation to form macropores for bony ingrowth (bony ingrowth remains to be tested). We have shown here that our new CPC–PLGA cement formulation can support the adhesion, spreading, proliferation and viability of osteoblast-like cells for up to 2 weeks in vitro. These studies suggest that the composite CPC–PLGA is biocompatible and can serve as a baseline for future work from which we can improve the cement.

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