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Cytotoxicity of three-dimensionally ordered macroporous sol–gel bioactive glass (3DOM-BG) ☆

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Abstract

In this study, 80% SiO_2–20% CaO (mole fraction) three-dimensionally ordered macroporous sol–gel bioactive glass (3DOM-BG, average pore size: 345 nm) particles were prepared and characterized. Since the 3DOM-BGs have a novel microstructure and ion-releasing profile, the cytotoxicity of 3DOM-BG particles was tested. The cytotoxicity tests were performed using MC3T3-E1 osteoblast-like cells: (1) Wst-1 assay for cell viability after culture in extracts from 3DOM-BG particles; (2) phase contrast microscopy for cell morphology after culture with 3DOM-BG particles; and (3) fluorescence microscopy for imaging cells cultured directly on 3DOM-BG particles. The results showed that 3DOM-BG particles were not cytotoxic, and that cells attached, spread and proliferated on and around 3DOM-BG particles.
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Keywords: Porosity; Sol–gel; Bioactive glass; Apatite; Cell culture

1. Introduction

The sol–gel bioactive glasses (BGs) are thought to be one type of third generation biomaterials, which are both bioactive and bioresorbable [1–6]. The bioactivity of sol–gel BGs has been demonstrated by the formation of bone-like apatite when incubated in a simulated body fluid (SBF) [1–6]. In addition, the resorption of sol–gel BGs is enhanced when compared to melt-derived BGs due to the presence of mesopores (size: 2–50 nm) and functional groups (i.e., Si–OH) [1–3]. As a result, sol–gel BGs have been tested as non-load bearing bone and periodontal fillers (e.g., applied as pastes by mixing with physiological buffer solutions), drug delivery agents and tissue engineering scaffolds [1–6].

Three-dimensionally ordered macroporous sol–gel BGs (3DOM-BGs) were recently developed by a sol–gel process combined with a colloidal crystal templating method [4–6]. The as-prepared 3DOM-BGs have pore sizes ranging from 300 to 800 nm with an estimated total porosity of over 60% [4–6]. For comparison, trabecular bone has a porosity of 50–90% with pore sizes in the order of hundreds of microns [7]. Compared to mesoporous sol–gel BGs, 3DOM-BGs are more readily resorbed and converted to apatite [4]. For example, 3DOM-BG particles with the composition of 75 SiO_2–21 CaO–4 P_2O_5 (different composition from the current study) were fully resorbable in SBF and completely converted to bone-like apatite within 10 d [6]. In addition, the apatite converted from the 75 SiO_2–21 CaO–4 P_2O_5 3DOM-BG particles can be further heated to develop biphasic calcium phosphates with controllable degradation properties [6].

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Direct interactions between mesoporous sol–gel bioactive ceramics and cells have been investigated [8–10]. Results indicated that the interactions depend on the cell type and the composition and structure of the ceramic. For example, Olmo et al. [8] showed that $CaO–SiO_2–P_2O_5$ and $CaO–SiO_2$ sol–gel BGs were not cytotoxic and that rat osteoblasts attached and proliferated on the BG pellets. However, Abiraman et al. [9] showed that L929 cells line did not adhere well on $CaO–SiO_2–P_2O_5–MgO$ sol–gel BG-ceramic due to the highly reactive ceramic surfaces.

Previous studies also investigated the effects of leachates from bioactive ceramics on cellular activity [11–15]. Extracts from bioactive glasses were found to be non-cytotoxic and enhanced cellular activity [11–15]. For example, rat primary osteoblasts showed higher proliferation and collagen secretion when cultured with ionic products from a melt-derived bioactive glass than when they were cultured with control medium [12]. Furthermore, Xynos et al. [14,15] demonstrated that the soluble extracts from 45S5 Bioglass can affect cell-cycle induction and progression.

Compared to dense and mesoporous bioactive ceramics, the 3DOM-BGs discussed herein have different microstructures and ion releasing profiles [4] that could affect their cytotoxicity [16–18]. Therefore, the present investigation continues the research of 3DOM-BGs with an emphasis on their cytotoxicity. The studies are broken down to two parts. First, $SiO_2–CaO$ 3DOM-BG particles were synthesized and characterized. The effect of heat treatment on the bone-like apatite that was converted from the 3DOM-BG particles was also investigated. Second, osteoblast-like cells (MC3T3-E1) were used to test the cytotoxicity of the 3DOM-BG particles with the Wst-1 assay, phase contrast and fluorescence microscopy.

2. Materials and methods

2.1. Preparation and characterization of 3DOM-BG

As previously described [4], 80% SiO_2–20% CaO (mole fraction) 3DOM-BG particles were prepared. The 3DOM-BG particles were obtained by heating the poly(methyl methacrylate) (PMMA) latex array templated gel at 600 °C in air for 1 h (heating rate: 2 °C/min). The calcined product, consisting of millimeter-scale particles, was ground in a mortar and sieved to a size range between 212 and 1000 μm. 3DOM-BG particles used in cell culture tests were sterilized in an autoclave.

Bone-like apatite development and 3DOM-BG degradation were evaluated by soaking 3DOM-BG particles in SBF (composition: 142.0 mmol/L Na^+, 5.0 mmol/L K^+, 2.5 mmol/L Ca^{2+}, 1.5 mmol/L Mg^{2+}, 148.8 mmol/L Cl^-, 4.2 mmol/L HCO_3^-, and 1.0 mmol/L HPO_4^{2-}) [19]. The 3DOM-BG particles were placed in polypropylene bottles with SBF (0.1 mg 3DOM-BG/mL SBF) and maintained at 37 °C. After 10 d, the SBF was sampled and solid products (bone-like apatite) were separated by vacuum filtration, washed with ethanol, and dried in air at 37 °C. The dried products were then heated at 800 °C (rate: 2 °C/min) in air for 1 h, in order to study the effects of heat treatment on the structure and composition of bone-like apatite.

The 3DOM-BG particles, c-A (i.e., the solid products after soaking 3DOM-BG particles in SBF for 10 d) and t-A (i.e., the products after heat treatment of c-A) were characterized by Fourier transform infrared spectroscopy (FTIR, Nicolet Magna 760 spectrometer), powder X-ray diffraction (XRD, Bruker-AXS microdiffractometer) and scanning electron microscopy (SEM, Hitachi S-900).

2.2. Cell culture

Culture of osteoblast-like cells (MC3T3-E1) was performed as previously reported [20]. Briefly, MC3T3-E1 cells (Riken Cell Bank, Hirosaka, Japan) were maintained in α-modification of Eagle's minimum essential medium (Biowhittaker Inc.) with a volume fraction of 10% fetal bovine serum (Gibco-BRL-Life Technologies) and 60 mg/L kanamycin sulfate (Sigma Inc.) in a fully humidified atmosphere with a volume fraction of 5% CO_2 at 37 °C. Medium was changed twice a week. Cultures were passaged with EDTA-containing (1 mmol/L) trypsin solution (mass fraction of 0.25%; Gibco Inc.) once a week.

2.3. 3DOM-BG extraction

3DOM-BG particles were placed in tissue culture flasks with medium (BD Biosciences) at ratios of (0.5, 5, and 25) mg/mL medium and incubated 24 h in a cell incubator (100% relative humidity and 5% CO_2). Positive controls contained media with Triton X-100 detergent (0.1% mass fraction) and negative controls contained only media in tissue culture plastic (TCPS). In parallel, a flask of 80% confluent MC3T3-E1 cells was passaged, cells were seeded into 24-well plates with 10,000 cells per well in 2 mL of media and the plates were incubated overnight. On the second day of the experiment, the medium from each well was removed and replaced with the 2 mL of extraction medium from one of the specimens (or with the positive or negative control media). Six wells were prepared for each extraction ratio, positive controls and negative controls. Cells were incubated in the extracts for 3 d before the Wst-1 assay.

2.4. Wst-1 assay

The Wst-1 assay is a colorimetric assay of cellular dehydrogenase activity where absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cell [21]. Wst-1 and 1-methoxy PMS were obtained from Dojindo Inc. Cells cultured in the extracts were 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 PMS in water) were then added to each well. After 2 h incubation at 37 °C, a 0.2 mL aliquot from each well was transferred to a 96-well plate and absorbance was measured with a platereader (Wallac 1420 Victor). Blank wells that contained only Tyrode's HEPES buffer and Wst-1 solution were also prepared, incubated and read. The blank value was subtracted from each of the experimental values as background.

2.5. Phase contrast and fluorescence microscopy

For phase contrast microscopy, 3DOM-BG specimens were cultured with cells for 1, 7 and 14 d (500,000 cells and 1 mg particles in 2 mL of medium in a 12-well plate) and imaged with digital photography using an inverted phase contrast microscope (Nikon TE300). Three specimen wells at each time point and control wells (cells only) were prepared and observed. Apatite particles prepared by soaking 3DOM-BG particles in SBF for 10 d (c-A) were also cultured for 7 d with cells under the same condition as 3DOM-BG particles.

For fluorescence microscopy, cells were stained 10 min in serum-free cell media with calcein-AM (live cells, green) and ethidium homodimer-1 (dead cells, red). Both stains (Molecular Probes Inc.) were used at a concentration of 2 μmol/L. Cells grown in the presence of 3DOM-BG particles for 7 and 14 d were stained and observed. Cells were also stained after culture directly on large 3DOM-BG particles for 1 d (particle size > 1 mm; 1 mg particles in 2 mL of medium in a 12-well plate).

2.6. Statistical analysis and disclaimer

Significant differences between specific groups were determined by ANOVA with Tukey's pairwise comparisons (95% confidence interval). Certain equipment, instruments and materials are identified in the paper to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology, nor does it imply that the materials are necessarily the best available for the purpose.

3. Results and discussion

3.1. 3DOM-BG

SEM studies (see Fig. 1A and B) confirmed that the amorphous (as demonstrated in Fig. 2A) $CaO–SiO_2$ 3DOM-BG particles have ordered macroporous structures [4], with an average pore size of 345 nm. After soaking in SBF for 10 d, 3DOM-BG was converted to a calcium-deficient, bone-like apatite (c-A, see Figs. 1C, 2B and 3B) [5,6]. The complete and rapid conversion from glass to bone-like apatite is a unique property of 3DOM-BGs [6].

After heating the converted bone-like apatite (c-A) at 800 °C for 1 h, a biphasic calcium phosphate (t-A) that was composed of both hydroxyapatite and beta-tricalcium phosphate was developed as a result of the decomposition of the calcium-deficient apatite [22]. The formation of hydroxyapatite is shown by XRD where the broad peak of c-A at 2θ of 32° (Fig. 2B) splits into the three characteristic diffraction peaks for hydroxyapatite (Fig. 2C). The formation of hydroxyapatite is also confirmed by the appearance of the hydroxyl band (631 cm^{-1}) in the FTIR spectrum (Fig. 3C). The formation of beta-tricalcium phosphate is shown by its characteristic XRD peaks at 2θ of approximately 28°, 31° and 34° (see Fig. 2C). The heat treatment provides a new method to adjust the properties (e.g., degradation) of the bone-like apatite converted from 3DOM-BG [6,22].

The present study showed that 3DOM-BG converts to bone-like apatite in SBF after several days. Previous studies have shown that the abundant Si–OH groups on the 3DOM-BG and its open, liquid-accessible 3DOM structure render 3DOM-BG with excellent degradability and bioactivity [4–6]. The structure and chemistry differences between sol–gel processed, $CaO–SiO_2$ 3DOM-BG and melt-derived, dense BGs such as sodium-containing 45S5 may cause the differences in their degradation. Furthermore, the current results for the first time show that the $CaO–SiO_2$ 3DOM-BG can be converted to biphasic calcium phosphates by heating c-A (the bone-like apatite made by incubating 3DOM-BG in SBF).

3.2. Wst-1 assay

Wst-1 results (Fig. 4) quantitatively showed the viability of cells cultured for 3 d with the leachates from 3DOM-BG particles. There was no statistical difference ($p > 0.05$) between negative control (TCPS) and extracts from different amount of 3DOM-BG particles [(0.5, 5, and 25) mg/mL medium]. However, all samples were significantly different ($p < 0.05$) from the positive control (Triton X-100). The results demonstrate that the leachates of 3DOM-BG are non-toxic.

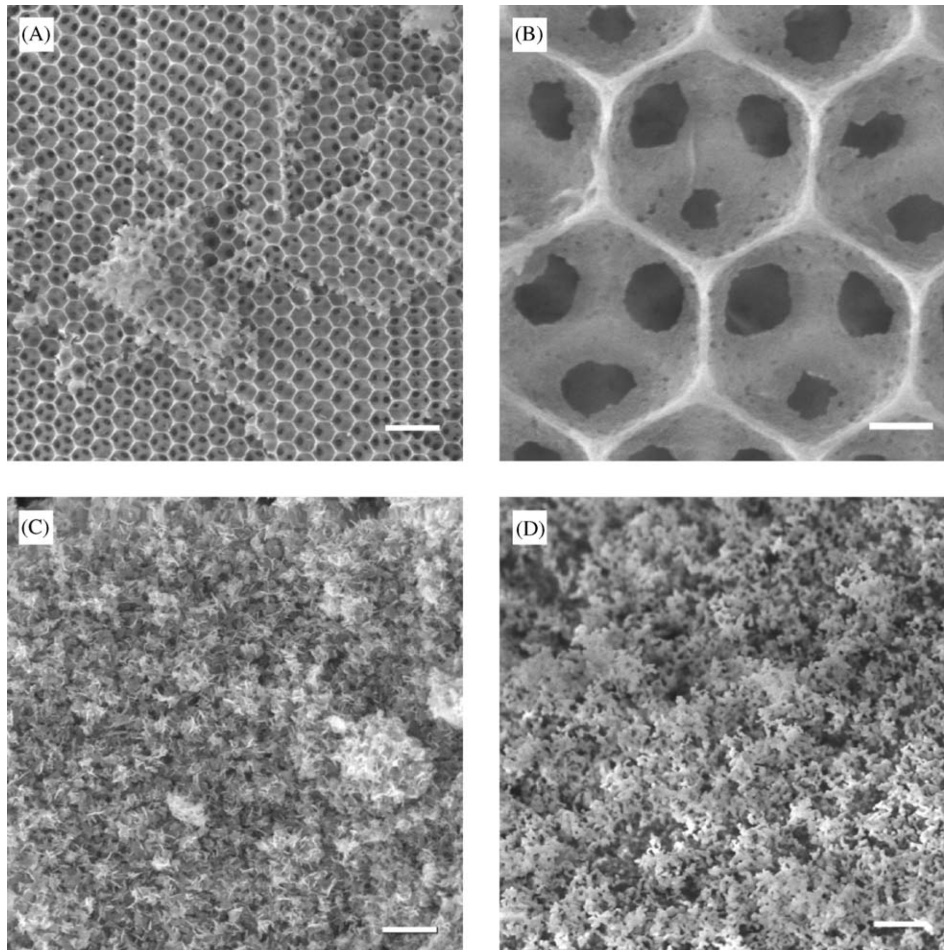

Fig. 1. SEM images of (A) and (B) 3DOM-BG particles, (C) the converted bone-like apatite (c-A) from 3DOM-BG particles, and (D) the biphasic calcium phosphates (t-A) after heating the converted bone-like apatite at 800 °C. Scale bar: 1 μm for A, C and D, 100 nm for B.

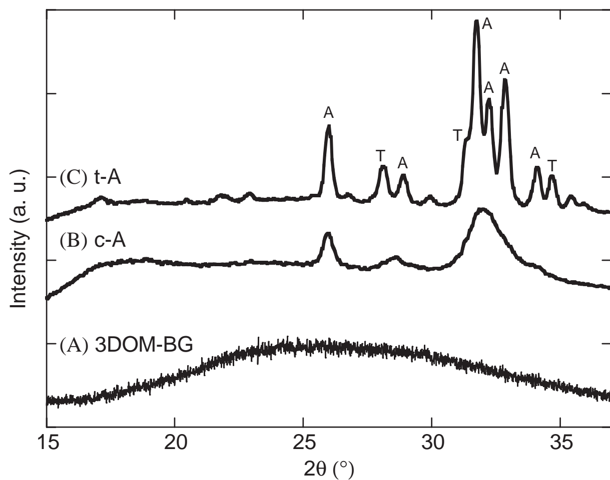

Fig. 2. XRD patterns for (A) 3DOM-BG, (B) c-A, and (C) t-A. A: hydroxyapatite and T: β-tricalcium phosphate (whitlockite).

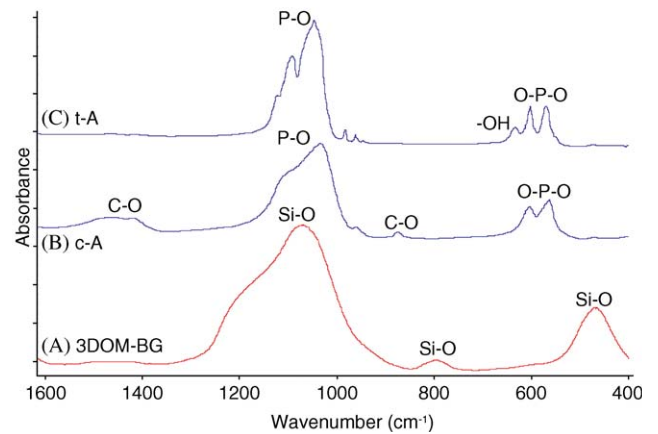

Fig. 3. FT–IR spectra for 3DOM-BG, c-A, and t-A.

These results from Wst-1 assay are consistent with previously reported data. An indirect toxicity evaluation by Dufrane et al. [13] showed that leachates from bioactive pseudowollastonite ceramic ($CaSiO_3$, composition is similar to the 3DOM-BG used in the current study) did not have cytotoxic effects on osteoblasts. Lai et al. also showed that the silicate leachates from bioactive glass particles were non-toxic in vivo [23].

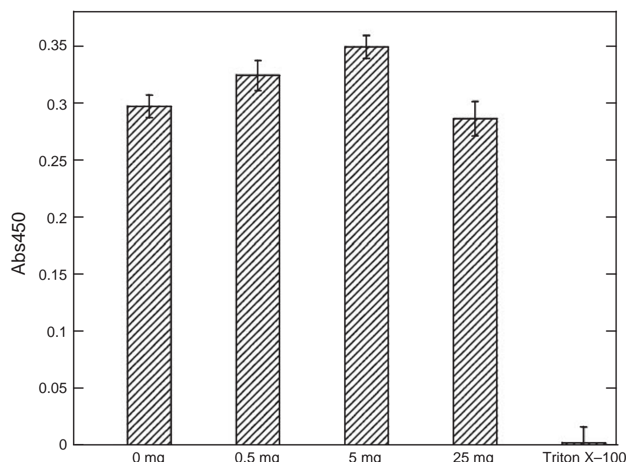

Fig. 4. MC3T3-E1 osteoblastic cell viability (Wst-1 assay) after 3 d culture in 3DOM-BG extracts (concentrations: 0.5, 5 and 25 mg/mL medium), negative (TCPS) and positive controls (Triton X-100). Standard deviation (indicated by bars) is taken as a measure of the standard uncertainty and $n = 6$.

Previous studies have also shown that 3DOM-BG particles not only release ions to solution but exchange ions with the solution [4–6]. Silicate groups were mainly leached out to the solution; calcium ions were exchanged between 3DOM-BG and the solution, and phosphate groups were adsorbed into 3DOM-BG to develop apatite [4]. In the tested concentrations, the products after ion release and exchange between 3DOM-BG particles and culture medium were not cytotoxic.

3.3. Phase contrast microscopy

Phase contrast microscopy studies showed that cells proliferated in the presence of 3DOM-BG particles. Cells reached confluence before 7 d and multilayers of cells were around and up against 3DOM-BG particles by 7 d (Fig. 5A). These multilayers of cells around 3DOM-BG particles formed reflective areas that were not seen in the controls (TCPS, Fig. 5D). Interestingly,

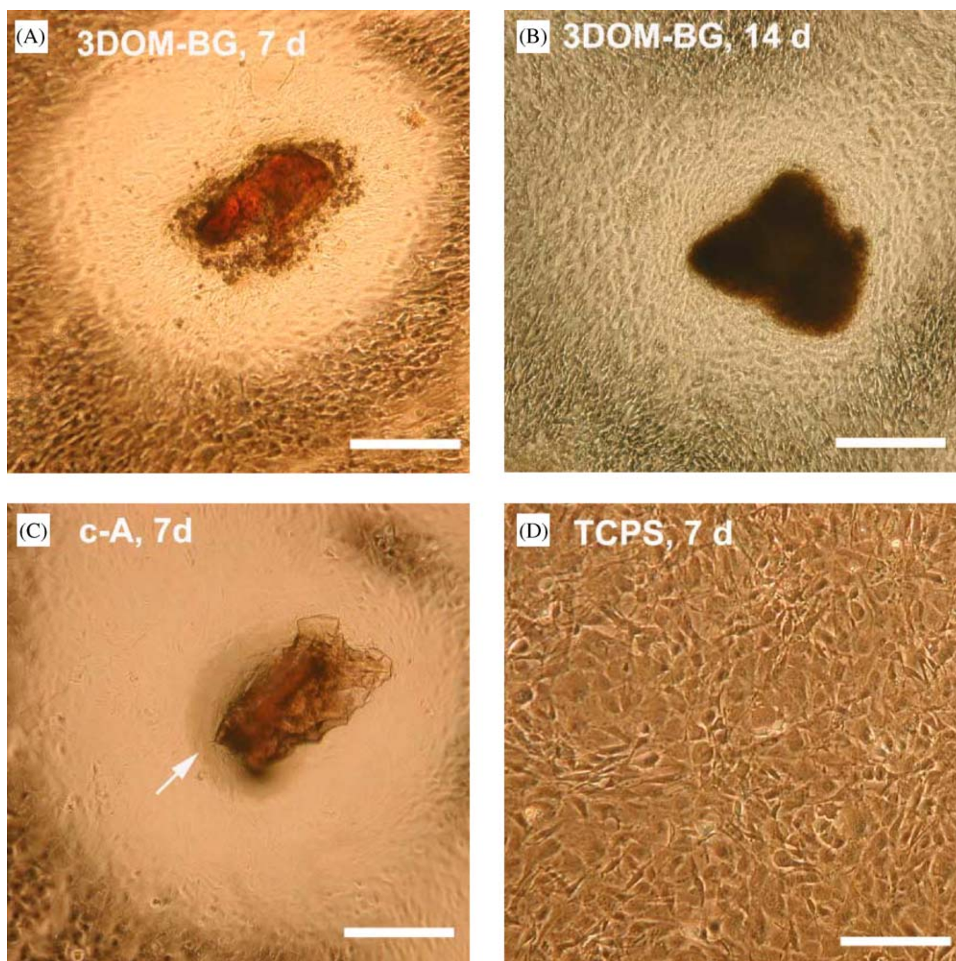

Fig. 5. Phase contrast microscopy images of MC3T3-E1 osteoblastic cells with 3DOM-BG and its converted apatite particles (concentration: 0.5 mg/mL medium). (A) 7 d with 3DOM-BG. (B) 14 d with 3DOM-BG. (C) 7 d with c-A. The arrow in the image shows that an opaque area appeared in the center of the reflective area and was in contact with the apatite particle. (D) 7 d on TCPS. Scale bar: 300 μm.

cells cultured with the converted apatite particles (c-A) for 7 d showed a distinctive behavior (Fig. 5C). Although a reflective area still developed around the converted apatite particles, a more opaque area appeared in the center of the reflective area and was in contact with the apatite particle (Fig. 5C). In addition, cells around the 3DOM-BG and c-A on day 7 had a smaller and less spread morphology than cells in TCPS controls. On day 14, similar multilayers of cells and reflective areas were found around the 3DOM-BG particles (Fig. 5B). However, no reflective areas were seen in the controls and the shape of cells in controls appeared different with 3DOM-BG particles. Loty et al. observed similar reflective and opaque areas in their experiments with BGs and suggested that this event leads to bone nodule formation [24].

Phase contrast microscopy allows for the observation of the morphological changes of in vitro cell culture. Cells appeared to be normal in culture with 3DOM-BG and c-A, indicating that 3DOM-BG and c-A are not cytotoxic. In addition, the smaller shape of cells around 3DOM-BG and c-A was similar to that of differentiated osteoblasts [25] suggesting that the cell differentiation process might be promoted in the presence of 3DOM-BG particles. The morphological difference between the 7 d culture of 3DOM-BG and that of c-A, characterized by the presence of an opaque area in the c-A samples, may be due to differences in the chemistry of the two

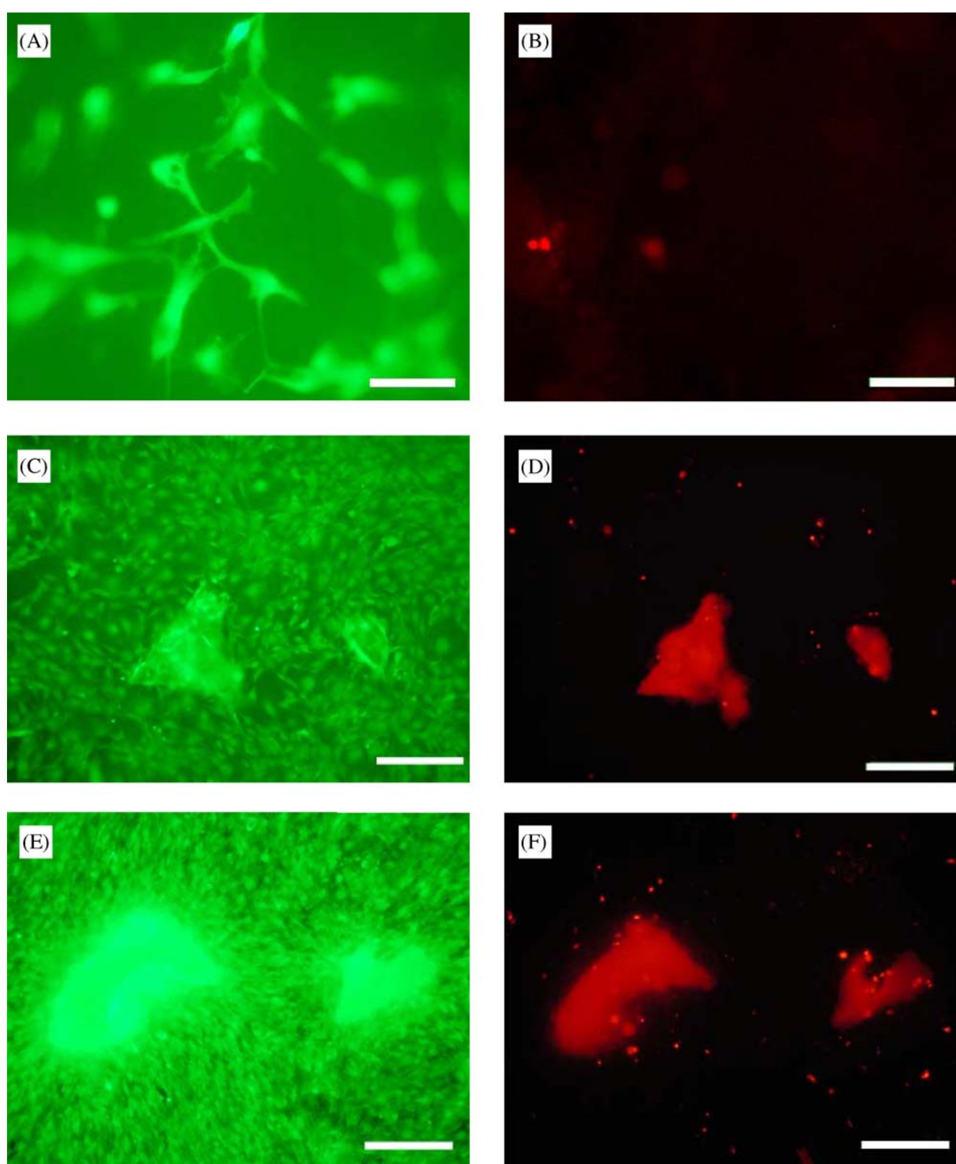

Fig. 6. Fluorescence microscopy of cells cultured on or in the presence of 3DOM-BG particles. (A, B) 1 d on 3DOM-BG particles. (C, D) 7 d in the presence of 3DOM-BG. (E, F) 14 d in the presence of 3DOM-BG. (A), (C) and (E) are live stained cells and (B), (D) and (F) are dead stained cells. Images on the left and right are the same field of view with different fluorescence filters (left: green, live; right: red, dead). Scale bar: 300 μm for (A)–(B) and 150 μm for (C)–(E).

materials. The favorable response of the MC3T3-E1 cells to the c-A is due likely to the similar compositions of c-A and real bone. c-A is composed of a calcium-deficient, carbonated apatite and the primary component (60–70%) of real bone is also calcium-deficient, carbonated apatite. In addition, other researchers have shown that the apatite formed on a sol–gel BG can enhance cell proliferation [8].

The degradation of 3DOM-BG appeared to be slower in cell media than in SBF. As 3DOM-BG converts to bone-like apatite (c-A), the 3DOM-BG loses the opalescence which results from diffraction of light in the 3DOM structure. In the current study, this opalescence was gone from 3DOM-BG after 4 d in SBF but was still present in 3DOM-BG incubated in cell media for 4 d. This phenomenon may be due to the presence of cells, culture medium and proteins. Duchyene et al. [26] demonstrated that the apatite conversion from BG and the BG dissolution were slower in cell culture medium than in SBF. This observation was attributed to the selective protein adsorption onto BG from cell culture [26].

3.4. Fluorescence microscopy

After 1 d of culture, cells attached and spread on large 3DOM-BG particles (size > 1 mm). The cells were viable, showing a normal, polygonal morphology (Fig. 6A). After culturing with 3DOM-BG particles (size: 212–1000 μm) for 7 d, fluorescence images (Fig. 6C) showed that cells around and on the particles reached confluence, were alive and had a normal shape. After 14 d, additional cell proliferation was evident since the cell density appeared to increase from 7 to 14 d (see Fig. 6E). In addition, visual assessment of Fig. 6B and D and F indicate that there were few dead cells on or around the 3DOM-BG particles.

Live/dead staining assay is a simple and rapid method to qualitatively evaluate the cytotoxicity of a biomaterial [20]. The assay uses two stains, calcein-AM and ethidium homodimer-1. Calcein-AM is a non-fluorescent and cell-permeant agent that can be converted to calcein by live cell enzymes. The converted calcein is fluorescent and cell-impermeant and its accumulation inside live cells causes fluorescent green color [21]. Ethidium homodimer-1 is excluded by intact plasma membranes of live cells. However, once it enters dead cells, ethidium homodimer-1 undergoes a 40-fold enhancement of fluorescence (red color) by binding nucleic acids [21]. The live/dead fluorescence microscopy study confirmed the results from Wst-1 and phase contrast microscopy studies. The cells were alive with 3DOM-BG particles up to 14 d. Cells attached and spread on 3DOM-BG particles after culturing for 1 d, and proliferated around and up against small 3DOM-BG particles up to 14 d.

4. Conclusions

3DOM-BG particles with the composition of 80% SiO_2–20% CaO (mole fraction) can develop biphasic calcium phosphates by heating c-A (bone-like apatite formed by incubating the 3DOM-BG in SBF). Wst-1 results showed that the released ionic products of 3DOM-BG particles in culture medium were not cytotoxic with MC3T3-E1 osteoblasts. Phase contrast microscopy demonstrated that cells proliferated and had a normal morphology up to 14 d in the presence of 3DOM-BG particles or c-A. The current results combined with the previous studies [23] indicate that the presence of 3DOM-BG may lead to bone nodule formation. Fluorescence microscopy studies using live/dead assay showed that cells attached onto 3DOM-BG particles after 1 d and were viable up to 14 d in the presence of 3DOM-BG particles. Taken together, the results show that 3DOM-BG particles are not cytotoxic and support the potential application of 3DOM-BG as a novel bone filler.

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