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Strong and bioactive composites containing nano-silica-fused whiskers for bone repair

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Abstract

Self-hardening calcium phosphate cement (CPC) sets to form hydroxyapatite with high osteoconductivity, but its brittleness and low strength limit its use to only non-stress bearing locations. Previous studies developed bioactive composites containing hydroxyapatite fillers in Bis-GMA-based composites for bone repair applications, and they possessed higher strength values. However, these strengths were still lower than the strength of cortical bone. The aim of this study was to develop strong and bioactive composites by combining CPC fillers with nano-silica-fused whiskers in a resin matrix, and to characterize the mechanical properties and cell response. Silica particles were fused to silicon carbide whiskers to roughen the whisker surfaces for enhanced retention in the matrix. Mass ratios of whisker:CPC of 1:2, 1:1 and 2:1 were incorporated into a Bis-GMA-based resin and hardened by two-part chemical curing. Composite with only CPC fillers without whiskers served as a control. The specimens were tested using three-point flexure and nano-indentation. Composites with whisker:CPC ratios of 2:1 and 1:1 had flexural strengths (mean $\pm$ SD; $n=9$) of (164 ± 14) MPa and (139 ± 22) MPa, respectively, nearly 3 times higher than (54 ± 5) MPa of the control containing only CPC fillers ($p<0.05$). The strength of the new whisker-CPC composites was 3 times higher than the strength achieved in previous studies for conventional bioactive composites containing hydroxyapatite particles in Bis-GMA-based resins. The mechanical properties of the CPC-whisker composites nearly matched those of cortical bone and trabecular bone. Osteoblast-like cell adhesion, proliferation and viability were equivalent on the non-whisker control containing only CPC fillers, on the whisker composite at whisker:CPC of 1:1, and on the tissue culture polystyrene control, suggesting that the new CPC-whisker composite was non-cytotoxic.
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Keywords: Bioactive composite; Nano-silica whisker fusion; Calcium phosphate cement; Strength; Elastic modulus; Bone repair

1. Introduction

Hydroxyapatite has been used for hard tissue repair because of its chemical and crystallographic similarity to the carbonated apatite in human teeth and bones [1–3]. Several calcium phosphate cements can self-harden to form hydroxyapatite in the bone cavity [4–7]. These moldable calcium phosphate cements avoid the problem of sintered hydroxyapatite implants that require the surgeon to fit the surgical site around the implant or to carve the implant to the desired shape [8]. One calcium phosphate cement, designated as CPC [4], consists of a mixture of tetracalcium phosphate [TTCP:$Ca_4(PO_4)_2O$] and dicalcium phosphate anhydrous (DCPA: $CaHPO_4$). CPC sets in an aqueous environment to form a solid consisting of nano-sized hydroxyapatite crystals with a diameter of approximately 50 nm and a length of about 300 nm [4,9] X-ray diffraction showed that hydroxyapatite was the only product formed in the set CPC [4,10]. Due to its excellent osteoconductivity, biocompatibility and bone-bonding ability, CPC has been used in a number of orthopaedic and dental procedures [11–16]. Unfortunately, the relatively low strength and susceptibility to brittle fracture of CPC have severely limited its use to non-load-bearing applications [11–15].

Previous studies have used hydroxyapatite particles and other bioactive materials as fillers in polymer-based bone cements to improve the mechanical properties [17–21]. Bis-GMA (bisphenol-*a*-glycidyl methacrylate)

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resin composites have been used as dental restorative materials [22,23]. Previous studies have added bioactive fillers into the resin matrix and imparted bioactivity to Bis-GMA-based composites for bone repair applications [24–27]. Studies have shown that Bis-GMA-based resins containing hydroxyapatite particles possessed flexural strength values ranging from about 40 to 60 MPa [28–30]. These flexural strength values, while being 4–6 times higher than that for pure CPC [31], were still lower than the 100–200 MPa reported for cortical bone [32,33].

Nano-sized silica particles were recently fused onto ceramic whiskers and used as fillers to reinforce Bis-GMA-based dental resin to extend their use to large stress-bearing restorations [23,34]. The whiskers possessed a high structural perfection resulting in superior strength values. While plain whiskers did not significantly strengthen the resin matrix, nano-silica fusion roughened the whisker surfaces and enhanced whisker retention in the matrix, resulting in strong composites with substantially increased strength and toughness [23,34].

In the present study, strong and bioactive composites were developed via the combination of pre-hardened bioactive CPC particles and nano-silica-fused whiskers in a Bis-GMA resin. Flexural and nano-indentation tests were used to characterize the mechanical properties of the composites. Because cell culture toxicity assays are the international standard for the initial screening of materials for biocompatibility [35], in-vitro cell culture was performed to evaluate the biocompatibility of the composites. It was hypothesized that combining bioactive CPC fillers and whisker fillers would render the composite significantly stronger than the composite containing only CPC fillers. It was further hypothesized that the composite containing CPC and whiskers would be as non-cytotoxic as the composite containing only CPC fillers.

2. Methods

2.1. Calcium phosphate cement (CPC) fillers

The powder used to make the CPC specimens consisted of a mixture of TTCP and DCPA with molar ratio of 1:1 [4]. The TTCP powder was synthesized from a solid-state reaction between $CaHPO_4$ and $CaCO_3$ (Baker Chemical Company, NJ), then ground and sieved to obtain TTCP particle sizes of 1–80 μm, with a median particle size of 17 μm. The DCPA powder was ground and sieved to obtain particle sizes of 0.4–3 μm, with a median particle size of 1 μm. The TTCP and DCPA powders were mixed in a blender (Dynamics Corporation of America, New Hartford, CT) to form the CPC powder, which was then blended manually by spatulation with distilled water to form a paste at a powder:water mass ratio of 3:1. The paste was placed into a $3 \times 4 \times 25$ mm mold sandwiched between two glass slides [9]. The assembly was incubated in a humidor at 100% relative humidity at 37°C for 4 h [36]. The hardened CPC was then demolded and immersed in distilled water at 37°C for 20 h. This resulted in hydroxyapatite being the only final reaction product of CPC [10]. The set CPC was manually ground by mortar and pestle into a powder having particle sizes ranging from about 2 to 15 μm with a mean of approximately 5 μm [31]. This powder, designated as CPC fillers, was silanized by mixing it with 4% mass fraction of 3-methacryloxypropyltrimethoxysilane and 2% mass fraction of *n*-propylamine in cyclohexane using a rotary evaporator in a 90°C water bath until dry [34,31]. The purpose of the silanization was to bond the fillers to the resin matrix [23]. Previous studies showed that silanization did not suppress the bioactivity of the hydroxyapatite fillers [17].

2.2. Nano-silica-fused whiskers

Silicon carbide whiskers (Advanced Refractory Technologies, Buffalo, NY) were used with diameters ranging from 0.1 to 3 μm with a mean of approximately 0.9 μm, and lengths ranging from about 2 to 100 μm with a mean of 14 μm. The whiskers were mixed with nano-sized silica having a particle size of approximately 80 nm (Aerosil OX50, Degussa, Ridgefield, NJ) at a whisker:silica mass ratio of 5:1 in ethyl alcohol stirred with a magnetic bar on a hot plate until dry. The purpose of this mixing was to disperse and separate the whiskers to prevent entanglement. The dried mixture was then heated in a furnace in air for 30 min at 800°C [23,34]. The purpose of the heating was to fuse the nano-silica particles onto the whiskers to facilitate silanization and enhance the whisker retention in the matrix by providing rougher whisker surfaces [23,34]. The nano-silica-fused whiskers were silanized in the same manner as for the CPC fillers. The silanized nano-silica-fused whiskers are hereinafter referred to as "whiskers".

2.3. Specimen fabrication

To study the effects of whisker:CPC ratio, the whiskers were mixed with CPC fillers at whisker:CPC filler mass ratio of 1:2, 1:1, and 2:1. Each filler powder was blended by spatulation with a resin monomer consisting of mass fractions of 48.975% Bis-GMA, 48.975% TEGDMA (triethylene glycol dimethacrylate), 0.05% 2,6-di-*tert*-butyl-4-methylphenol (BHT), and 2% benzoyl peroxide (BPO) to form paste one, the initiator paste, of a two-part chemically activated composite [23,34]. The filler level, i.e. (whisker + CPC)/(whisker + CPC + resin), was fixed as 60% mass fraction for all the composites [23]. Paste two, the accelerator paste,

consisted of the same amount of powder mixed with a resin made of mass fractions of 49.5% Bis-GMA, 49.5% TEGDMA, and 1% *N*,*N*-dihydroxyethyl-*p*-toluidine (DHEPT) as the polymerization accelerator [23,34]. Equal masses of the two pastes were blended by spatulation and filled into a $2 \times 2 \times 25$ mm mold. Each specimen was chemically cured at 37°C for 15 min, demolded, and immersed in distilled water at 37°C for 24 h prior to testing [23,34]. The composite with whisker:CPC ratio of 0:1 was also fabricated using the same filler mass fraction of 60% to serve as a control which contained hydroxyapatite (CPC fillers) in the resin matrix without whiskers.

2.4. Flexural testing

A standard three-point flexural test [37] with a span of 10 mm was used to fracture the specimens at a crosshead speed of 1 mm/min on a computer-controlled Universal Testing Machine (model 5500R, Instron Corp., MA) [23,34]. The flexural strength and work-of-fracture (the energy required to fracture the specimen, obtained from the area under the load–displacement curve divided by the specimen's cross-sectional area) were measured [38].

2.5. Nano-indentation

A nano-indentation system (Nano Instruments, Knoxville, TN) with a diamond Berkovich indenter was used to produce the indentations [39,40]. The indentation loads and the corresponding displacements were recorded continuously throughout a loading–unloading cycle, enabling the measurement of the elastic modulus of the indented specimen. The calculation of hardness and elastic modulus was made according to a method described previously [39]. The method involves the extrapolation of a tangent to the top of the unloading curve to determine the depth (a combination of elastic and plastic displacement) over which the indenter tip is in contact with the specimen at the maximum load, P_{max}. This depth, and the knowledge of the indenter geometry, gives the contact area, A; hardness H then follows directly from [39,41]: $H=P_{max}/A$. The slope of the unloading curve also provides a measure of the contact stiffness, which can be used with the contact area to determine the elastic modulus [39,41]. Indentations were made in the composites with whisker:CPC ratios of 1:2, 1:1, and 2:1 and also in the control composite with only CPC fillers. Six indentations were made for each composite. P_{max} of 1 N was used to yield an indentation contact area of about 1500 $(\mu m)^2$ to 2400 $(\mu m)^2$, depending on the hardness of the material. This method ensures that the measured hardness and elastic modulus approximate those of the composite bulk, rather than the resin phase or filler particles.

2.6. Cell culture and fluorescence microscopy

Established protocols for the culture and passage of MC3T3-E1 cells were followed [21]. 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 modified Eagle's minimum essential medium (Biowhittaker, Walkersville, MD). The medium was supplemented with 10% (volume fraction) fetal bovine serum (Gibco, Rockville, MD) and kanamycin sulfate (Sigma, St. Louis, MO). The medium was changed twice weekly, and the cultures were passaged with 2.5 g/l trypsin (0.25% mass fraction) containing 1 mmol/l ethylenediaminetetraacetic acid (EDTA, Gibco, Rockville, MD) once per week. Cultures of 90% confluent MC3T3-E1 cells were trypsinized, washed and suspended in fresh media. Composite specimens of dimensions of approximately $2 \times 2 \times 8$ mm were horizontally laid one each on the bottom of a tissue culture polystyrene well of a 24-well plate (BD Biosciences, Bedford, MA). Five specimens for each of three materials were tested: bioactive composite with a whisker:CPC ratio of 1:1; the non-whisker control composite containing only CPC fillers; and the tissue culture polystyrene control (or "TCPS control"). All the specimens were autoclaved at 121°C for 20 min prior to cell culture studies. Fifty thousand cells diluted into 2 ml of media were added to each well containing the specimen, or to an empty well in the case of TCPS control, incubated for 1 d or 14 d (2 ml of fresh media every 2 d).

After 1 d or 14 d, the media was removed and the cells were washed with 1 ml fresh media. Cells were then stained and viewed by epifluorescence microscopy (Eclipse TE300, Nikon, Melville, NY). Staining of cells was done for 5 min 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). 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 composites allows simultaneous examination of both live and dead cells on the specimens.

2.7. Wst-1 cell viability quantification

Cells grown on the specimens for 14 d were analyzed for cell viability using the Wst-1 assay which measures mitochondrial dehydrogenase activity [42]. Wst-1 refers

to 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (Dojindo, Gaithersburg, MD). Specimens with cells were transferred to clean wells in a 24-well plate and rinsed with 1 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). 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-5-methylphenazinium methylsulfate in water) were added to each well and incubated at 37°C for 2 h. Blank wells were also prepared that contained only buffer and Wst-1 solution. After 2 h, 0.2 ml of each reaction mixture was transferred to a 96-well plate and the absorbance at 450 nm, which was related to the mitochondrial dehydrogenase activity, was measured with a microplate reader (Wallac 1420 Victor^2, PerkinElmer Life Sciences, Gaithersburg, MD). The assay was performed on five wells for the whisker composite at whisker:CPC of 1:1, and on five wells for the non-whisker control containing only CPC fillers. The assay was also performed on 5 blank wells, and the absorbance for the blank wells was subtracted from the data for the composites.

2.8. SEM and statistical analysis

Scanning electron microscopy (SEM, model JSM-5300, JEOL, Peabody, MA) was used to examine gold-sputtered specimens. Cells cultured for 1 d on specimens were rinsed with saline, fixed with 1% glutaraldehyde, subjected to graded alcohol dehydrations, rinsed with hexamethyldisilazane, and then sputter coated with gold. One standard deviation was given in this paper for comparative purposes as the estimated standard uncertainty of the measurements. These values should not be compared with data obtained in other laboratories under different conditions. One-way ANOVA was performed to detect significant differences in data. Tukey's Multiple Comparison procedures were used to compare the data at a family confidence coefficient of 0.95.

3. Results

Fig. 1 shows SEM micrographs of the nano-silica-fused whiskers after fusion at 800°C. In (A), the nano-sized silica particles were indicated by the arrow on the whisker surface. Some areas of the whisker surfaces were relatively densely coated with the silica particles (e.g., A), while other areas were less densely coated (e.g., B). Some clustering of the silica particles also occurred when fused on the whiskers.

Fig. 2 shows flexural strength and work-of-fracture of the bioactive composites at different whisker:CPC ratios. Each value is the mean of nine measurements, with the error bar showing one standard deviation (SD). One-way ANOVA identified significant effects of whisker:CPC ratio on strength and work-of-fracture ($p<0.001$). The bioactive composites with whisker:CPC ratios of 2:1 and 1:1 had flexural strengths (mean $\pm$ SD; $n=9$) of (164 ± 14) MPa and (139 ± 22) MPa, respectively, not significantly different from each other; the strength at 2:1 ratio was significantly higher than (122 ± 13) MPa at whisker:CPC = 1:2 (Tukey's multiple comparison test; family confidence coefficient = 0.95). All the three whisker-containing bioactive composites had strengths significantly higher than (54 ± 5) MPa of the non-whisker control containing only CPC fillers ($p<0.05$). The work-of-fracture was also significantly increased with the addition of whiskers. While the

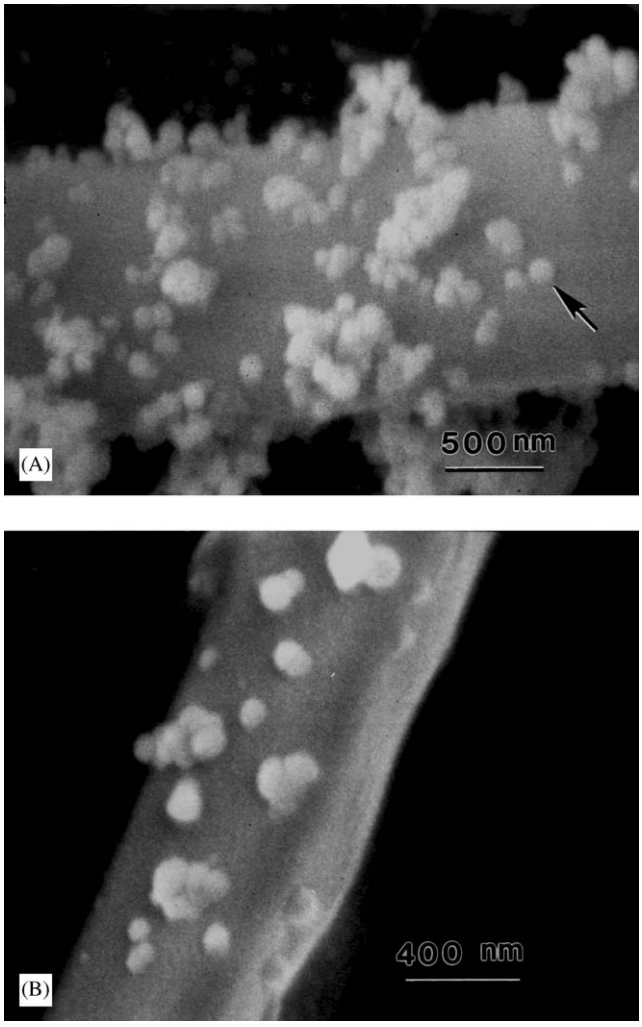

Fig. 1. SEM of nano-sized silica particles thermally fused onto the whisker surfaces to roughen the whiskers for enhanced retention in the resin matrix. (A) An area of whisker surface relatively densely coated with silica particles. Arrow indicates the nano-sized silica particles on the whisker surface. (B) An area of whisker surface less densely coated with silica particles.

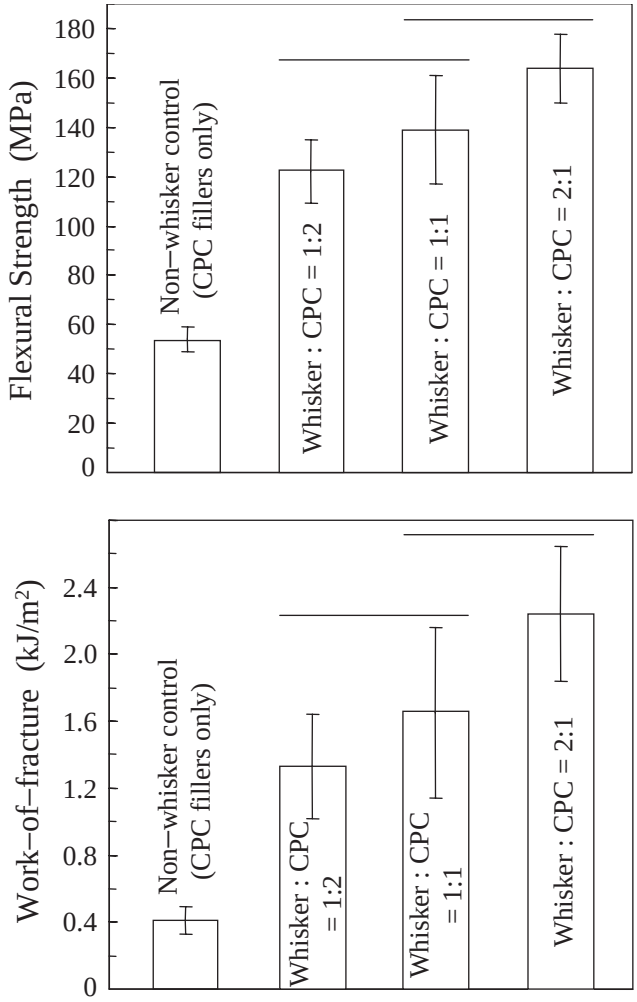

Fig. 2. Flexural strength and work-of-fracture of the bioactive composites at different whisker:CPC ratios. Each value is the mean of nine measurements, with the error bar showing one standard deviation (mean ± SD; $n = 9$). Horizontal lines indicate values that are not significantly different (Tukey's multiple comparison test; family confidence coefficient = 0.95). The strength was increased by 3-fold with the addition of whiskers, while the work-of-fracture was increased by 4- to 5-fold, over the non-whisker control.

increase in strength was about 3-fold, the increase in work-of-fracture was 4–5 times over that of the non-whisker control.

SEM micrographs of fracture surfaces are shown in Fig. 3 for (A) non-whisker control, (B) composite at whisker:CPC = 1:1, and (C) composite at whisker: CPC = 2:1. The fracture surface of the non-whisker control was relatively flat, typical for brittle materials. In contrast, the fracture surfaces of the whisker-containing composites were noticeably rougher, with whisker pull-out (upper arrow in B) and fracture steps (lower arrow in B). CPC particles were observed on the fracture surfaces, an example of which is indicated by the right arrow in (C), while a whisker is indicated by the left arrow.

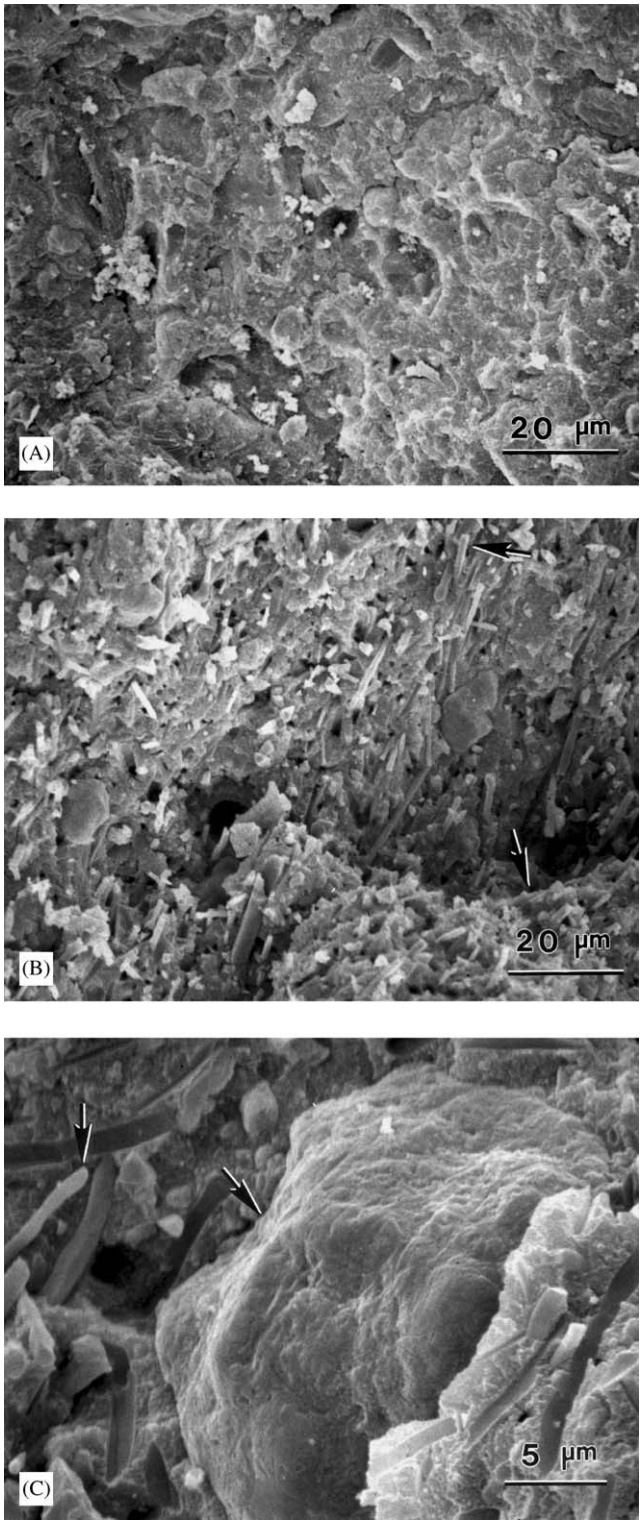

Fig. 3. The specimens were fractured in three-point flexure and the fracture surfaces were examined in the SEM: (A) non-whisker control, (B) composite at whisker:CPC = 1:1, and (C) composite at whisker: CPC = 2:1. The non-whisker control had relatively flat fracture surfaces, typical for brittle materials. The whisker composites had rough fracture surfaces, with whisker pullout (upper arrow in B) and fracture steps (lower arrow in B), indicating fracture resistance and energy dissipation. The right arrow in (C) indicates an example of a CPC particle, and the left arrow indicates a whisker.

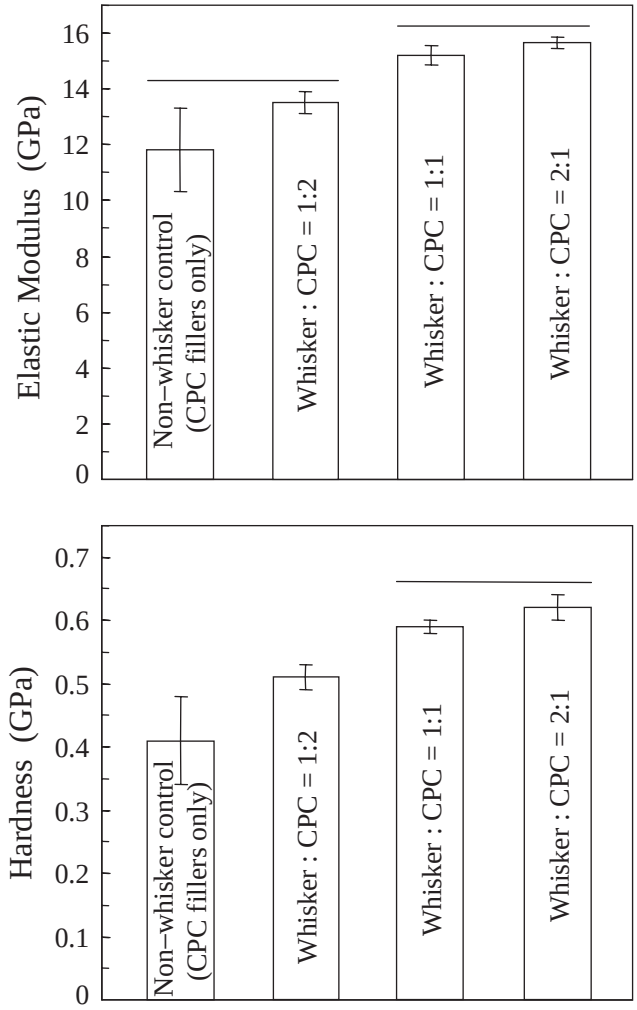

Fig. 4. Elastic modulus and hardness of the composites measured by nano-indentation. Each value is the mean of six measurements with the error bar showing one standard deviation. Both the elastic modulus and hardness significantly increased with the addition of whiskers. Horizontal lines indicate values that are not significantly different (Tukey's multiple comparison test; family confidence coefficient = 0.95).

Elastic modulus and hardness measured by nano-indentation are plotted in Fig. 4. The elastic modulus increased with the addition of whiskers, from (11.8 ± 1.5) GPa for the non-whisker control to a significantly higher value of (15.2 ± 0.4) GPa at whisker:CPC = 1:1. The hardness increased from (0.41 ± 0.07) GPa for the non-whisker control to a significantly higher value of (0.59 ± 0.01) GPa at whisker:CPC = 1:1 (Tukey's multiple comparison test; family confidence coefficient = 0.95).

Cells cultured for 1 d on the non-whisker control containing only CPC fillers, the whisker composite at whisker:CPC = 1:1, and the TCPS control were viewed with fluorescence microscopy and shown in Fig. 5. The live cells (stained green) appeared to have adhered and attained a normal, polygonal morphology when seeded on all three materials. Visual examination indicated that the density of live cells adherent to each material appeared to be similar. All three materials had very few dead cells (stained red).

Fig. 6 shows SEM micrographs of cells cultured for 1 d. In (A), cells attached on the surface of composite with whisker:CPC = 1:1. Arrows in (A) point to examples of cells well spread on and attached to the specimen surface. Cell spreading is shown at a higher magnification in (B) on composite with whisker:CPC = 1:1. Long cytoplasmic extensions are shown in (C) on TCPS control. The lengths of cytoplasmic extensions were observed to range from about 20 to 60 μm. The cytoplasmic extensions are regions of the cell plasma membrane that contain a meshwork or bundles of actin-containing microfilaments, which permit the movement of the migrating cells along a substratum [43]. Cell–cell interactions on the non-whisker control containing only CPC fillers are shown in (D). Arrows in (D) indicate cell–cell adhesion and junctions that have been shown to consist of clusters of cell adhesion molecules [43]. These features were observed to be similar on all the three materials tested. Therefore, after 1 d culture, cell adhesion and viability on the whisker composite were similar to that on the non-whisker control and on the TCPS control.

Fig. 7 shows cells cultured for 14 d on the composite with whisker:CPC = 1:1, the non-whisker control, and the TCPS control. The live cells (stained green) appeared to have formed a confluent monolayer on these materials by 14 d. The three materials appeared to have a similar live cell density, indicating that cells proliferated equally well on them. The density of live cells at 14 d was much greater than the density of live cells at 1 d (Fig. 5), showing that the cells had greatly proliferated. Dead cells (stained red) were few on all three materials at 14 d. These results suggest that cell proliferation and viability after 2 weeks of culture on the whisker composite were similar to those of the non-whisker control and the TCPS control, demonstrating that the whisker-CPC composite was as biocompatible as the TCPS control and the non-whisker control containing only CPC fillers. The cell viability was quantitatively assessed at 14 d using the Wst-1 assay and the results are plotted in Fig. 7E. The Wst-1 assay on cells cultured on TCPS was not performed because the growth area of the 24-well TCPS plates was not equivalent to the growth area on the composite specimens and would not allow an accurate comparison. The amount of dehydrogenase activity in cells cultured on the non-whisker control containing only CPC fillers and the whisker composite at whisker:CPC = 1:1 was not significantly different (Student's t; $p > 0.1$). This is consistent with the live and dead cell examinations showing that the whisker-CPC composite was as non-cytotoxic as the non-whisker control containing only CPC fillers.

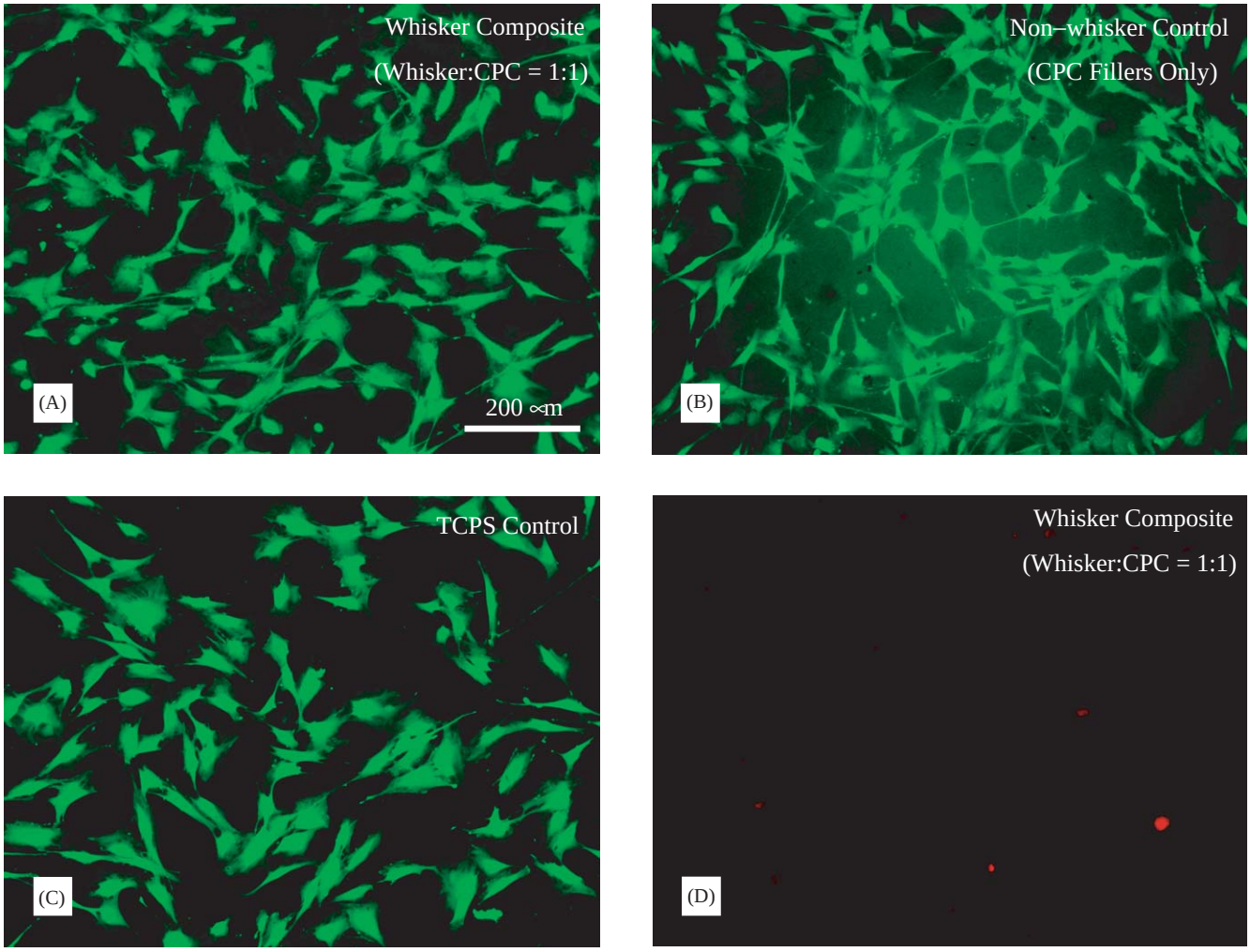

Fig. 5. Fluorescence microscopy of osteoblast-like cells cultured for 1 d. (A) Live cells on the whisker composite at whisker:CPC = 1:1. (B) Live cells on the non-whisker control containing only CPC fillers. (C) Live cells on the tissue culture polystyrene control (or "TCPS control"). (D) Dead cells on the whisker composite at whisker:CPC = 1:1. The live cells (green) appeared to have adhered and attained a normal, polygonal morphology on all three materials. The dead cells were stained red and there were very few dead cells on all three materials.

4. Discussion

Hardened CPC forms nano-crystalline hydroxyapatite and has excellent biocompatibility and osteoconductivity [4,11]. As a result, CPC has been used in a number of orthopaedic and dental procedures, including the reconstruction of frontal sinus and the augmentation of craniofacial skeletal defects [12–14]. However, its brittleness and low strength have severely limited its use to only non-load-bearing applications [11,14]. One way to overcome this weakness may be to use pre-hardened CPC fillers in a resin matrix. In the present study, the control composite containing CPC fillers in a resin matrix possessed a flexural strength of 54 MPa, much higher than the 10 MPa for pure CPC without whiskers or fibers reported in previous studies [38]. The flexural strength of 54 MPa is consistent with previous studies on bioactive composites with hydroxyapatite particles in Bis-GMA-based resins [28–30]. In the present study, combining the bioactive CPC fillers with strong silica-fused whisker resulted in novel composites with flexural strength reaching 120–160 MPa. These strength values were 3 times higher than the strength achieved in previous studies for bioactive composites containing hydroxyapatite particles in Bis-GMA-based resins [28–30], and they approached the flexural strength of 100–200 MPa for cortical bone [32,33].

SEM examinations appeared to indicate the reinforcement mechanisms to be whiskers deflecting and bridging the cracks, and friction from whiskers being pulled out of the matrix resisting crack propagation. The high strength and toughness of the whiskers contributed to the reinforcement efficacy, because strong whiskers are less likely to be broken during crack deflection and during frictional pullout of the whiskers. The strength (about 50 GPa, an order of

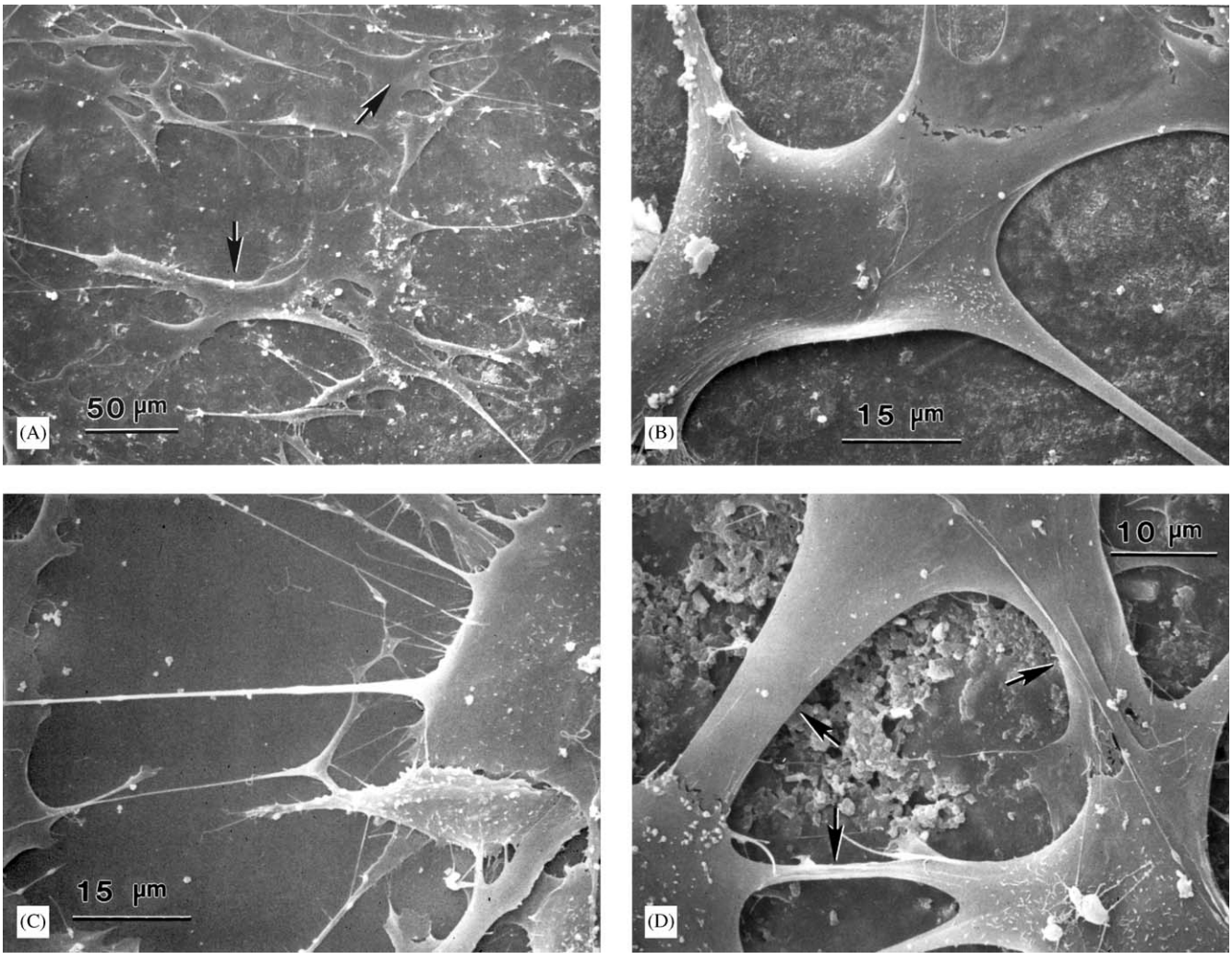

Fig. 6. SEM examination of cells cultured for 1 d. Cell adhesion and spreading were observed to be similar on all three materials: whisker composite at whisker:CPC = 1:1, non-whisker control with only CPC fillers, and TCPS control. Examples are shown in the SEM micrographs. (A) Cell attachment on the surface of composite with whisker:CPC = 1:1. (B) Cell spreading on composite with whisker:CPC = 1:1. (C) Cytoplasmic extensions on TCPS control. (D) Cell–cell interactions on the non-whisker control containing only CPC fillers.

magnitude greater than that of glass fibers) and fracture toughness (approximately 2.5 MPa · $m^{1/2}$, 3 times that of glass fibers) [44,45] of the whiskers are likely responsible for the high strength of the CPC-whisker containing composites.

Besides matching the flexural strength of cortical bone, the elastic modulus and hardness of the whisker-CPC composites also approximated those of bone. The elastic modulus of cortical bone was measured by nano-indentation to range from 16.6 to 25.7 GPa; that of trabecular bone ranged from 13.4 to 19.4 GPa [46,47]. In the present study, the non-whisker control containing bioactive CPC fillers had a lower elastic modulus of 11.8 GPa. However, the whisker composite at whisker: CPC of 1:1 had an elastic modulus of 15.2 GPa, within the range for trabecular bone and approaching the lower end for cortical bone. Previous studies reported that the nano-indentation hardness of cortical bone ranged from 0.56 to 0.74 GPa, and the hardness of trabecular bone ranged from 0.52 to 0.62 GPa [46,47]. In the present study, the non-whisker control had a lower hardness of 0.41 GPa. The hardness of whisker-CPC composites ranged from 0.51 to 0.66 GPa, overlapping those for trabecular bone and cortical bone. Natural bone consists of collagen fibers, hydroxyapatite crystals and other organic tissues. The bioactive composite of the present study contained fibers (silica-fused whiskers) and hydroxyapatite (CPC fillers) in a polymeric matrix. These novel composites may be more biomimetic than previous composites containing only hydroxyapatite fillers in a polymer matrix without a fiber phase. Further studies should incorporate collagen fibers into the CPC composite and examine the effects on mechanical properties as well as on cell attachment and cell viability.

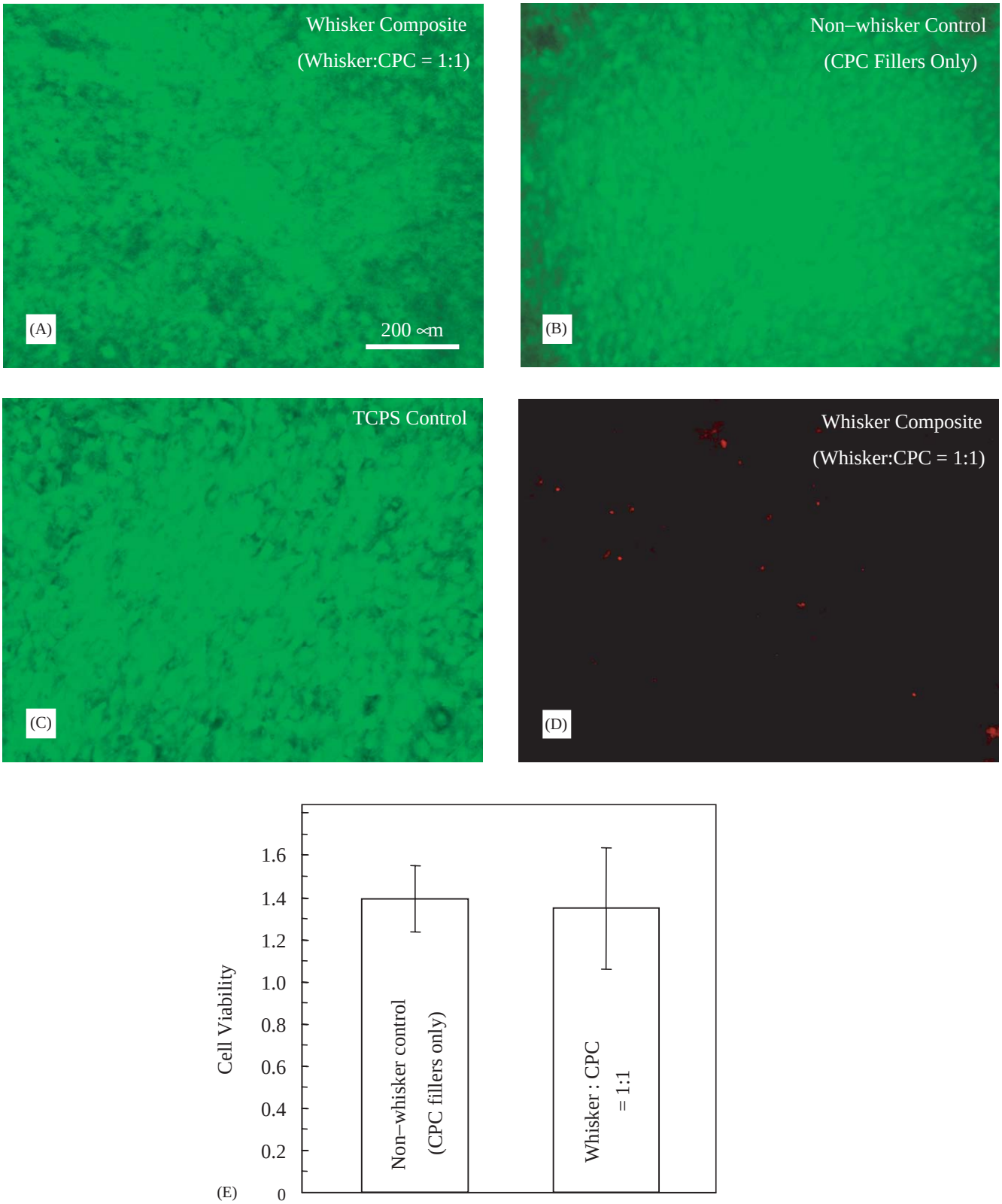

Fig. 7. Fluorescence microscopy of cells cultured for 14 d. (A) Live cells on whisker composite at whisker:CPC = 1:1. (B) Live cells on non-whisker control containing CPC fillers. (C) Live cells on the tissue culture polystyrene (TCPS control). (D) Dead cells on whisker composite at whisker:CPC = 1:1. The live cells were stained green and appeared to have formed a confluent monolayer. The three materials appeared to have a similar live cell density, indicating that cells proliferated equally well on them. The density of live cells at 14 d was much greater than the density of live cells at 1 d, showing that the cells had greatly proliferated. Dead cells were stained red and were very few on all three materials. (E) Quantitative measurement of cell viability using the Wst-1 assay for cells culture for 14 d. The amount of dehydrogenase activity in cells cultured on non-whisker control containing CPC fillers and on whisker composite with whisker:CPC of 1:1 was not significantly different (Student's t; $p > 0.1$). Each value is the mean of five measurements with the error bar showing one standard deviation.

The cell culture and live-dead stain experiments showed that both the non-whisker control containing only CPC fillers and the whisker composite (whisker:CPC of 1:1) were as non-cytotoxic as the tissue culture polystyrene control. After 1 d cell culture, osteoblast-like cells (MC3T3-E1) were able to adhere, spread and remain viable on all three materials when observed by fluorescence microscopy. At 14 d cell cultures, fluorescence microscopy and the quantitative Wst-1 assay showed that cell adhesion, proliferation and viability were equivalent on these materials. Therefore, these in vitro cell culture results suggest that the new CPC-whisker composite is non-cytotoxic.

When the bioactive fillers are imbedded in a resin matrix, it is possible that the bioactivity and bone-integrating ability of the implant may decrease [27]. Previous studies have demonstrated that there are at least two ways to avoid such suppression of bioactivity [25,27]. First, when the resin composite paste is implanted in vivo and polymerized in situ, the hardened composite could contain an uncured surface, because the oxygen present in blood inhibits radical polymerization reactions [27]. This was shown to expose the bioactive fillers at the implant surfaces and thus induce bone bonding [27]. Second, the Bis-GMA-based composites can be used as pre-fabricated implants with abraded surfaces. The abrasion was demonstrated to expose the bioactive fillers at the implant surfaces with increased bone-bonding ability [25]. Another concern is that coating the bioactive fillers with a silane coupling agent may affect the bioactivity. The silane agent was used on the whiskers and CPC fillers for bonding with the Bis-GMA-based resins. The mechanical properties of bioactive composites were modestly increased when the hydroxyapatite fillers were silanized [19,30]. Previous studies investigated whether the silanization of hydroxyapatite particles would suppress the bioactivity, and found that the materials maintained their apatite formation ability in a simulated body fluid [17].

Increasing the whisker:CPC ratio significantly increased the composite strength, work-of-fracture, elastic modulus and hardness. However, this reduced the content of the bioactive CPC fillers in the composite and hence may reduce the bioactivity of the composite. With the total filler mass fraction being fixed at 60% and at the whisker:CPC ratios varying over 1:2, 1:1 and 2:1, the resulting CPC mass fraction in the composite became 40%, 30% and 20%, respectively. The present study's 60% filler level resulted in relatively flowable pastes suitable for filling irregularly shaped cavities. One possible way to increase the bioactivity may be to increase the total filler level. A previous study on whisker composites achieved a total filler mass fraction of 79%, which would have resulted in higher CPC mass fractions of 52.7%, 39.5% and 26.3%, at whisker:CPC ratios of 1:2, 1:1 and 2:1, respectively. Further studies are needed to investigate the bioactivity (i.e., surface apatite formation in a simulated body fluid, or bone-bonding strength) with varying whisker:CPC ratios and total filler mass fractions for these strong and bioactive composites.

5. Summary

1. Novel bioactive composites were developed containing nano-silica-fused whiskers and highly osteoconductive CPC fillers. The strength and work-of-fracture of the whisker-CPC composites were an order of magnitude higher than pure CPC, and 3–5 times higher than the composite containing only CPC fillers without whiskers. The strength of the new whisker-CPC composites was 3 times higher than the strength achieved in previous studies on bioactive composites containing hydroxyapatite particles in Bis-GMA-based resins.
2. The flexural strength, elastic modulus and hardness of the bioactive whisker-CPC composite nearly matched those of cortical bone and trabecular bone.
3. The whisker:CPC ratio was a key microstructural parameter that significantly affected the composite properties. Compared to whisker:CPC of 2:1, the composite with whisker:CPC of 1:1 contained a higher level of bioactive CPC fillers without significantly compromising the mechanical properties.
4. Osteoblast-like cell adhesion, proliferation and viability were equivalent on the non-whisker control containing only CPC fillers, the whisker composite at whisker:CPC of 1:1, and the tissue culture polystyrene control, suggesting that the new CPC-whisker composite is non-cytotoxic.

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Disclaimer

Certain commercial materials and equipment are identified in this paper 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.

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