Osteoblast responses to tape-cast and sintered bioactive glass ceramics

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Abstract: The advantage of tape-cast bioactive glasses lies in the manufacturing procedure, which allows the build-up of layers and, therefore, the production of complex shapes. This, therefore, has applications to tissue engineering, where specific shapes are required such as repair of craniofacial defects. The bioactivity of tape-cast discs sintered at temperatures ranging from 800°C to 1000°C and for 3 or 6 h was analyzed by FTIR. Tape-cast discs were used to culture primary human osteoblasts, and cell attachment, cell death, collagen production, nodule formation, and mineralization were studied. These responses were dependent upon Si and Na release profiles of the tape-cast discs, and development of the hydroxyapatite layer. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 69A: 621–628, 2004

Key words: bioactive; tape cast; primary human osteoblasts; tissue engineering

INTRODUCTION

Bioactive glasses have applications to tissue engineering and bone repair due to their ability to bond to bone *in vivo* via a hydroxyapatite surface layer.[1,2] The formation of this surface layer has been reported on tape-cast and sintered bioactive glass ceramics by incubation in simulated body fluid (SBF).[3] The hydroxyapatite layer formed, as determined by FTIR, was better and more rapidly established on the sample sintered at 900°C, as summarized in Table I. Formation of such layers in Tris buffer (contains no Ca or P) also has been analyzed,[4] and it was found that tape-cast bioactive glass ceramic sintered at 900°C and 1000°C (3 and 6 h) demonstrated a strong bioactive response after 24 h, as demonstrated by FTIR. Lack of densification of the sample sintered at 800°C and subsequent ion release levels appeared to hinder HA formation. Si and Na release also were found to differ depending on sintering temperature and also are summarized in Table I.

The samples sintered at 800°C had the highest ion release profile, with samples sintered at 1000°C having the lowest ion release profile. Due to these differences in rates and concentrations of ionic release and hydroxyapatite formation, we analyzed the osteoblast response after culture from 90 min up to 14 days. Rates of hydroxyapatite formation on the surface of the discs after incubation in culture medium containing serum were established due to the controversy as to whether serum proteins promote or inhibit HA formation. Ion release into volumes of culture medium typical of a cell culture experiment also were determined.

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METHODS

Material processing

Tape-cast and sintered bioactive glass discs were produced as described previously.[3] Tape-casting slurries were prepared by thoroughly mixing 42.3 weight percent particulate bioactive glass 45S5 (USBiomaterials Corp., Alachua, FL), 6.3% polyvinyl butyral, 2.8% phthalic acid, 37.9% toluene, and 10.7% ethanol. Slurries then were poured onto a moving carrier film, and 100-μm films were formed using a doctor blade. Circular blanks were punched from the dried tapes, stacked, and pressed under low heat and pressure such that 2.5-mm-thick green discs were formed. The green discs then were heated between 200–500°C for 24 h to remove the organic phases, at which point the temperature was ramped slowly (1°C/min) to 800°C, 900°C, or 1000°C. The maximum temperature was maintained for 3 h. An additional set of samples was processed at 1000°C for 6 h.

Tape-cast and sintered bioactive glass-ceramic samples of approximately 14-mm diameter and 2.0-mm thickness were

TABLE I
Summary of Hydroxyapatite (HA) Layer Formation on, and Ionic Dissolution Release From, Tape-Cast and Sintered Bioactive Glass After Incubation in SBF or Tris Buffer (see refs. 3 and 4)[a]

Sample	SBF	Tris
800		
FTIR	Poor HA layer formation	Poor HA layer formation. Needle-like clusters after 2 h
Ion release	Not measured	Highest ion release 23 ppm Si after 1 h, 85 ppm after 24 h, 122 ppm after 14 days. 82 ppm Na 1 h, 350 ppm 24 h, 650 ppm 14 days
900		
FTIR	Most rapid HA layer formation after 2 h	HA (continuous layer) began forming after 2 h. Strong bioactive response, crystalline HA after 24 h
Ion release	Not measured	Medium level ion release 5 ppm Si 1 h, 58 ppm 24 h, 90 ppm 14 days. 6 ppm Na 1 h, 100 ppm 24 h, 575 ppm 14 days
1,000 (3)		
FTIR	Developed HA layer more slowly, but by 24 h was well established	HA (continuous layer) began forming after 2 h Strong bioactive response, crystalline HA after 24 h
Ion release	Not measured	Lowest ion release, comparable to 1000(6) 5 ppm Si 1 h, 48 ppm 24 h, 70 ppm 14 days 6 ppm Na 1 h, 50 ppm 24 h, 200 ppm 14 days
1,000 (6)		
FTIR	Developed HA layer more slowly, but by 24 h was well established	Strong bioactive response, crystalline HA after 24 h
Ion release	Not measured	Lowest ion release comparable to 1000(3) 6.5 ppm Si 1 h, 47 ppm 24 h, 70 ppm 14 days 6 ppm Na 1 h, 50 ppm 24 h, 200 ppm 14 days

[a]The different samples are referred to by their sintering temperature only. Numbers in parentheses after the 1000 samples refer to sintering time. Release of both Si and Na followed trend 800 > 900 > 1000 in both SBF and Tris buffer.

soaked in cell culture medium at 37°C for 2 h, 24 h, or 14 days. Following removal from the culture medium, samples were dipped in acetone and allowed to dry prior to FTIR analysis.

Fourier transform infrared spectroscopy (FTIR)

Development of the hydroxyapatite layer was determined using FTIR using a Spectronic Unicam Genesis II after mixing samples with KBr (1:100 by wt.). Analysis was performed in reflectance mode between 1,400–400 cm^{-1} in order to characterize the formation of a hydroxyapatite surface layer.

Preparation of conditioned medium containing ionic dissolution products

Conditioned medium was prepared to culture cells and determine if ion leaching caused cell death. Discs were incubated in culture medium (DMEM, as detailed below) for 24 h at 37°C.

Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Ionic concentrations of released silicon (Si), calcium (Ca), sodium (Na), and phosphorous (P) after immersion of samples in culture medium (DMEM) for 24 h at 37°C in static conditions, to mimic the cell culture environment, were determined using ICP-OES (3580B ICP Analyser, Applied Research Laboratories, IM35xx ICP Manager software, Micro-Active Australia Pty Ltd). The samples were diluted 1:10 in a 2N HNO_3 matrix before analysis. Three integrations were performed per sample and the mean and standard deviation were calculated.

Cell culture

Human primary osteoblast cells (HOBs) were isolated, as described previously, from the femoral head.[5,6] Samples of bone were obtained after total hip replacement surgery and cut into fragments of approximately 3 × 3 mm. Fragments were washed several times in phosphate buffered saline (PBS) to remove blood cells and debris with a final wash in culture medium. Fragments then were placed in 30-mm Falcon culture dishes in complete Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum with 1% glutamine, 2% penicillin/streptomycin, and 50 μg/mL ascorbic acid-2 phosphate. The fragments were incubated at 37°C in a humidified incubator with 5% CO_2. After culture for 7–10 days, fragments were subjected to trypsin (0.02%) and collagenase (0.162 U/mL) digestion for 20 min at 37°C on a roller mixer. The resulting cell suspension then was centrifuged at 1000 rpm and enzyme digestion of the fragments repeated. The whole process was performed a total of

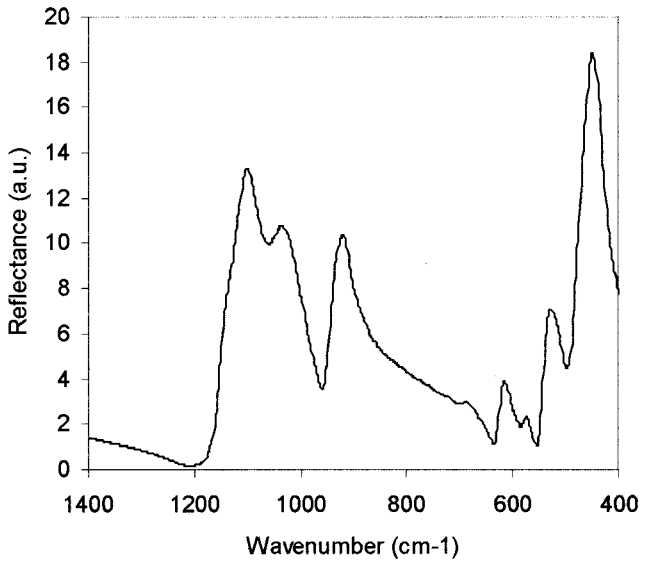

Figure 1. FTIR spectrum of tape-cast and sintered bioactive bioactive glass following sintering at 800°C. Spectra of 900, 1000(3), and 1000(6) samples were nearly identical.

five times and cells were pooled. HOBs were cultured at a density of 40,000 cells/cm^2.

Cell attachment

Cell attachment was quantified after fixing in 4% paraformaldehyde and staining with 10 μg/mL propidium iodide in PBS for 2 min at room temperature. Numbers of cells attached were counted by visualization using a fluorescence microscope, using a random field of view counting technique.

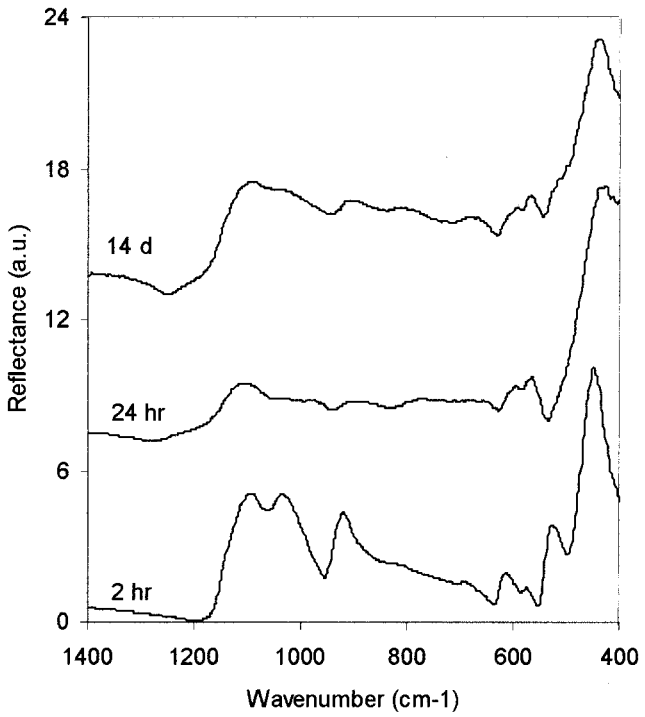

Figure 3. FTIR spectra of 900°C TCS bioactive glass following 2 h, 24 h, and 14 days immersion.

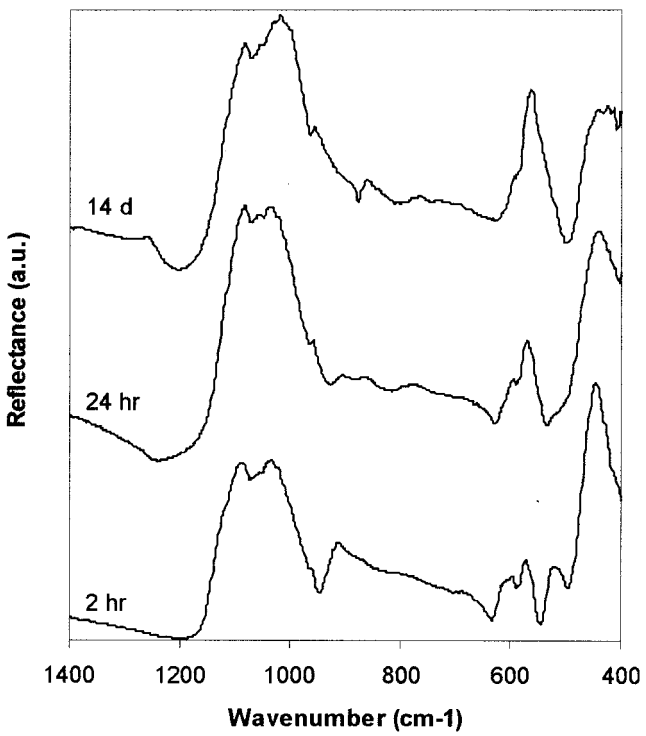

Figure 2. FTIR spectra of 1000°C(6) TCS bioactive glass following 2 h, 24 h, and 14 day immersion.

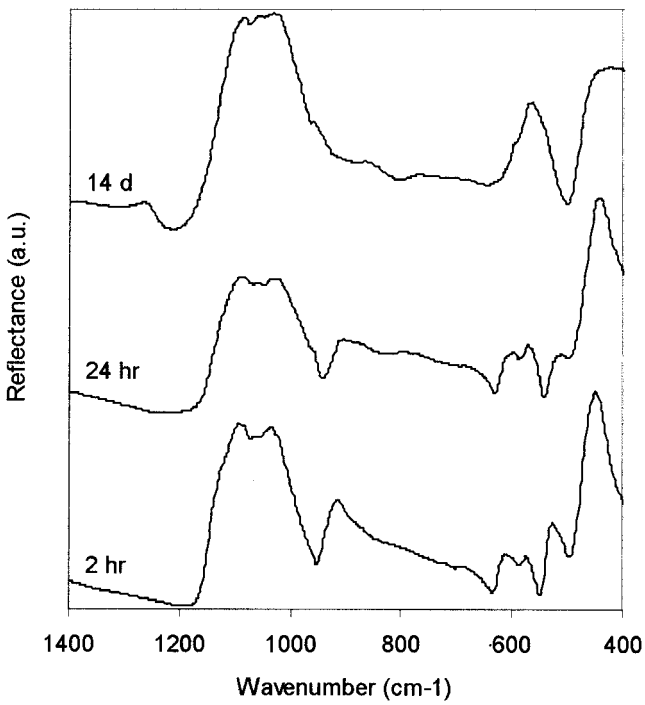

Figure 4. FTIR spectra of 1000°C(3) TCS bioactive glass following 2 h, 24 h, and 14 days immersion.

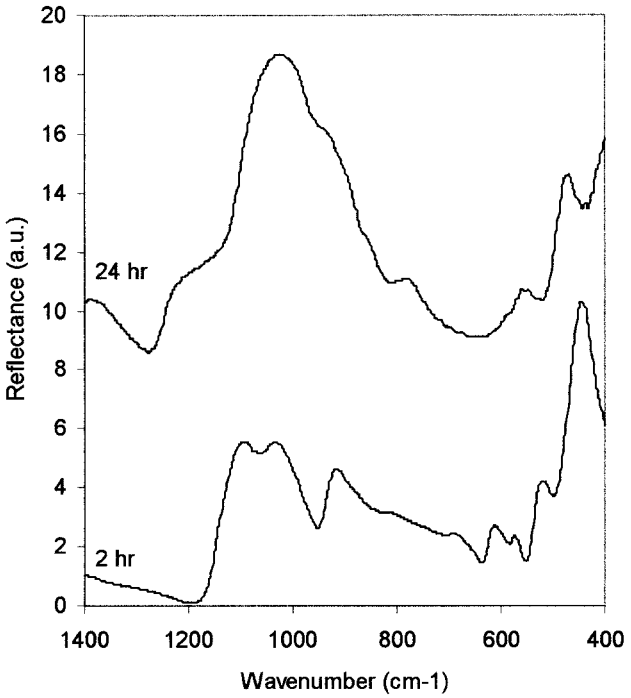

Figure 5. FTIR spectra of 800°C TCS bioactive glass following 2- and 24-h immersion.

Determination of apoptosis and necrosis

Cells were fixed in 4% paraformaldehyde and stained with 10 μg/mL propidium iodide in PBS for 2 min at room temperature. Cells were washed in PBS and viewed under a fluorescence microscope. Cells with uniform staining were noted as viable, cells with condensed nuclear staining in concordance with typical apoptotic morphology[7,8] were noted as apoptotic, and cells with sparse ghost-like nuclear staining were noted as necrotic in concordance with typical necrotic morphology.

Nodule formation

Determination of bone nodule formation, an *in vitro* phenotypic characteristic of osteoblasts, was performed by observation using light microscopy and by staining with alizarin red for mineralization. Determination of collagen presence in the nodules was performed by staining with propidium iodide as above and anti collagen-1 antibody staining (Sigma, Dorset, UK). Cells were fixed as above and incubated in primary antibody for 1 h at 37°C. Cells were washed in 1% bovine serum albumin in PBS and incubated in secondary antibody (Dako, Ely, UK) for 1 h at 37°C. Collagen-1 staining and nodule formation were visualized using a BioRad confocal microscope.

RESULTS

HA formation in culture medium after 2 h, 24 h, and 14 days

After 2 h in culture media, FTIR spectra of the samples sintered at 800°C, 900°C, and 1000°C(3) sam-

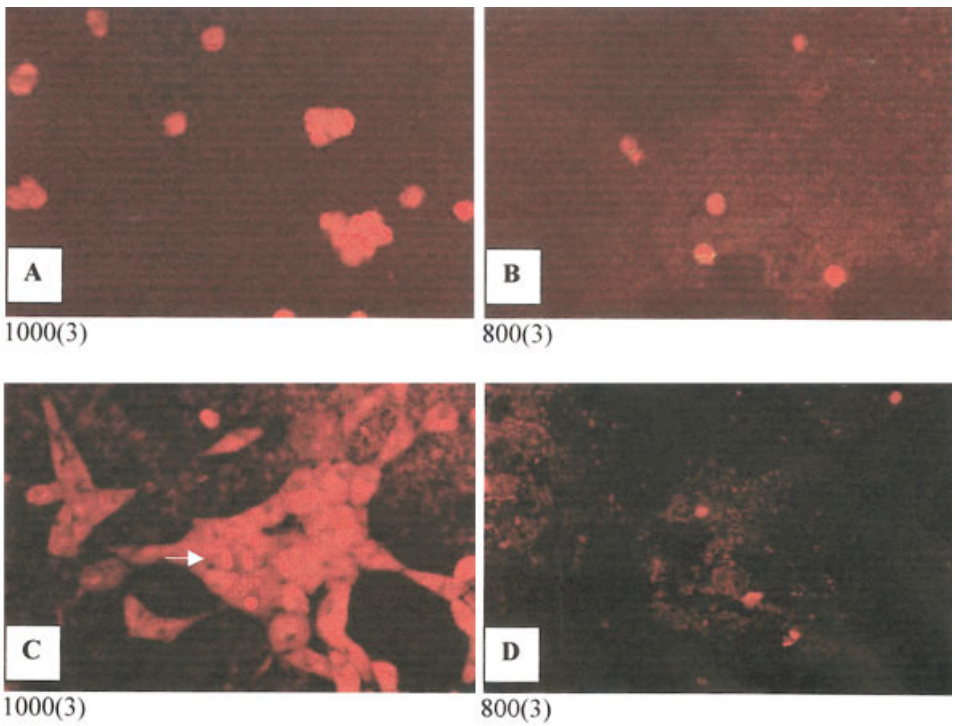

Figure 6. Fluorescence micrographs of cell attachment after 90 min (A,B) and 24 h (C, D) in culture. Cells were stained with propidium iodide for cell nuclei. Arrow shows cells undergoing mitosis (proliferation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

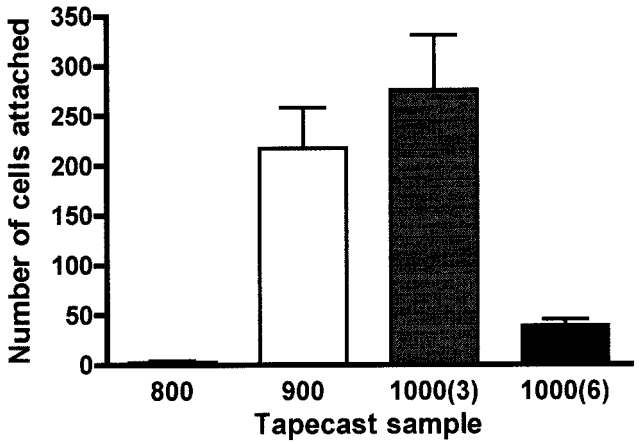

Figure 7. Levels of cell attachment after 24 h culture on the tape-cast discs sintered at different temperatures. All samples are significantly different from each other at $p < 0.01$, using one way ANOVA, $n = 3$.

ples each appeared nearly identical to the as-processed spectra, indicating no detectable HA formation (Fig. 1). In contrast, FTIR spectral analysis indicated that an HA layer began forming on the 1000°C(6) sample surface after only 2 h in TCM (sharp peaks near 598 and 573 cm^{-1}) (Fig. 2).

Hydroxyapatite formation clearly was evident in the samples sintered at 900°C, 1000°C(3), and 1000°C(6) samples after 24 h (598, 573, and 565 cm^{-1}) (Figs. 2–4). A

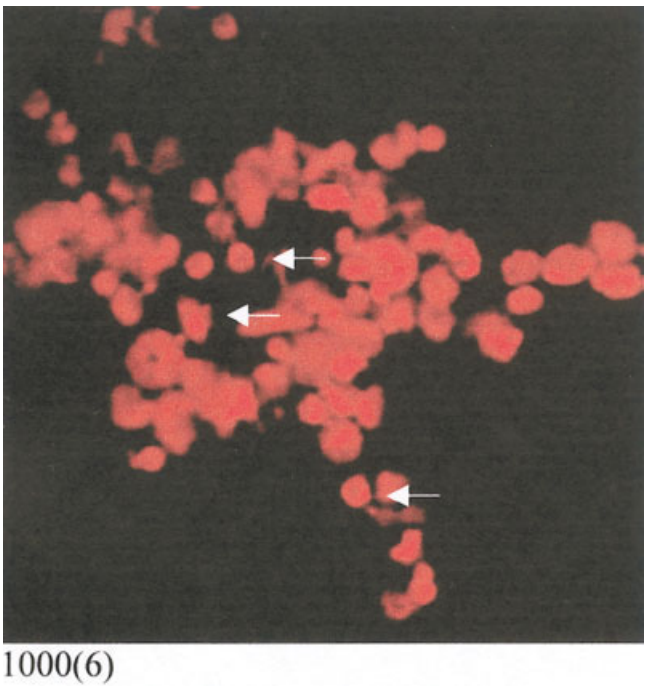

Figure 8. Fluorescence micrograph of apoptotic cells after culture in conditioned medium from 1000°C(6) tape-cast sample. Cells were stained with propidium iodide for cell nuclei. Arrows show examples of apoptotic cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II
Apoptosis, Necrosis, and Viability of Cells Cultured in Conditioned Medium

Sample	% Viable	% Apoptotic	% Necrotic
DMEM control	96	4	0
800(3)	0	22	78
900(3)	13	79	8
1000(3)	87	13	0
1000(6)	3	91	6

semi-crystalline calcium phosphate phase formed on the sample sintered at 800°C surface, after 24 h (Fig. 5).

The formed surface layers continued to increase in thickness after 14 days, as indicated by the decrease in the Si-O bending peaks near 450 cm^{-1}[13]

Cell attachment and apoptosis

Cell attachment and apoptosis were analyzed simultaneously using propidium iodide staining of cell nuclei. After 90 min of culture, cells were observed attaching to the bioglass surfaces (Fig. 6 shows cells attaching to the samples sintered at 800°C and 1000°C (3) and a greater number of cells were seen after 24 h). Very few cells attached to the 800°C sample at 90 min, whereas significantly more cells attached to the other samples. Quantification of cell attachment after 24 h is shown in Figure 7, where the greatest cell attachment was seen on the 900°C and 1000°C(3) samples. Cells on some surfaces and conditioned medium from some of the samples were observed to undergo apoptosis, as demonstrated by classical apoptotic nuclear morphology (Fig. 8). The number of apoptotic, necrotic, and viable cells observed after culture in conditioned medium was quantified and is shown in Table II. Samples at 900°C and 1000°C (6) resulted in the greatest levels of apoptosis, whereas the 800°C sample resulted mainly in necrosis.

Nodule formation

The ability of cells to form bone nodules on the surface of the tape-cast bioactive glass discs was ana-

TABLE III
Number of Nodules Observed on Each Tape-Cast and Sintered Disc

Sample	No. Nodules
0	0
800	0
900	13
1000(3)	85
1000(6)	0

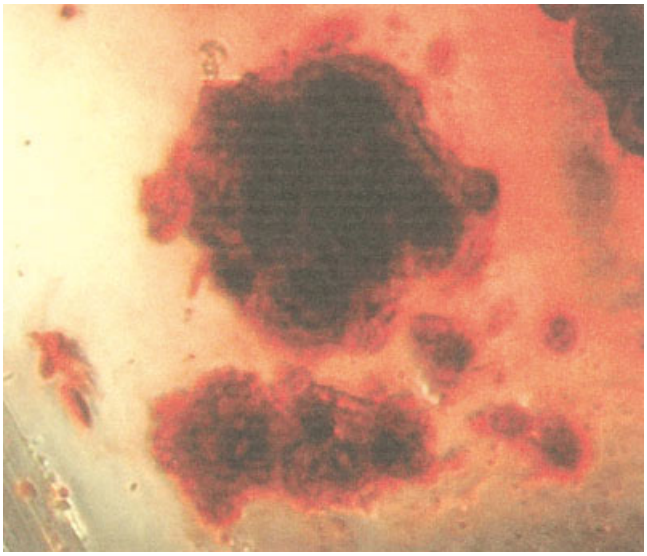

Figure 9. Light micrograph of alizarin red stained nodule after culture on 1000(3) tape-cast disc for 15 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

lyzed microscopically. Nodule formation was observed mainly [on the sample sintered at 1000°C(3)], as shown in Table III, and these nodules were found to be mineralized by alizarin red staining (shows presence of calcium), as shown in Figure 9. Nodules also were stained with collagen-1 antibody to demonstrate production of collagen by the cells as shown in Figure 10.

ICP measurements through culture time

Although ion release from tape-cast bioactive glass discs has been reported previously, ICP was used to measure release of Na, Ca, P, and Si in a typical cell culture, i.e., using the same surface area:volume of cell culture medium, therefore relating ion release directly to *in vitro* osteoblast responses. Figures 11 and 12 show Si and Na release profiles, respectively (Ca and P did not significantly change), of the tape-cast and sintered discs. The samples sintered at 1000°C(3) and 1000°C(6) samples were almost identical in Si and Na release. The 900°C sample was slightly higher, with the 800°C sample showing the highest release over the time period studied.

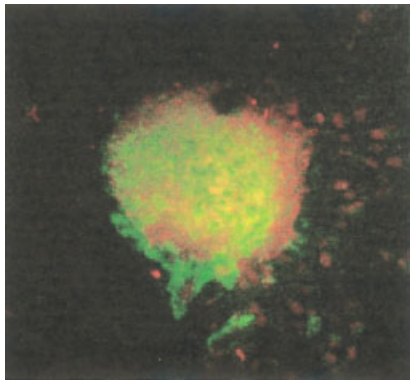

Figure 10. Fluorescence micrograph of a nodule after culture on 1000°C(3) for 14 days. Cells were stained with anti-collagen 1 antibody and propidium iodide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

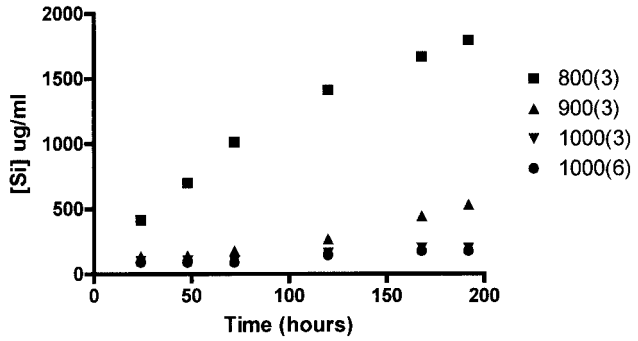

Figure 11. Si release profile of each disc determined by ICP. Values are ug/mL and represent mean values where $n = 3$.

pH of culture medium

The pH of culture medium was measured after 24 and 48 h incubation of tape-cast bioglass disc, as shown in Table IV. All samples were more alkaline than the culture medium and followed the trend 800°C > 900°C > 1000°C(3) > 1000°C(6).

DISCUSSION

This current study showed very high Na and Si release profiles from the sample sintered at 800°C,

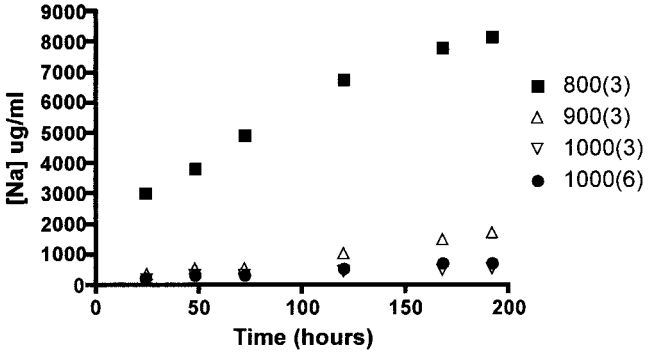

Figure 12. Na release profile of each disc determined by ICP. Values are ug/mL and represent mean values where $n = 3$.

TABLE IV
pH Values of Conditioned Medium After 24 and 48 h Incubation

	Time	
Sample	24 h	48 h
DMEM control	7.2	7.2
800(3)	9.03	8.86
900(3)	8.23	8.16
1000(3)	8.12	8.03
1000(6)	8.1	7.98

which resulted in the occurrence of necrosis of the cells, which tends to occur from severe toxic injury. The release profiles into DMEM followed the trend: 800°C > 900°C > 1000°C(3) > 1000°C(6), and this same trend was found previously in Tris buffer.[4] If ion release alone was responsible for effects on cell responses, then one would assume the best cell response would be found after culture on the 1000°C(6) sample, which had the least release. However, this was not the case and in fact culture on this sample resulted in very high levels of apoptosis.

Si has already been associated with both apoptosis, as well as increased bone formation,[9,10] depending on ionic concentration. It also has been found that certain dissolution product concentrations from bioactive glasses have a highly beneficial effect, as demonstrated by Xynos et al.[11] and Peaker et al.[12], but higher concentrations appear to cause programmed cell death, as shown here. The mechanism of action of Si is unknown, but it is thought that it is in the form of silicic acid.[13] Oligomers of inorganic silica also may be present in the culture medium containing the bioactive tape-cast samples.

Hydroxyapatite formation in DMEM was similar to that formed in Tris buffer[4] and SBF,[3] although the process was slightly slower in culture medium. The most rapid HA formation was observed on the 1000°C(6) sample, in contrast to the 900°C sample in Tris and SBF. As mentioned above, the 1000°C(6) sample resulted in the highest level of apoptosis. Therefore, speed of HA formation appears to effect cell responses, as well as release of Si and Na. The role of proteins in promoting or inhibiting (or even both) the formation of hydroxyapatite and the processes of biomineralization are not clear,[14] although it has been reported that the presence of serum proteins can slow or inhibit HA formation.[15]

The pH of a solution in which cells are cultured is vital. The pH of culture medium is maintained at 7.2–7.4 and any alteration on pH may alter a cell's ability to function normally. The potential across the cell membrane may be altered and ion exchange systems may be inhibited such as Ca^{2+} and Na+/H+, which may result in a build-up of intracellular H+ and denaturation of proteins. Changes in extracellular pH are, therefore, likely to cause apoptosis or necrosis. It is likely that the pH of the systems studied contributes to the apoptosis or necrosis observed. In a previous study, the authors noted a 9% decrease in levels of apoptosis after culture in dissolution products from bioactive glass that had been neutralized (original pH was 8).

Surface topography of a substrate on which cells are cultured also is crucial to their responses and survival. The samples used in this study were imaged using scanning electron microscopy in a previous study,[3] and it was found that surface roughness increased with decreasing sintering temperature. The results of our current study suggest that the cell responses are not a result of the difference in surface topography.

The results suggest that hydroxyapatite formation should not be too rapid, nor should excessive ion leaching from the bioactive substrates occur. A medium-level rate of bioactivity may be ideal for osteoblast survival, proliferation, and nodule formation.

Further investigations are underway determining the effects of Si alone on cell responses and gene expression.

CONCLUSION

Hydroxyapatite formation in DMEM was similar to that formed in Tris buffer and SBF, as reported previously,[3,4] although the process was slightly slower in culture medium. The most rapid HA formation was observed on the 1000°C(6) sample, which also resulted in the highest levels of apoptosis, a form of programmed cell death. This pattern of cell death appears to be related to levels of ion release. The 800°C sample released the highest rates of Na and Si, and it was determined that this caused high levels of necrosis. Necrosis is a form of cell death that is not controlled and tends to result from exposure to high levels of toxins, where lower levels are likely to result in apoptosis. The author has demonstrated previously Si release from 58S bioactive glass foams and apoptosis.[10] The greatest number of viable cells and nodules formed were on the 1000°C(3) sample. The results suggest that hydroxyapatite formation should not be too rapid, nor should excessive ion leaching occur, i.e., a medium-level bioactivity rate may be ideal for osteoblast survival, proliferation, nodule, and, ultimately, bone formation.

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